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Laboratory evaluation of citrus cultivars susceptibility and influence of fruit size on Fortune mandarin to infection by *Alternaria alternata* pv. *citri*

A. Vicent, J. Badal, M.J. Asensi, N. Sanz, J. Armengol and J. García-Jiménez
Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022-Valencia, Spain (Phone: +34963879254; Fax: +34963879269; E-mail: anvici@eaf.upv.es)

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Abstract

Young leaves of 62 citrus cultivars were inoculated with conidia of three Spanish isolates of *Alternaria alternata* pv. *citri*, the causal agent of brown spot of citrus. Hybrids with Dancy mandarin, King mandarin or their derivates as a parent, grapefruit cultivars and the mandarin cultivars Guillermina, Emperor, Clemenpons and Esbal were highly susceptible to the pathogen. Satsuma cultivar Clausellina and orange cultivars, with the exception of Sanguinelli, were slightly susceptible. Lemon and lime cultivars were not susceptible, with the exception of Mexican lime (*Citrus aurantifolia*), which was slightly susceptible. Although this study shows a range of potential hosts for this pathogen, to date the only affected cultivars in Spain are Fortune and Nova mandarins, and Minneola tangelo. The susceptibility of Fortune fruits decreased as diameter increased, being susceptible through the whole season. This was confirmed with field observations in autumn where fruit infections have been detected when the diameter reaches 6–7 cm.

Introduction

Two distinct pathotypes of *Alternaria alternata* have been described as the causal agents of Alternaria brown spot of citrus based on their host specificity and toxin production. The 'rough lemon pathotype' is specific for rough lemon (*Citrus jambhiri*) and Rangpur lime (*Citrus limonia*), and produces the host-specific ACRL-toxin. *A. alternata* pv. *citri* (*Aac*) is specific for tangerines (*Citrus reticulata*) and tangerine × grapefruit (*C. reticulata* × *Citrus paradisi*) hybrids and produces the host-specific ACT-toxin (Kohmoto et al., 1979; Peever et al., 2002).

Alternaria brown spot caused by *Aac* was first described on Emperor mandarin in Australia in 1903. It appeared in Florida in 1974 and subsequently was identified in other citrus production areas including South Africa, Colombia (Timmer et al., 2000), Cuba (Herrera, 1992), Brazil and Argentina (Peres et al., 2003). In the Mediterranean basin, the disease was first detected in Israel in 1989 (Solel, 1991), in Turkey in 1995 (Canihos et al., 1997) and was reported in Spain in 1998 (Vicent et al., 2000) and in Italy in 2000 (Bella et al., 2001).

The presence of *Aac* in Spain has become a serious problem to Fortune mandarin production. This is one of the most important late-maturing cultivars grown, which produced more than 123 000 t in Comunidad Valenciana region (eastern Spain) in the year 2001 (CAPA, 2002). Symptoms in young leaves are irregular brown necrotic areas with characteristic yellow halos. Apices of young shoots are completely defoliated in severely affected trees. On fruits, symptoms include light brown, slightly depressed spots to circular and dark brown areas on the external surface. Infected young fruits and leaves often fall and the mature fruits are unmarketable due to lesions, resulting in important economic losses (Vicent et al., 2000). Extensive surveys conducted from 1998 revealed a few infected orchards of Nova mandarin and Minneola tangelo (Vicent et al., 2001).

The objectives of this study were (i) to determine the susceptibility of citrus cultivars to Spanish isolates of *Aac* by artificial inoculation in order to evaluate the potential risk of the disease for other citrus cultivars, and (ii) to evaluate the influence of fruit size of Fortune mandarin on infection, information that could allow a reduction of fungicide spraying at late stages of fruit development.

Materials and methods

Plant and fruit material

Young leaves were obtained in the spring flush from 62 genotypes from the fields of the Citrus Germplasm Bank of the Instituto Valenciano de Investigaciones Agrarias (I.V.I.A., Moncada, Valencia, Spain) and commercial fields planted with certified material. Fruits of Fortune mandarin were collected from May to December in a 12-year-old field in Ribarroja (Valencia). Bioassays were completed immediately after harvest.

Fungal isolates and inoculum production

Three highly virulent isolates of *Aac* isolated from Fortune mandarin and representing different geographical origins were used for inoculations: AF2999-1 from Alzira (Valencia), AF3499-1 from Ribarroja (Valencia) and AF5199-2 from Pilar de la Horadada (Alicante), respectively. The isolates were single-spored prior to use and stored in oat agar (OA) slants at 4 °C.

Abundant conidia were obtained by a method adapted from Everts and Lacy (1996). Isolates were grown on potato dextrose agar (PDA) plates at 25 °C in darkness for 8–10 days, later illuminated with fluorescent lamps (Philips TLD 18W/33) at 25 °C for 8 h to initiate conidiophore formation, and placed in the dark at 18 °C for 12 h. Conidial suspensions were prepared by pouring sterile water over the colonies and gently rubbing the surface with a sterile glass rod. The suspension was filtered through two layers of cheesecloth and the conidial concentration was determined with a hematocytometer and adjusted by dilution. Suspensions with less than 90% germination of conidia were discarded.

Susceptibility of citrus cultivars

Young leaves (about 50% of leaf development) were inoculated with 10^5 conidia ml^{-1} (Kohmoto et al., 1991). Two drops of this suspension (40 μl) were placed on the lower surface of each leaflet using five leaves per isolate and cultivar. Controls were inoculated with sterile-distilled water. Leaves were incubated in a moist chamber in the dark at 27 °C and results were evaluated 48 h after inoculation. The severity of the infection was recorded as: − = no necrotic lesions; ± = slight necrosis limited to below the drop; + = necrotic lesions spread over the leaves. The experiment was repeated.

Susceptibility of citrus fruits

Fruits of Fortune mandarin were inoculated with conidial suspensions of 10^4 and 10^5 conidia ml^{-1} at different stages of development: 1, 2, 3, 4, 5, 6 and 7 cm diameter. Four filter paper squares (0.5 × 0.5 cm) were dipped in the inoculum and placed equatorially on the rind of each fruit. Inoculated fruits were incubated in a humid chamber in the dark at 27 °C and results were evaluated 48 h after inoculation (Solel and Kimchi, 1997). Five replicates of two fruits were used for each conidial suspension and fruit size.

The severity of the infection was assessed below each filter paper according to the following grades: 0 = no necrotic lesions were observed; 1 = 1–5 necrotic lesions were observed; 2 = more than five necrotic lesions were observed, lesions coalesced. The experiment was repeated once. A disease severity index (DSI) was calculated as the mean of the eight inoculation areas for each of the five replicates. Severity data were $\log_{10}$ transformed before analysis to improve the homogeneity of the variance. Regression analysis was performed separately for each inoculum level and isolate using the software Statgraphics Plus 4.0 (Statistical Graphics Corp, Englewood Cliffs, NJ, USA).

Results

Susceptibility of citrus cultivars

Leaves of 62 citrus cultivars were tested for their reaction to inoculation by three isolates of *Aac* (Table 1). For better comprehension, cultivars were grouped as mandarins, oranges, lemons, limes, grapefruits and hybrids according to Saunt (1992) and Agustí (2000).

Most of the mandarin cultivars were not susceptible to *Aac*. The exceptions were the Clementine cultivar Guillermina and the mandarin cultivar Emperor (infected by the three isolates tested) and the Clementine cultivars Clemenpons and Esbal (infected

Table 1. Reaction of citrus cultivars to inoculation with three isolates of Aac (AF2999-1, AF3499-1 and AF5199-2)

Citrus cultivars[a]		Lesion formation[b]		
		Isolates		
		AF2999-1	AF3499-1	AF5199-2
Mandarin				
Clementine (*Citrus clementina*)	Arrufatina	–	–	–
	Beatriz	–	–	–
	Clemenpons	–	+	+
	Clementard	–	–	–
	Clementina fina	–	–	–
	Clemenules	–	–	–
	Esbal	–	+	+
	Guillermina	+	+	+
	Hernandina	–	–	–
	Loretina	–	–	–
	Marisol	–	–	–
	Nour	–	–	–
	Orogrande	–	–	–
	Oronules	–	–	–
Satsuma (*C. unshiu*)	Clausellina	–	±	±
	Hashimoto	–	–	–
	Okitsu	–	–	–
	Satsuma	–	–	–
Other				
C. nobilis	Campeona	–	–	–
C. deliciosa	Willowleaf	–	–	–
C. reticulata	Emperor	+	+	+
C. tangerina	Parson's Special	–	–	–
C. temple	Temple	–	–	–
Orange (Sweet orange *C. sinensis*)				
Navel group	Lane Late	±	±	±
	Navel Foyos	±	±	±
	Navel Powel	±	±	±
	Navel Valencia Late	±	±	±
	Navelate	–	±	±
	Navelina	±	–	±
	Washington Navel	±	±	±
Blood group	Sanguinelli	–	–	–
Blancas group	Salustiana	±	±	±
	Valencia Late	±	±	±
	Valencia Seedless	±	±	±
Lemon				
C .limon	Eureka	–	–	–
	Fino	–	–	–
	Lisbon Frost	–	–	–
	Verna	–	–	–
C. jambhiri	Rough lemon	–	–	–
Lime				
C. latifolia	Bears	–	–	–
C. limettioides	Palestine	–	–	–
C. aurantifolia	Mexican	±	±	±
C. limonia	Rangpur	–	–	–

Table 1. (*Continued*)

Citrus cultivars[a]		Lesion formation[b]		
		Isolates		
		AF2999-1	AF3499-1	AF5199-2
Grapefruit				
C. paradisi	Duncan	+	+	+
	Marsh	+	+	+
	Red Blush	+	+	+
	Rio Red	+	+	+
	Star Ruby	+	+	+
Hybrid				
Tangor (*C. reticulata* × *C. sinensis*)				
	Ellendalle	−	−	−
	Murcott	+	+	+
	Ortanique	−	−	−
Tangelo (*C. paradisi* × *C. reticulata*)				
Duncan grapefruit × Dancy mandarin	Minneola	+	+	+
Duncan grapefruit × Dancy mandarin	Orlando	+	+	+
(*C. paradisi* × *C. deliciosa*)				
Duncan grapefruit × Willowleaf mandarin	Mapo	−	−	−
Other mandarin hybrids				
King × Willowleaf	Encore	+	+	+
Clementine × Dancy	Fortune	+	+	+
Owari × King	Kara-Kara	+	+	+
Clementine × King	Palazzelli	+	−	±
C. unshiu × *C. deliciosa*	Simeto	−	−	−
Other				
Clementine mandarin × Orlando tangelo	Fairchild	+	+	+
Clementine mandarin × Orlando tangelo	Nova	+	+	+
Minneola tangelo × Clementine mandarin	Page	+	+	+

[a]Source for citrus species and cultivars (Saunt, 1992; Agustí, 2000). [b]Reactions: +, severe necrotic lesions; ±, slight necrosis; −, no necrosis.

by isolates AF3499-1 and AF5199-2). The Satsuma cultivar Clausellina was slightly susceptible to isolates AF3499-1 and AF5199-2.

All orange cultivars were slightly susceptible to the pathogen, except Sanguinelli that was not susceptible to the pathogen. Some orange cultivars showed different responses to the inoculation with one or two of the isolates. These include cultivars Navelate and Navelina that were not susceptible to isolates AF2999-1 and AF3499-1, respectively.

Lemon and lime cultivars were not susceptible to *Aac* with the exception of Mexican lime (*Citrus aurantifolia*) which showed an intermediate reaction. In contrast, grapefruit cultivars were all susceptible.

In the hybrid group, all cultivars of Dancy mandarin, King mandarin or their derivatives (Minneola or Orlando tangelos) were susceptible. Cultivar Palazzelli showed variable reactions being susceptible to isolate AF2999-1, slightly susceptible to isolate AF5199-2 and not susceptible to isolate AF3499-1. The cultivar Murcott was also susceptible to *Aac*.

Susceptibility of citrus fruits

The polynomial equation that best described the logarithm of DSI in relation to fruit diameter (D) for each inoculum level and isolate was $\log_{10}(\mathrm{DSI} + 1) = b_0 + b_1\,\mathrm{D} + b_2\,\mathrm{D}^2$, in which b_0, b_1 and b_2 are parameters (Table 2, Figure 1). In all cases, P values indicated a significant correlation between the diameter and the susceptibility of the fruit at $P = 0.01$. Coefficient of determination (R^2) values ranged from 0.54 for isolate AF5199-2 to 0.67 for isolate

Table 2. Estimated parameters of equations for relationship between log-transformed DSI on Fortune mandarin fruit size for three Aac isolates (AF2999-1, AF3499-1 and AF5199-2) at two inoculum doses (10^4 and 10^5 conidia ml^{-1}) with coefficient of determination (R^2) and P-value.

Inoculum dose/isolate	Regression terms[a]			R^{2c}	P-value
	b_0	$D^b(b_1)$	$D^2(b_2)$		
10^4 conidia ml^{-1}					
AF2999-1	0.44998	0.01215	−0.00848	0.67	0.0000
AF3499-1	0.33113	0.05106	−0.01222	0.57	0.0000
AF5199-2	0.47625	−0.07058	0.00178	0.55	0.0000
10^5 conidia ml^{-1}					
AF2999-1	0.41173	0.06183	−0.01161	0.83	0.0000
AF3499-1	0.37939	0.08668	−0.01482	0.68	0.0000
AF5199-2	0.42934	0.04563	−0.00963	0.78	0.0000

[a] b_0, b_1, b_2, regression coefficients.
[b] Diameter of fruit.
[c] Coefficient of determination.

AF2999-1 at 10^4 conidia ml^{-1}. R^2 values were higher at 10^5 conidia ml^{-1} for all isolates, that ranged from 0.68 for isolate AF3499-1 to 0.82 for isolate AF2999-1. Isolates AF5199-2 and AF3499-1, at 10^4 conidia ml^{-1}, presented low R^2 values (0.55 and 0.57, respectively) indicating a poor fit of the model, while for isolate AF2999-1 at 10^4 conidia ml^{-1} and all isolates at 10^5 conidia ml^{-1}, a negative relationship was observed between DSI and D, showing the decline in fruit susceptibility as diameter increased. The DSI at the highest diameters was no lower than 1 rated on the 0–2 scale.

Discussion

Inoculation tests with Spanish isolates of *Aac* showed a wide variation in susceptibility among the citrus cultivars. Gardner et al. (1986) indicated that the apparent resistance and/or susceptibility to the ACT host-selective toxin can be influenced by the bioassay method. The inoculation method used in this work (Kohmoto et al., 1991) is similar to the method used by Solel and Kimchi (1997) which is considered useful as a means of discarding potential hosts.

In general, our results were consistent with those obtained by other authors, but were some relevant differences. There is little information about the susceptibility of mandarin cultivars. Hutton and Mayers (1988), Kohmoto et al. (1991) and Solel and Kimchi (1997) demonstrated that Clementina fina was not susceptible to *Aac* when inoculated with isolates from Australia, Florida and Israel, respectively, and Satsuma was susceptible when inoculated with one isolate from Israel (Solel and Kimchi, 1997). In the present work, Satsuma was not affected by the three isolates used in this study. On comparison to other mandarin cultivars, Emperor was highly sensitive to *Aac*. This was the first cultivar affected by the disease, recorded in 1903 in Australia (Hutton and Mayers, 1988). These authors also found that *Citrus temple* and *Citrus deliciosa* were both resistant to the disease. *Citrus deliciosa* was also resistant when inoculated with an isolate from Israel (Solel and Kimchi, 1997).

In our study, oranges showed a slight reaction. This result is in agreement with the study of Solel and Kimchi (1997) who observed that the cultivars Washington Navel, Valencia Late and Shamouti were susceptible, although Kohmoto et al. (1991) found that Washington Navel was resistant to the inoculation with one isolate from Florida.

Limes and lemons were resistant to *Aac* in previous studies (Hutton and Mayers, 1988; Kohmoto et al., 1991; Solel and Kimchi, 1997). In our case the only exception was Mexican lime (*C. aurantifolia*) which showed an intermediate reaction. This species is one of the most sensitive to the Mancha Foliar de los Cítricos caused by *Alternaria limicola,* a pathogen which does not produce any host-specific toxins (Palm and Civerolo, 1994). It is interesting to note that both *C. jambhiri* and *C. limonia* are severely affected only by the 'rough lemon pathotype', but not by *Aac*. Thus, our results provide additional confirmation that Spanish isolates of *A. alternata* belong to *Aac*.

All grapefruit cultivars tested were susceptible to Spanish isolates of *Aac*, confirming previous findings by artificial inoculation. There are also some reports about its occurrence in grapefruit orchards, although natural infections caused by *Aac* seem not to be severe (Gardner et al., 1986; Schutte, 1996; Solel and Kimchi, 1997; Timmer and Peever, 1997; Timmer et al., 2000).

All the hybrids with Dancy mandarin as a parent (Orlando, Minneola, Fortune, Fairchild, Page and Nova) were susceptible to the pathogen. These cultivars, including Murcott, which has unknown parents (Saunt, 1992), are the more affected by *Aac* worldwide (Hutton and Mayers, 1988; Timmer et al., 2000) and some of them are the cultivars affected in Spain.

Kohmoto et al. (1991) obtained a resistant reaction when King mandarin and its hybrids (Encore and Kara-Kara) were inoculated with Florida isolates of *Aac*. Kara-Kara cultivar showed also a resistant reaction when it was inoculated with two Australian isolates of

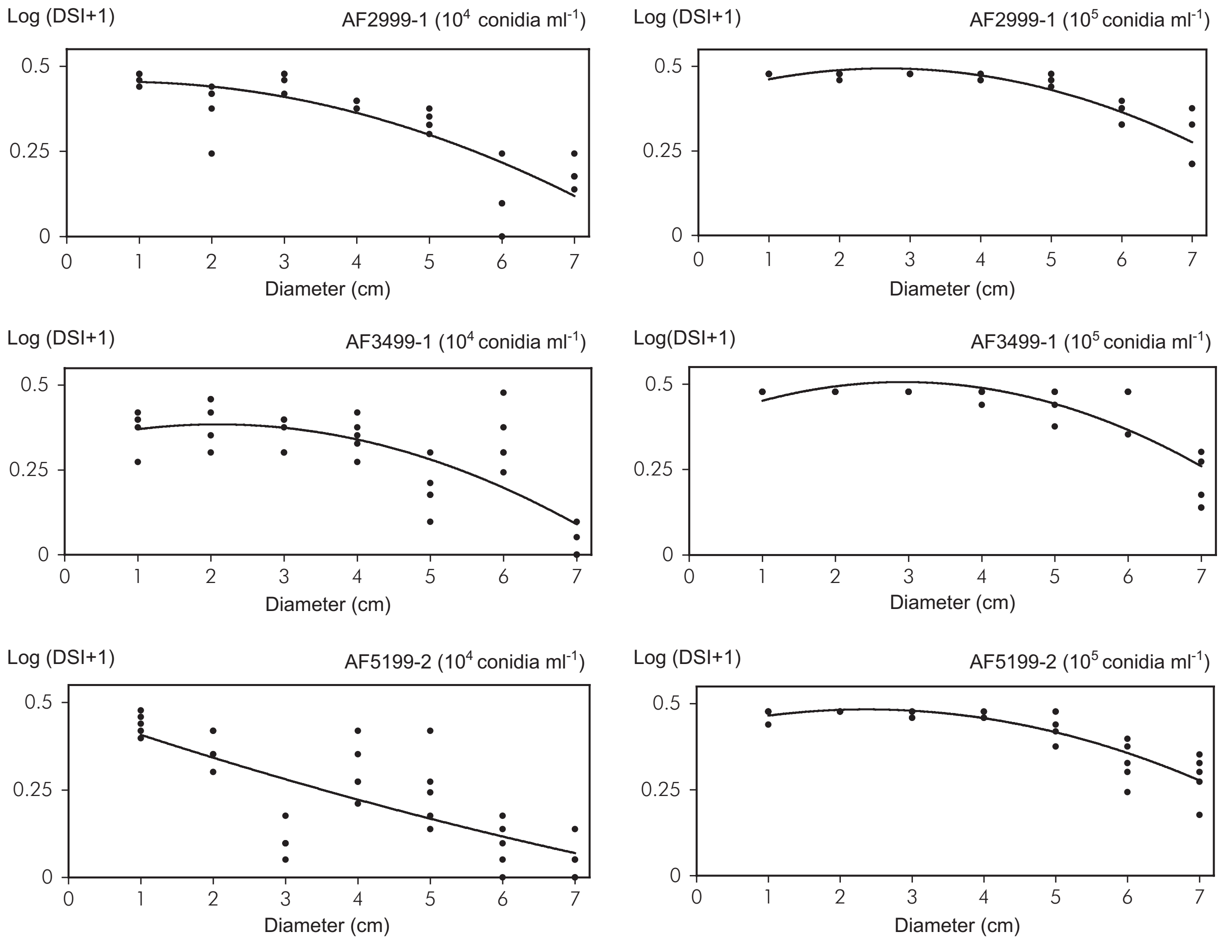

Figure 1. Changes in susceptibility of Fortune mandarin fruit at different stages of development to three isolates of *Aac* (AF-2999-1, AF-3499-1 and AF-5199-2) at two inoculum doses (10^4 and 10^5 conidia ml^{-1}).

Aac (Hutton and Mayers, 1988). Nevertheless, Solel and Kimchi (1997) demonstrated that the King mandarin and its hybrids, Wilking and Kara-Kara cultivars, were susceptible to an isolate of *Aac* from Israel. However, natural infections of these cultivars have never been reported. In our study, the hybrids of King mandarin (Encore, Kara-Kara and Palazzelli) were susceptible to the inoculation with *Aac*.

Recently, Peever et al. (2002) investigated the worldwide phylogeography of *Aac* and revealed some differences in virulence among isolates from different geographic origins. This could help to explain the differences in susceptibility noted in the present study.

When considering the relationship between fruit size and susceptibility to *Aac*, the DSI obtained at the highest diameters show that fruits of Fortune mandarin become less susceptible with age, but are never resistant. This result has been confirmed with field observations in autumn, where fruit infections have been detected when the diameter reaches 6–7 cm. In other studies, Whiteside (1976) showed that susceptibility of Dancy tangerine fruits decreased with age becoming resistant to infection by *Aac* at 3 cm diameter. Canihos et al. (1999) and Timmer et al. (2000) indicated that several applications of fungicides are required to produce acceptable control of the disease until the fruit becomes resistant when they reach a diameter of about 3 cm. On the other hand, Solel and Kimchi (1998) indicated that fruits of Minneola tangelo were highly susceptible throughout the whole season in Israel.

In conclusion, fruits of Fortune mandarin should be considered susceptible to infection caused by Spanish isolates of *Aac* at all growth stages, being most susceptible during the early stages of development in late spring–early summer and least susceptible in autumn at the time of fruit maturation. Control strategies throughout the crop season are necessary for an adequate control of the disease.

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