INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
21–1070/2022/27–4–295–300
DOI: 10.17957/IJAB/15.1929
http://www.fspublishers.org

Full Length Article

Field and *In Vitro* Evaluation of Mandarin Cultivars Resistance to *Alternaria alternata*

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Received 31 December 2021; Accepted 23 March 2022; Published 30 April 2022

Abstract

Alternaria brown spot (ABS) of tangerines, caused by the necrotrophic fungus *Alternaria alternata* (Fr.) Keissl, is one of the most destructive diseases affecting mandarins worldwide, especially under the Mediterranean climate. The present study aimed to assess the susceptibility of mandarins to the *A. alternata*, through *in vivo* and *in vitro* inoculation for 10 mandarins varieties from a Moroccan collection at INRA-Kénitra including Ananas, Bergamota, Dancy, Murcott Honey, Carvhalal, Satsuma Wase, Vohanisahy Ifranica, Temple, Nadorcott and Lée. Field inoculation trials were performed in parallel with the laboratory experiments by inoculation of fungal spores in fruits and young detached leaves. *In vitro* leaves inoculations were conducted in two successive years to confirm results and ABS-resistant hybrids were selected. The severity of disease in fruits and leaves was determined by following a specific diagrammatic scale of ABS and calculating the disease progress curve (AUDPC). The results indicated that all the cultivars showed disease symptoms on fruits and leaves either in the field or in the laboratory. The AUDPC values of the ABS of fruits and leaves inoculated in the field ranged from 87.63 to 201.2 and 32.88 to 74.04, respectively. For the *in vitro* inoculations, the minimum values of AUDPC were essentially obtained by the Temple and Nadorcott varieties. Temple, Nadorcott, Lée and Vohanisahy Ifranica have low AUDPC values and seem to be resistant to the disease, whereas others namely Dancy, Carvhalal, Ananas, Murcott Honey, Satsuma Wase exhibited a greater disease susceptibility.

Keywords: *Alternaria alternata*; Mandarins; Resistance

Introduction

Citrus fruit production has seen notable growth in recent years. According to the recent statistics reported by USDA (2021), tangerines and their hybrids constitute the most important and predominant group with a production of 1.2 Million tons (Mt) and exports exceeded 500000 tons. In 2019, Morocco holds the third place on tangerines and mandarins exportation (USDA 2019). Despite its importance, the orchards suffer from a range of fungal diseases, especially, Alternaria brown spot (ABS) caused by the tangerine pathotype, *Alternaria alternata* (Fr.) Keissl. (Stewart *et al.* 2014; Azevedo *et al*. 2019). Recently, there was report of occasional association of *A. arborescens* pathogen with Alternaria brown spot on citrus (Moosa *et al.* 2020; Wang *et al.* 2021; Zelmat *et al.* 2021). The ABS is widely distributed throughout the world (Elena 2006) and is considered distinct from other *Alternaria* diseases on citrus including Alternaria black rot (ABR) and Alternaria leaf spot of rough lemon. This disease causes necrotic lesions on leaves, twigs and fruits in the pre-harvest stage (Aiello *et al*. 2020). Moreover, the ABS can be expressed after fruit harvesting and provokes different symptoms as stem-end rot infection and leads to serious value loss of the mandarins (Garganese *et al.* 2016; Saito and Xiao 2017). The produced necrotic areas are commonly associated with the ACT host-specific toxin production which distorts profoundly the plasma membrane permeability of the plant hosts cells (Tsuge *et al.* 2013; Ma *et al.* 2019).

Until now, the main strategy used to control the ABS over the world is based essentially on the fungicide applications depending on the genotype and climate (Vega *et al*. 2012; Kim *et al*. 2017). In Morocco, copper, copper oxychloride and copper hydroxide were applied heavily to managed the *Alternaria* diseases on citrus (Lahlali *et al*. 2021). However, current studies conducted on Florida and Brazilian orchards of tangerines and their hybrids to assess the *A. alternata* sensitivity to chemical compounds demonstrate that a large number of *A. alternata* isolates from citrus were resistant to the fungicides based on copper and quinone outside inhibitor (QoI) (Chitolina *et al.* 2021). In addition to the emergence of resistant *A. alternata* strains, intensive applications of fungicides have harmful and hazardous consequences for animals, humans and the environment (Vicent *et al.* 2009). In fact, several studies have been carried out to find a sustainable solution and develop a long-term strategy to combat this disease, in particular, by researching new ABS-resistant cultivars (Campos *et al*. 2017; Turgutoğlu and Baktir 2019; Costa *et al.* 2020). The genotypes are classified mainly according to their level of resistance. Dancy, Fortune and Murcott were reported as susceptible to ABS, whereas Afourer and Carvalhais were considered among the resistant cultivars (Arlotta *et al*. 2020). In fact, the tolerance of the resistant cultivars to ABS is controlled by a single recessive locus (ABSr) (Gulsen *et al*. 2010; Cuenca *et al*. 2013). The present study aimed to determine the susceptibility or resistance of 10 cultivated varieties of mandarins to *A. alternata* fungus isolated from citrus basing on the *in vitro* and *in vivo* inoculation.

Materials and Methods

Procurement of *A. alternata* Isolate

A Moroccan single isolate of *A. alternata* (MW616576) originating from symptomatic orange citrus fruit (Zelmat *et al*. 2021), was used in this study (Fig. 1). The virulence of this fungal isolate was evaluated by inoculating detached leaves of susceptible genotype, *Citrus jambhiri* ‘Rough lemon’. This examination was performed in order to determine the capacity of the *A. alternata* isolate to cause lesions and develop necrosis around the inoculated point (Fig. 1).

Plant Materials

The resistance to ABS was assessed for 10 varieties of mandarins/hybrids belonging to the INRA experimental orchards (Table 1). These plants were selected based both on their potential commercial value and on the availability of mature fruits during the period of inoculation.

Preparation of Inoculum

The *A. alternata* inoculum was prepared from young cultures of five days as described by Costa *et al.* (2020) with some modifications. The conidia were collected by adding 10 mL of sterilized distilled water containing Tween 20 (0.02%, v/v) to each plate and scraping gently using a sterile scalpel. Then, the obtained suspension was filtered through two layers of sterile paper to eliminate the mycelium fragments and adjusted to 10^5 conidia mL^{-1} using a hemocytometer technique.

Field Inoculation

Field bioassays were carried out over previously selected trees of mandarins. Three plants of each cultivar were used in this experiment. A total of nine young leaves (5–7 cm) and nine mature fruits from each tree were marked before and inoculated manually by spraying appositively 2 mL of the conidia suspension per leaf/fruit (Pacheco *et al*. 2012). Controls were inoculated with the same volume of sterile distilled water. Thus, the inoculated samples were covered with a transparent polyethylene bag whose interior was previously sprayed with sterile distilled water to serve as a humid chamber (Reis *et al*. 2007; Souza *et al*. 2009). The assessments were performed four to seven days after inoculation (Souza *et al.* 2009).

Inoculation of Detached Leaves

In vitro inoculations were performed in two consecutive years (2019 and 2020) for all the tested cultivars. Young leaves measured 5–7 cm and 2–3 cm in length were sampled from the plants and inoculated immediately after their harvest. Briefly, nine leaves per variety were disinfected in 1% of hypochlorite sodium and placed individually in Petri dishes with a humid filter paper and inoculated using the same volume of suspensions mentioned above. Control leaves were sprayed only with distilled water and the plates were kept at the temperature of 27°C under a 12 h photoperiod. Disease assessments were performed 24 h, 48 h and 72 h after inoculation (Reis *et al*. 2007).

Assessment Method

The severity of symptoms on the fruits was referred using a specific scale described by Pacheco *et al.* (2012) as shown in Table 2. For leaves, the severity of symptoms was determined using a specific diagrammatic scale developed by Martelli *et al.* (2016) to assess the ABS leaf symptoms (Table 3). The results were recorded by calculating the area under the disease progression curve (AUDPC) (Campos *et al.* 2017):

$$AUDPC = \sum [((y1+y2)/2*(t2-t1)]$$

Where y1 and y2 are two consecutive evaluations carried

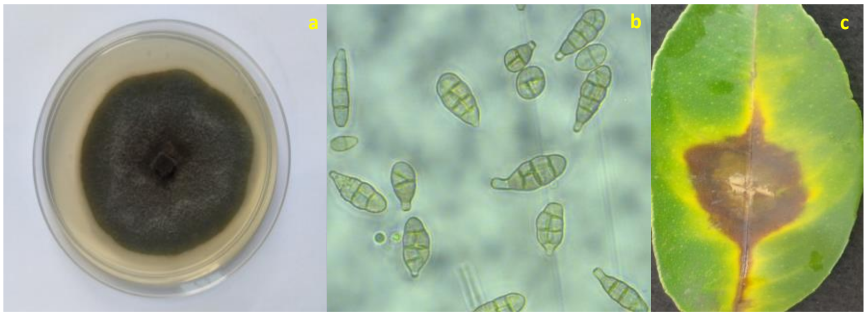

Table 1: List of the mandarin cultivars used in this study, from INRA of Morocco

Accession	Description	System/References
Murcott Honey	*C. reticulata* × *C. Sinensis*	Gogorcena *et al.* (1990)
Bergamota	*Citrus reticulata*	Swingle system
Dancy	Seedling of Moragne tangierine	Hodgson (1967)
Carvalhal	*Citrus reticulata Blanco*	Tanaka system
Ananas	*Citrus reticulata Blanco*	Tanaka system
Satsuma wase	*Citrus unshiu*	Tanaka system
Vohanisahy Ifranica	*Citrus reticulata*	Swingle system
Lee	*C. paradisi* × *tangerine*	Tanaka system
Temple	*C. reticulata* × *C. sinensis*	Tanaka system
Nadorcott	*Murcott tangor* × *Mandalina mandarin*	Nadori (2004)

Table 2: Fruit disease severity rating scale

Score	Infection
0	0%
1	0.1%
2	1%
3	2.5%
4	5%
5	11%
6	25%

Table 3: Leaf disease severity sating scale

Score	(%) of infection
0	0%
1	0.3%
2	3.5%
3	8%
4	15%
5	34%
6	61%
7	80%
8	90%
9	97%

out at times t1 and t2 respectively.

Results

Field Evaluation of Alternaria Brown Spot

In this experiment, the resistance level of ten varieties of mandarins to *A. alternata* was evaluated for both fruits and leaves. Field results showed that all studied varieties were affected and responded to this pathogen. Symptoms were typically characteristic of ABS disease as shown in the Fig. 2. On fruit, small necrotic spots were dispersed randomly in their surface while the leaf lesions included large area brown to black areas according to the severity incidence. In fact, the AUDPC values given in Table 4 indicate that lesions on fruits were more severe than on leaves. The minimum values of AUDPC were recorded by Vohanisahy Ifranica, Lée, Temple and Nadorcott cultivars, with 127.75, 125.88, 122.8, 87.63 on fruits, and with 40.69, 46.06, 32.88, 38.63 on leaves, respectively. The higher values of AUDPC were observed on fruits of Murcott Honey, Bergamota and Dancy and on leaves of Carvhalal, Ananas and Satsuma Wase.

In vitro Evaluation of Alternaria Brown Spot

The detached-leaf method was repeated in two successive years depending on the leaves size. The ABS severity of each cultivar was calculated as AUDPCs (Table 4). Compared to the field inoculations, lesions on leaves inoculated under controlled conditions (*in vitro*) were slightly severe. On the other hand, results showed that the young leaves measuring 3 to 4 cm in size were more susceptible to ABS than those measuring 5–7 cm. The symptoms were generally typical and appeared as irregular areas with a dark brown color (Fig. 3). Based on the *in vitro* results, Nadorcott and Temple seem the most resistant cultivars to *A. alternata*, whereas high values of AUDPC were observed for Murcott, Dancy, Carvalhal and Ananas.

Discussion

ABS is one of the severest diseases of mandarins (*C. reticulata*) and their hybrids leading to significant economic

Fig. 2: Fruits showing the typical symptoms of ABS after 7 days of field inoculation (**a**); Temple (**b**); Ananas (**c**); Carvhalal (**d**); Vohanisahy Ifranica

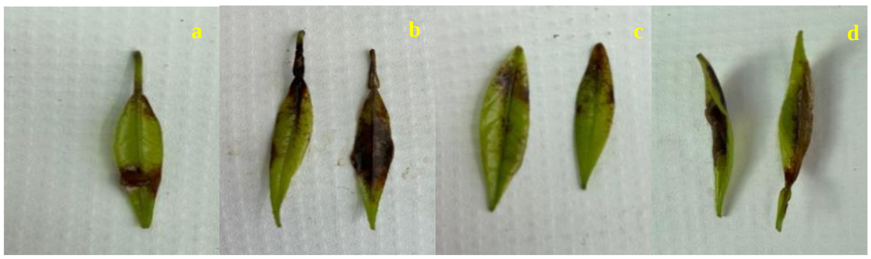

Fig. 3: Leaves showing the typical symptoms of ABS after 48 h of *in vitro* inoculation (**a**); Carvhalal (**b**); Dancy (**c**); Ananas (**d**); Satsuma wase

Table 4: Area under the disease progress curve (AUDPC) of ABS in 2019 and 2020

Cultivars	AUDPC			
	In field		In laboratory	
	Fruits	Leaves (5–7 cm)	Leaves (5–7 cm)	Leaves (2–3 cm)
Murcott Honey	201.2	51.42	149.34	222.18
Bergamota	182.66	57.57	75.27	158.64
Dancy	175.26	56.34	78.99	264.78
Carvhalal	157.39	74.04	74.68	295.05
Ananas	154.27	70.32	76.52	200.06
Satsuma Wase	149.89	70.33	88.87	254.9
Vohanisahy Ifranica	127.75	40.69	86.39	296.88
Lée	125.88	46.06	49.35	150.59
Temple	122.8	32.88	33.93	68.5
Nadorcott	87.63	38.63	41.33	140.09

losses. Discovering and screening for resistant cultivars to this disease became recently a major objective for several researchers. In the present study, the evaluation of ABS resistance was carried out in the field and on detached leaves of ten mandarins varieties using a virulent *A. alternata* isolate. Results showed a different degree of susceptibility among the tested cultivars. The ABS symptoms were clearly expressed on fruits and young leaves inoculated artificially with *A. alternata* pathogen. The produced lesions were observed as brown to black-colored areas dispersed in the leaf surface. Similar symptoms were detected on leaves of resistant and susceptible mandarin hybrids inoculated with *A. alternata* spores (Campos *et al.* 2017; Arlotta *et al.* 2020). In the literature, it has been widely reported that the development of lesions is mainly due to the host-selective ACT-toxin production by *A. alternata* tangerines pathotype (Akimitsu *et al.* 2014). On the other hand, the detached leaves were found highly susceptible to the disease than those inoculated in the trees. This is in agreement with several studies reporting the fast development of severe symptoms under controlled conditions compared with the *in vivo* experiments (Souza *et al.* 2009; Turgutoğlu and Baktir 2019). Azevedo *et al.* (2010) noted that the physiological defense of leaves against pathogens is decreased and lost gradually after their detachment. On the other hand, the resistance level assessment of the studied cultivars based on the AUDPC, showed the high susceptibility to *A. alternata* by Murcott Honey, Bergamota, Dancy, Carvhalal, Ananas, Satsuma Wase and Vohanisahy Ifranica. Lee, Temple and Nadorcott were recorded low severity and to be resistant to ABS on leaves and fruits. In accordance, Dancy and Murcott were reported in previous studies as highly susceptible genotypes to this disease and used as controls (Cuenca *et al.* 2013; Campos *et al.* 2017). A current study demonstrates genetically that Nadorcott mandarin (syn. Afourer) was moderately resistant to *A. alternata* fungus (Arlotta *et al.* 2020). Likewise, Reis *et al.* (2007) and Souza *et al.* (2009)

describe the Temple as slightly susceptible to this fungus. This variety was also found resistant to ABS in previous data (Cuenca *et al*. 2016).

Conclusion

Among the ten mandarin cultivars evaluated in this study, Temple, Nadorcott and Lée were revealed a medium resistance to *A. alternata* pathogen. Furthermore, our findings indicate the greater susceptibility of the detached leaves inoculated in the laboratory to the ABS disease. Hence, new mandarins varieties should be tested with different *A. alternata* isolates show a high pathogenic ability to cause disease in the citrus crops.

Acknowledgments

This study was supported by the grant from KAFACI (Project Number: KAH 20180108), Rural Development Administration of Korea.

Author Contributions

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

No potential conflict of interest was reported by the authors.

Data Availability

Accession No MW616576 is already available in NCBI

Ethics Approval

Not applicable

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