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Evaluation of Citrus Cultivars Susceptibility to Leaf Spot Disease Caused by *Alternaria alternata* under *in vitro* Conditions

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ABSTRACT
Alternaria brown spot is a serious disease of fruit and foliage of citrus. This disease caused by *Alternaria alternata* and causes serious economical losses on susceptible cultivars in Iran. In this research, susceptibility of seven citrus cultivars including Fortune, Orlando tangelo, Mineola tangelo, Page, Clementine, Sour orange and Sweet orange (Thomson) fruits to *A. alternata* was investigated. The result revealed that the Fortune, Orlando tangelo, Mineola tangelo, Page and Thomson fruits are highly susceptible and showed severe symptoms of brown spot, especially in "unwounded" fruits. In contrast, Clementine and Sour orange are resistant to this pathogen, and *A. alternata* could not grow on unwounded fruits. However, weak symptoms were observed in Clementine and Sour orange when cuticle was broken and flavedo or flavedo + albedo was removed.

Keywords: *Alternaria alternata*, Alternaria Brown Spot, Clementine, Resistance, Sour orange

INTRODUCTION

Alternaria brown spot pathogen of *Alternaria alternata*, formerly named as *A. citri*, was first reported in 1966 from Queensland, Australia on Emperor Mandarin (Pegg, 1966) and it has expanded worldwide distribution considerably in recent years. It has been known for many years in the United States (Peever *et al.*, 2002), South Africa (Swart *et al*., 1998), Turkey (Canihos *et al.*, 1997), Spain (Vicent *et al.*, 2000), Italy (Bella *et al.*, 2001), China (Wang *et al.*, 2010), Brazil and Argentina (Peres *et al.*, 2003). This disease was first reported in Iran in 2006 on the tangerine hybrid cultivars (Minneola, Page and Fortune) and then on other citrus cultivars (Golmohammadi *et al.*, 2006).

Alternaria species cause several diseases in *Citrus* species, resulting in a substantial loss of production and a lower value product, which in many cases leading to serious financial loss. There are seven pathotypes of *A. alternata* including two pathotypes which produce host-selective ACT-toxin causing Alternaria brown spot and host-selective ACR-toxin causing Alternaria leaf spot (Akimitsu *et al.*, 2003; Yago *et al.*, 2011). These two pathotypes are involved in citrus diseases due to the production of ACT- and ACR-toxin and retain clearly distinct host ranges because of producing distinct host-selective toxins. Alternaria black rot of citrus is caused by *A. citri*. This pathogen is morphologically similar to the causal agents for Alternaria brown spot and leaf spot (Masunaka *et al.,* 2005). However, *A. citri* does not produce host-selective toxins and the black rot symptoms were mainly caused by endopolygalacturonase (Isshiki *et al*., 2001). Alternaria brown spot and leaf spot pathogens cause necrotic lesions on leaves and twigs, and lesions may expand rapidly due to the production of a host-specific toxin by the pathogens, often resulting in leaf drop and twig dieback (Akimitsu *et al.,* 2003). On fruit, lesions vary from small dark necrotic spots to large sunken pockmarks, thereby reducing the value of the fruit for the fresh market. Citrus fruits are susceptible to infection until about midsummer in many areas (Timmer *et al*., 2000), but fruits may be susceptible for much longer in cooler climates. To prevent the development of this pathogen and to limit losses, treatment with chemical fungicides is a widely used procedure. However, such chemicals may produce serious problems, with residues on the fruit, appearance of fungicide resistant strains of *A. alternata* and their possible accumulation in human adipose tissue constituting an additional health threat (Vicent *et al.,* 2009).

An alternative way to fight against these infections might be to modulate the natural defense mechanisms of the plant. Although few in number, Some studies have described the mechanisms involved in the defense response of citrus fruit against fungal infection, among which are the induction of the expression of defense-related genes, (PR proteins), phytoalexins and other antifungal secondary metabolites that induce protection (Afek *et al.,* 1999; Feng and Zheng, 2007). In addition, some studies showed a possible role that phenolic compounds might play as phytoalexins in some *Citrus* species (Arcas *et al.,* 2000; Ortuno *et al.,* 2002; Del Rio *et al.,* 2004). The susceptibility to *A. alternata* depends on the citrus species. Many studies have demonstrated that cultivars of *C. reticulata* and its hybrids, including Minneola tangelo and Orlando tangelo, the Tangor Murcott, and the hybrids Fortune, Nova and Lee are susceptible to the tangerine pathotype of *A. alternata* (Kohmoto *et al*., 1991; Vicent *et al.,* 2004). In contrast the Satsumas (*C. unshiu* Mark. Marc.) and the Clementines (*C. clementina* Hort. ex Tan.) show a certain degree of resistance and other species, such as *C. sinensis*, *C. limon* (L.) Burm., and *C. margarita* (Lour.) Swing. are resistant to the pathogen (Gardner *et al.,* 1986). Some citrus cultivars such as Clementine has a resistance gene to Alternaria brown spot and RAPD fragments showing a linkage to resistance against the disease can be amplified by specific PCR primers (Dalkilic *et al.,* 2005). Different citrus species are cultivated in Iran and some of them have certain degrees of resistance to this pathogen (Golmohammadi *et al.,* 2006).

In this research, the reaction of some citrus fruits to *A. alternata* was evaluated and the role of citrus peel in resistance of fruits to this pathogen was studied.

MATERIALS AND METHODS

Plant materials

In this study, mature fruits (around 205-days-old) of citrus cultivars including Fortune (*C. tangerina* × *C. clementina*), Orlando tangelo, Mineola tangelo (C. paradisi × C. *reticulata*), Page (Minneola × *C. clementina*), Clementine (*C. clementina)*, Sour orange (*C. aurantium*) and Sweet orange (Thomson, *C. sinensis*) were used. These cultivars were provided form Laboratory of horticulture, Isfahan University of Technology and Iranian Citrus Research Institute.

Fungal isolates and inoculum production

A pathogenic single-spore isolate of *A. alternata* isolated from infected Clementine fruit was used for inoculation. This isolate was identified morphologically and cultivated on PDA medium at 25 ºC to serve as inoculum. The pathogenicity of selected isolate was determined by spray inoculating detached leaves from greenhouse-grown Page, Mineola tangelo and Clementine trees. Detached leaves were placed in 2 ml microcentrifuge tube filled with water by inserting petioles into water and sealing with parafilm. Conidia (1×10^5 /ml) were sprayed and leaves were placed into moist chamber at 25°C for 4 days.

Fruit inoculation and measurement of growth

To study the *in vivo* growth of the fungus, fruits were sprayed with 90% ethanol and placed on trays to be inoculated. Twenty similar fruits were used in each of the inoculation assays. Briefly, the mycelium of 15-day old *A. alternata* was deposited on a) intact, unwounded fruit, b) fruit with broken cuticle, using for this a scalpel to make cross cuts measuring 5 mm in length by 0.5 mm deep, c) fruit with the flavedo removed, and d) fruit with flavedo + albedo removed, using a hollow glass tube of 5 mm external diameter to mark the external diameter of the lesion, then eliminating the corresponding tissues (flavedo or flavedo + albedo) by a scalpel. For the inoculation assays, the mycelia of 15-day-old *A. alternata* was removed from the surface of 5 mm diameter of PDA culture medium by scalpel and placed in each case (unwounded or wounded fruits) and these were sealed by adhesive plastic tape. The inoculated fruits were then kept in a growth chamber at 20ºC with 85% relative humidity and examined at different times post-inoculation to measure fungal growth as diameter of the lesion in millimeter.

Statistical analysis

Statistical analyses of the data were performed with SPSS statistical software (SPSS for Windows v.11.5).

RESULTS

In pathogenicity test, *A. alternata* was significantly pathogenic on Page and Mineola tangelo leaves but not shown disease symptoms on Clementine leaves. Therefore, it seems that, this isolate was accidentally on the damaged Clementine fruit. In pathogenicity test, *A. alternata* was significantly pathogenic on Page and Mineola tangelo leaves but not shown disease symptoms on Clementine leaves. Therefore, it seems that, this isolate was accidentally on the damaged Clementine fruit. The results obtained following artificial inoculation of the citrus fruits with *A. alternata* showed that the degree of fungal development depended on the barriers that existed in the respective peels. After ten days in Fortune, for example, the fungus had developed even if no lesion had been made in the peel (6 mm diameter), the extent of growth depending on the number of tissues removed: 9.5, 13.7 and 16.5 mm diameter, respectively The cuticle had been broken, or the flavedo or flavedo + albedo had been removed, representing increases of 58.3, 128 and 175% over the growth observed in unwounded fruit, respectively (Fig. 1, A).

Similarly, these results were observed in Mineola tangelo, Orlando tangelo, Page and Sweet orange (Thomson). In Thomson the respective diameter for “no wounding” is 7 mm and for the three ‘‘wounding’’ types were 14, 17 and 19.8 mm (Fig. 1, B). The results obtained for Sour orange point to its lower degree of susceptibility to *A.alternata*. since in this case no fungal growth occurred in unwounded fruit, while fungus diameters of 1.5, 4.6 and 6 mm were obtained for the respective ‘‘wounding’’ types (Fig. 2, A). The results of Mineola tangelo, Orlando tangelo and Page were not published. The results revealed that in tolerant species (mature fruits of Clementine) and resistant species (Sour orange) no fungal growth occurred in unwounded fruit but the brown spot developed. Although very slightly in Sour orange, when the cuticle had been broken, or the flavedo or flavedo + albedo had been removed (Fig. 2, B), the symptoms were observed. This result was similar to those observed by other researches (Kohmoto *et al.*, 1991; Dalkilic *et al.*, 2005).

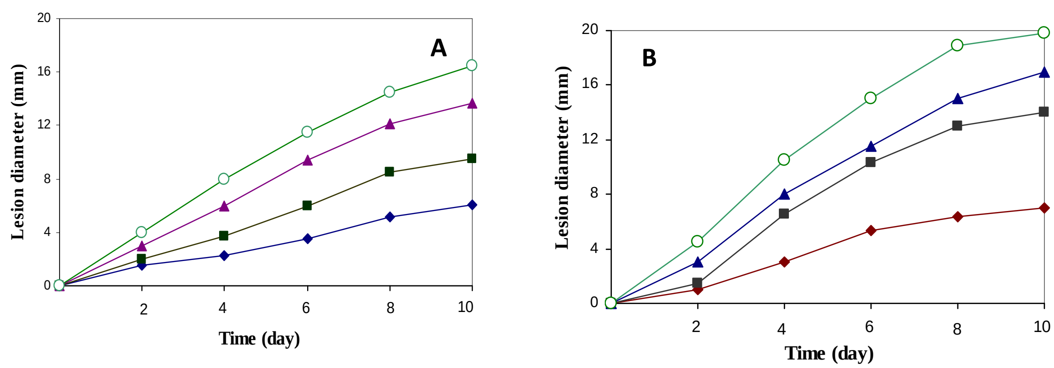

Figure 1. Lesion development during infection of mature fruits of Fortune (A) and Thomson (B) by *Alternaria alternata*. Artificial inoculation of fruit: without wounding (◆); with the cuticle broken (■); with flavedo removed (▲); with flavedo + albedo removed (○). Data represent mean values of lesion diameter (mm) at different days post-inoculation

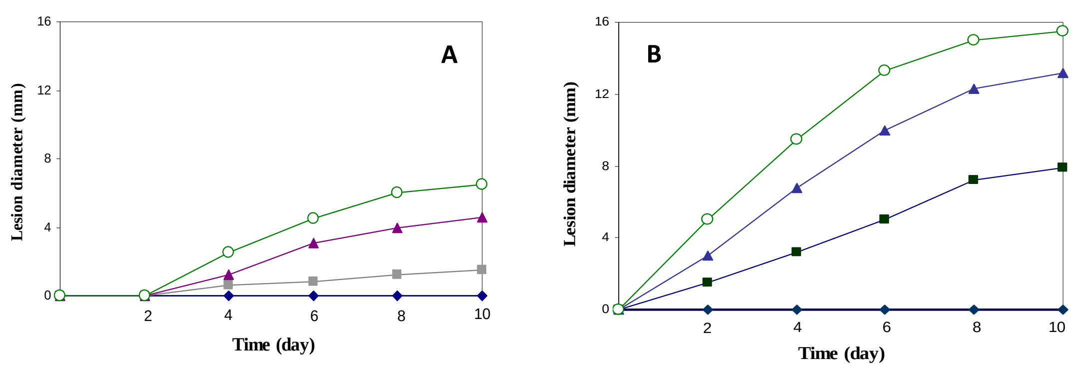

Figure 2. Lesion development during infection of mature fruits of Sour orange (A) and Clementine (B) by *Alternaria alternata*. Artificial inoculation of fruit: without wounding (◆); with the cuticle broken (■); with flavedo removed (▲); with flavedo + albedo removed (○). Data represent mean values of lesion diameter (mm) at different days post-inoculatio

DISCUSSION

Alternaria brown spot pathogen of *A. alternata* produce Host-selective toxin ACT-toxin I and the HST has the same selectivity for plants as do pathogens that produce them (Kohmoto *et al.*, 1991). ACT-toxin has high specificity and toxicity and induces cell death at 10^{-9} to 10^{-8} M when applied to susceptible plants. The selective toxicity of HSTs is correlated with the pathogenicity reactions of the fungi that produce them and HSTs are demonstrated pathogenicity factors (Masunaka *et al.*, 2005). Kohmoto *et al.*, (1991) proposed that many citrus cultivars are very sensitive to toxins but their susceptibility to the pathogen is varied. In this research, *A. alternata* could not growth on unwounded fruits of Clementine and Sour orange but symptoms of brown spot were shown. One possible explanation of these results could be that these cultivars are resistant or slightly susceptible to pathogen but they are highly sensitive to HST and symptoms may were caused by HST or by other factor such as endopolygalacturonase. Others cultivars (Mineola tangelo, Orlando tangelo, Page, Sweet orange and Fortune) are susceptible to the pathogen and highly sensitive to its toxin (Kohmoto *et al.*, 1991).

Other explanations of them are biochemical and physical barriers in citric peels. The existence of a biochemical barrier at flavedo level in resistant fruits and that this barrier might be weakening as soon as the cuticle damaged. It is well known that substantial differences exist in citrics as regards the composition of the secondary metabolites they contain, especially those of a terpenic or phenolic nature and flavanoides (Albach *et al.*, 1969; Ortuno *et al.*, 2008).

Furthermore, the presence or concentration of these compounds differs in different peel tissues (Cheng *et al.*, 1985). A study of the distribution of the flavanoids was carried out on the flavedo, albedo and pulp of mature citric fruits. The results pointed an irregular distribution of flavanones and flavones in fruits. Thus, the flavanones (hesperidin and eriocitrin) are found in higher concentrations in the albedo followed by the flavedo and pulp. For all this and taking into account that some of these have been proposed as possible phytoalexins (Arcas *et al.*, 2000; Del Rio and Ortuno 2003; Del Rio *et al.*, 2004; Ortuno *et al.*, 2006).

It would seem to be of interest to establish whether the presence or absence of one or more of these secondary compounds, as well as concentration of them, might affect the tolerance or susceptibility of the species/hybrids under study.

The plant cell wall is a potential barrier to the penetration and spread of phytopathogenic bacteria and fungi, and many plant pathogens make extracellular enzymes such as Polygalacturonase (PGs) that can degrade cell wall polymers. Polygalacturonase inhibiting proteins (PGIPs) inhibit fungal polygalacturinases and release pectic oligomers, which are able to elicit plant defense responses (Lin *et al.*, 2011). PGIPs have been detected in the cell walls of vegetative and fruit tissues.

Constitutive expression of PGIPs was detected in fruits but not leaves, stem or roots of rough lemon. In addition to, when leaves were wounded or were infected by *Alternaria* sp., the expression of PGIPs was induced (Akimitsu *et al.*, 2003).

In fact, PGIPs are resistance factor in citrus fruits against *A. alternata* and resistant levels of citrus are directly related to amount of PGIPs gene expression. The result this research revealed that Clementine and Sour orange fruits are resistant and pathogen could not growth on unwounded fruits, as well as Clementine's leaves are slightly susceptible and show disease symptoms in pathogenicity test.

One possible explanation of these results could be that PGIPs are produce in fruits and not in leaves (Isshiki *et al.*, 2001).

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