Purification of Host-Specific Toxin from Iranian Isolates of *Alternaria alternata*, Causal Agent of Brown Spot Disease of Tangerine

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ABSTRACT

Brown spot disease caused by *Alternaria alternata* (Fr.: Fr.) Keissl is a serious problem for production of tangerines and tangerine hybrids in Iran. The Tangerine pathotype causes brown spot disease on young leaves and immature fruits of limited varieties of mandarins and tangerines (*Citrus. reticulata* Blanco). Specificity in the interaction between tangerine and the pathogen is determined by a host-specific toxin (HST), called ACT-toxin. Culture filtrates of isolates of *Alternaria alternata* were selectively toxic to tangerine leaves; related toxic compounds were isolated from culture filtrates of *Alternaria alternata*. The biological characteristics of ACT-toxin matched the criteria for HST. ACT-toxin was purified by chromatography analysis, and H-NMR data showed the presence of ACT-toxin related structures. These results suggest that collected isolates produce ACT-toxin which is toxic to specific host plants (tangerines). This host specific toxin rapidly affects plasma membrane integrity of susceptible genotypes and plays a critical role in the infection process, pathogenicity and host specificity of the pathogen.

Keywords: Brown spot disease; Tangerine; Host-Specific toxin; ACT-toxin; Purification; Pathogenicity.

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INTRODUCTION

**Alternaria** Nees is a cosmopolitan fungal genus that includes saprophytic, endophytic and pathogenic species. Plant pathogenic species of *Alternaria* infect a number of economically important plants such as tangerine (*Citrus reticulata* Blanco), apple (*Malus domestica* Borkh.), pear (*Pyrus pyrifolia* Nakai), and tomato (*Lycopersicon esculentum* Mill.). Different species and pathotypes of the pathogen cause four diseases on citrus including *Alternaria* black rot of many citrus cultivars, *Alternaria* brown spot of rough lemon (*Citrus jambhiri* Lush), Manch foliar of Mexican lime (*C. aurantifolia* Swingle) and brown spot of tangerines (Peever et al., 2004). *Alternaria* brown spot was first described on Emperor Mandarin in Australia in 1903 (Pegg, 1996). It appeared in Florida in 1974 and subsequently was identified in South Africa, Colombia, Cuba, Brazil and Argentina (Whiteside, 1976). In Mediterranean basin, the disease was detected in Israel in 1989, in Turkey in 1995, in Spain in 1998, and in Italy in 2000. In Iran, the disease affects tangerines, grapefruit (*C. paradisi* Macf) and their hybrids resulting in lesion on leaves on, and abscission of immature leaves and fruits (Reis et al., 2007). The presence of this disease in Iran has become a serious problem to tangerines production and was reported in 2001 (Golmohamadi et al., 2005). Tangerine and pathotype rough lemon are two distinct pathotypes of *A. alternata* and are known as agents of brown spot disease on citrus fruits and leaves. These pathogens previously were included in the species *Alternaria citri*, that clearly they have different host (Kohmoto et al., 1979).

Tangerine pathotype is pathogenic to Dancy tangerine (*Citrus reticulata* Blanco) and Emperor Mandarin (*C. reticulata* × *C. paradisi*), but is not pathogenic to rough lemon (*C. jambhiri* Lush) and other citrus plants. In contrast, the rough lemon pathotype is pathogenic to rough lemon but not to Dancy tangerine and Emperor Mandarin. Both of these pathotypes are morphologically similar and produce a chemically distinct host-specific toxin (HSTs). Specificity in the interaction between rough lemon and the rough lemon pathotype is determined by ACR- toxin. The primary action site for ACR- toxin is mitochondria of susceptible rough lemon. The toxin causes uncoupling of oxidative phosphorylation and changes in membrane potential. The major form of HST produced by tangerine pathotype is known as ACT- toxin. The effects of ACT- toxin appear to be more complex; ACT- toxin induces rapid increases in electrolyte loss from susceptible tissues and invagination of the plasma membranes (Kohmoto et al., 1992).

The objectives of this research are to determine if the Iranian isolates of *A. alternata* collected from tangerine orchards produce host-specific toxins. Purification and characterization of toxins probably produced in culture filtrates of the fungus.

MATERIAL AND METHODS

**Plants**

Young leaves of five citrus species included Page tangerine, Minneola tangelo, Thomson navel, Sour orange and Mexican lime, were used in the experiments. Plants were maintained in a greenhouse and young leaves (about 50% of leaf development) were used for experiments (Kohmoto et al., 1992).

**Pathogen**

40 isolates of *Alternaria alternata* were isolated from Minneola tangelo and Page tangerine orchards and were used for inoculations. The isolates were purified and single- spored prior to use and stored in water agar (WA) slants at 4°C.

**Preparation of spores**

Isolates were grown on potato dextrose agar (PDA) medium at 25°C in darkness for 8- 14 days. Conidial suspensions were prepared by washing the mycelia with sterilized distilled water. The suspension was filtered through two layer of filter paper and the conidial concentration was determined by a hemocytometer slide and adjusted by dilution. Suspensions with less than 90% germination of conidia were discarded. For long time storage the suspensions were filtered, air
dried on the filter paper at room temperature and preserved at -20° C in the dark until use (Pegg, 1996)

**Pathogenicity test**

10 young leaves from each cultivar were scratched at the center of the lower surface with a needle. The leaves were turned lower surface up and drops (50 μl) of spore suspensions (5× 10^5 spores / ml) of *A. alternata* were placed on the wounded site of each leaf. The leaves were incubated in a moist chamber in the dark for 48h at 25° C and the necrotic area on each leaf was measured by image analyzer and the severity of the infection in each sample was compared with the other samples. Controls were inoculated with sterile- distilled water (Akimitsu *et al*., 1989).

**Extraction of toxin from culture filtrate**

40 isolates of *A. alternata* were isolated from Minneola tangelo and Page tangerine and cultures were maintained on PDA slants in test tubes. Small pieces of the mycelial mats from cultures were transferred to 500 mL incubation bottles, each containing 200 mL of a modified Richards' medium (Kohmoto *et al*., 1992). After the stationary cultures were grown for 24 days at 25° C, they were harvested by filtration through four layers of gauze and a filter paper (Munkell, Germany) to eliminate mycelia. The culture filtrate (200 ml) was adjusted to pH 5.5 with 10% NaH_2PO_4 and was stirred with 50 ml of Amberlite XAD-2 resin (Merck, Germany) for 2 h to adsorb toxins. The Amberlite XAD-2 resin was packed in a column and eluted with 200 ml methanol. The eluate was evaporated under reduced pressure at 40° C till all methanol was gone. The residual concentrate was extracted five times with ethyl acetate and the extract was dehydrated with anhydrous sodium sulfate and evaporated under reduced pressure at 40° C. The final residue was dissolved in methanol and subjected to thin- layer chromatography (TLC) using Kieselgel 60 F- 254 (E. Merck, Darmstadt, Germany) plates (0.5 mm). After development with hexan/ ethyl acetate/ methanol (40/ 40/ 20, v/v), two activate zone were detected; one of them which was highly toxic to Page tangerine, Minneola tangelo and caused electrolyte loss from leaf disks, but not to Sour orange and Mexican lime ( Rf 0-0.11) was scraped from the plate and eluted with ethyl acetate. The eluted ethyl acetate was subjected directly to a high-performance liquid chromatography (HPLC) on a C18 column using acetonitrile, deionized water and acetic acid (60: 40: 1 v/v in 40 min) as a mobile phase at a flow rate of 1 ml/min. the absorbance of the effluent was monitored at 290 nm. One major peak fraction was detected; evaporated and dissolved in ethyl acetate.

**Leaf necrosis assay for ACT- toxin**

The biological activity and host specificity of ACT- toxin in sample solutions were determined by a leaf necrosis assay using host and non-host plant leaves. The midribe of young leaves (midrib length 2 to 4 cm) were removed, and the lower surface of the leaf lamina was scratched near the center with a needle. A drop (50 μl) was placed on each wounded site. Ethyl acetate of HPLC yield was removed and the concentrate was tested at a concentration of 1 μg/ml. The treated leaves were incubated in a moist chamber for 48 h at 25° C in dark. After incubation; the necrosis appearing around wounded site was recorded (Kohmoto *et al*., 1991). The assay was adapted for use with the solutions from TLC and HPLC.

**Measurement of electrolyte loss**

Twenty leaf disks, 0.7 cm in diameter, were cut from leaves by a leaf punch. The disks were vacuum-infiltrated with toxin solution and serial dilutions of the culture filtrate, or deionized water (as control) for 45 min. Disks were rinsed with deionized water, were placed in flasks, each containing 20 ml of deionized water and were incubated on shaker (120 strokes per minute) at 25° C. Conductance of ambient solutions were measured at intervals (every 60 minutes) with a conductivity meter (GLP 32, CRISON, Germany) (Kohmoto *et al*., 1992).
Spectral analysis
1H-NMR spectra were obtained with a FT-NMR 300 (Bruker; Germany) spectrometer and UV spectra were recorded (Kohmoto et al., 1992).

RESULTS AND DISCUSSION
Pathogenicity and toxin production
Five citrus types were examined for susceptibility to tangerine pathotype of *A. alternata* and for sensitivity to ACT-toxin. Isolates samples from Page tangerine and Minneola tangelo were pathogenic only on their respective hosts, which is consistent with previous reports (Kohmoto et al., 1991). Only two kinds of citrus, Page tangerine and Minneola tangelo were susceptible to tangerine pathotype. The other plants examined (Thamson navel, Sour orange and Mexican lime) were resistant to both the toxin and the pathogen. These results were repeated by purified HST (ACT-toxin) at a concentration of 1 μg/ml.

Isolation and purification of ACT-toxin
After TLC analysis one activate zone was obtained which was highly toxic to Page tangerine and Minneola tangelo, but did not cause necrosis on resistant genotypes. Based on TLC analysis, and biological activity, this group was judged to contain ACT-toxin. This group was purified further with HPLC and one major peak (Rt 22.5- 28.5 min) and several minor peaks were detected (figure, 1), this fraction was applied to biological tests and a selective toxic compound to Page tangerine and Minneola tangelo was obtained.

The presence of ACT-toxin related structures and HPLC purification quality were shown by 1H-NMR analysis.

![Fig.1: High-performance liquid chromatography (HPLC) of eluted ethyl acetate from TLC activate zone, using a C18 column using acetonitrile, deionized water and acetic acid (60: 40: 1 v/v in 40 min) as a mobile phase at a flow rate of 1 ml/min. the absorbance of the effluent was monitored at 290 nm.](www.SID.ir)
Toxicity of ACT-toxin to citrus
The isolated ACT-toxin and caused a rapid increase electrolyte loss from leaf disks (detected within 60 min after toxin exposure) in susceptible citrus at concentrations of more than 1 μg/ml, respectively.

The leaf necrosis assay showed that ACT-toxin induced veinal necrosis around the point of application on Page tangerine and Minneola tangelo leaves at a concentration of 1 μg/ml. ACT-toxin gave no reaction on leaves of resistant plants (Sour orange and Mexican lime) at 2 and 4 μg/ml concentrations (figure 2).

CONCLUSION

*Alternaria* brown spot is a major fungal disease of Tangerine hybrids all through the world. Pathogenesis is thought to be due to the ability of the fungus to produce the host-selective ACT-toxin (Kohmoto *et al.*, 1979). Toxin at a concentration of $2 \times 10^{-8}$ M causes veinal necrosis on leaves, with a rapid loss of electrolytes from host cells. ACT-toxin and its derivatives are the only major products detected in fluids purified from germinating conidia (Kohmoto *et al.*, 1992).

In the present research, 40 Iranian isolates of Tangerine pathotype of *A. alternata* were studied. ACT-toxin was obtained from culture filtrates of virulent isolates.

Previous ultrastructural and physiological studies indicated that the initial site of action by ACT-toxin is on plasma-membrane (Kohmoto *et al.*, 1992).

In this study, five common citrus cultivars were examined for ACT-toxin sensitivity. Inoculation tests with Iranian isolates of Tangerine pathotype of *A. alternata* showed variation in susceptibility among the citrus cultivars. ACT-toxin was highly toxic to young leaves of susceptible Page tangerine and Minneola tangelo. The apparent discrepancy may be related to the fact that ACT-toxin is not very stable (Kohmoto *et al.*, 1979).

Resistant citrus tolerates 4 μg/ml ACT-toxin. The data indicated that electrolyte leakage might be used as the basis of a quantitative bioassay, provided conditions are carefully standardized and the effect of ACT-toxin on loss of electrolyte from leaf tissues paralleled development of necrosis in susceptible leaf tissues. Toxin sensitivity correlates in all cases with susceptibility to the pathogen (Kohmoto *et al.*, 1991). These results and modes of action by ACT-toxin are compatible with the hypothesis that plasma-membrane disorders caused by host-specific toxins are a key and central event in pathogenesis, allowing the producing fungus access to host cells (Kohmoto *et al.*, 1992). The 1H-NMR spectrum of toxin showed that the structure of ACT-toxin produced by Iranian isolates was closely related to structures which had described previously (Kohmoto *et al.*, 1992). These studies were done for the first time in Iran and the results showed that pathogenicity
of *A. alternata* is related to the amount of toxin for each of the isolates and it is needed to check the correlation between the two factors. This method will hopefully help us to select more resistant Tangerine hybrids in Iranian citrus breeding programs.

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**REFERENCES**


