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DETECTION AND IDENTIFICATION OF ALTERNARIA SPECIES CAUSING DISEASES OF CARROT IN ANKARA PROVINCE, TURKEY

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Abstract

Carrot (Daucus carota var. sativus) is widely planted in the Ankara provinces. In order to identify species of Alternaria causing disease on root and foliage, surveys of carrot production areas in Ayaş and Beypazarı districts of Ankara province between June and November in 2008–2009 were undertaken. Sixty isolates of Alternaria spp. have been obtained from necrotic lesions on the leaves and roots. Alternaria radicina, A. alternata, A.tenuissima and A.dauci were isolated from symptomatic plants collected in our survey and the pathogenicity of fungi have been tested. Species identification was done based on culture and conidial morphology, growth rate and rDNA sequences. Pathogenicity test such as hypocotly test, carrot disc method, 6–8 weekly seedling andplant test were conducted Isolates of A.radicina, A.dauci were shown high virulence although Alternaria alternata were found as moderately or low virulence.

Key words*: Carrot, root rot, Alternaria blight, Alternaria spp., Turkey.*

INTRODUCTION

Carrot (*Daucus carota* ***var.*** *sativus* Röhl.) is one of the most popular and commonly consumed vegetables (Rubatsky, 2002). Commercial carrot production is an economically important industry worldwide. In the Turkey, the most productive land is in the Central Anatolia Region and Eastern Mediterranean Region. Carrot is widely planted in the Anatolia region that includes Ankara producing nearly 60% of Turkey's carrots. In 2013, carrot cultivated area is about 104,404 da and annual production is 557,977 tons in Turkey (Anonymous, 2014). Root and foliar diseases are among the most important factors limiting carrot production worldwide.

Fungi are the most common pathogens of *D. carota*. Species of the genus *Alternaria* such as *Alternaria carotiincultae* E. G. Simmons, *Alternaria dauci* (J. G. Kuhn) J. W. Groves & Skolko, *Alternaria. petroselini* (Neerg.) E. G. Simmons, and *Alternaria radicina* Meir, Drechsler &E. D. Eddy, have been reported on *D. carota* for several countries (Farrar et al., 2004).

Alternaria Nees is an widely spread mould genus which can be found on plants, in soil, food and indoor air. Most frequent species are *A. alternata* and *A. tenuissima* (Lõiveke et al., 2004). The pathogenic species *Alternaria radicina* and *Alternaria dauci* are isolated from diseased carrot plants in all growing stages (Stranberg, 2002). Alternaria leaf blight caused by *A. dauci* and Alternaria black rot caused by *A. radicina* are widespread on carrot crops in the world where are reported to cause considerable damage (Davis and Raid, 2002).

Black rot (*Alternaria radicina*) is found in all the main carrot-production areas. Although this disease is important as a storage disease of carrots, it also causes seedling damping-off, foliar and crown infection ((Koike et al., 2009).

Alternaria leaf blight (*Alternaria dauci*) is one of the most important foliar diseases of carrot and occurs worldwide. Severe epidemics reduce carrot root size and yields (Koike et al., 2009). In Turkey, *A.dauci* was first described as leaf blight caused on carrot in the Hatay province of Turkey (Soylu et al., 2005).

The objective of the present study was to determine the *Alternaria* species causing

diseases in carrot growing areas in Ankara province, Turkey.

MATERIALS AND METHODS

Survey and fungal isolation

In order to identify species of *Alternaria* causing diseases on carrot root and foliage, surveys were carried out in production areas in Ankara province between June and November in 2008-2009 growing seasons (Figure 1). Samples were taken from fields in Ayaş and Beypazarı districts of Ankara.

Infected carrot leaves and root pieces were surface-sterilized (1,0% (w/v) sodium hypochlorite) for 2-3 min then rinsed in sterile water three times before they were placed onto potato dextrose agar (PDA, Merck) containing streptomycin and incubated at 23±1°C with a 12-h photoperiod for 7-10 days. Single spore isolates were stored on PDA slant tubes at 4°C.

Figure 1. Survey area for Alternaria diseases of carrot in Turkey

Identification of fungus

For identification, culture morphology, growth rate and conidial morphology were observed from 12-15 day-old cultures grown on PDA and PCA (Ellis 1970, 1971; Rotem 1994; Simmons 1995). The shape, length and width of 50 conidia for each isolate were determined and mean length and width were calculated. In addition, the number of transepta per conidium and the production of conidia in catenate arrangement was determined.

Pathogenicity tests

The pathogenicity of isolated fungi from diseased plants was assessed. Carrot disc pathogenicity test (modified from Pryor et al., 1994), the 6-8 week old seedling test (Coles and Wicks 2003) and plant test (Pryor and Gilbertson 2002) were used.

Carrot disc pathogenicity tests

Mature "Maestro" carrots were assessed for pathogenicity of *Alternaria* spp. Mature carrot roots were washed in tap water and sliced into disks approximately 5 mm thick. The disks were surface-disinfested by soaking in 0,1% sodium hypochlorite for 5 min. then triple rinsed with water and placed on a paper towel for 1hr to dry. The four carrot discs were then placed in each petri dishes (20 x 100 mm) containing two Whatman No. 1 filter papers moistened with 2 ml of streptomycin sulphate solution (100mg/l) Twenty discs were used for each isolate. Carrot discs were inoculated with mycelial plugs (4 mm diameter) cut from the margins of actively growing culture (Figure 2). Controls were treated similarly using similar sized pieces of water agar. The dishes containing inoculated disks were incubated on wire racks in clear plastic trays for 10 days at 24 ± 2 ° C, 12 h light with 12-hour dark cycle. After 10 days, pathogenicity was evaluated on a scale of 0 to 4 (Coles and Wicks, 2003). A total of 60 isolates of Alternaria. was tested, and each test was replicated four times.

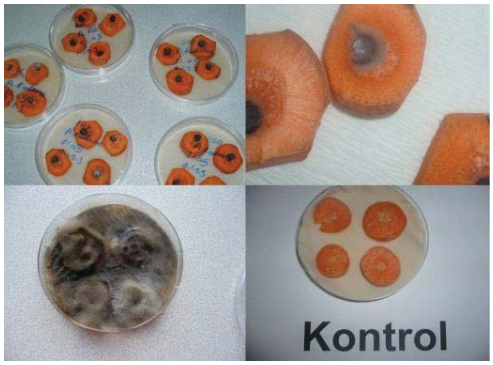

Figure 2. Carrot disc pathogenicity tests

Six-eight week old seedling test

The second method used fresh 6 and 8-week old carrot seedlings of the commercial cultivar “Nantes”. Seedlings were placed on surface sterilised aluminium foil sheets in prewashed plastic trays with pre-moistened absorbent paper. Five seedlings per treatment were inoculated by taking 1.0 x 0.5 cm water agar pieces from mature colonies of *A. radicina* and placing the mycelial surface down on to the hypocotyl region near the crown of each seedling (Figure 3). Controls were treated similarly using similar sized pieces of water

agar. The trays were enclosed in a clear plastic bag and incubated on the laboratory bench at room temperature for 10 days. The level of disease was assessed by measuring the extent of necrosis from the point of inoculum. Fungi causing blackening, or soft decomposition of the hypocotyl region, or death of the upper stem and petioles were classed as pathogenic. After 10 days, fungal growth and pathogenicity were evaluated on each plant based on 0-4 scale (Coles and Wicks 2003). Each treatment was replicated three times.

Figure 3. Six-eight week old seedling test

Plant test

Eight seeds from Nantes variety were sown in 10.5 cm diameter plastic pots containing a sterilized mixture of carrot field soil, peat and sand (1:1:1,v/v/v). Pots were maintained under optimum greenhouse conditions at temperatures ranging from 23–26 °C, and 35–40% humidity.

Conidial suspensions were prepared in sterile distilled water using 14-day-old cultures. Spore suspensions were adjusted to $2x10^3$ conidia/mL for *A. radicina* (Pryor and Gilbertson, 2002) and other *Alternaria* spp. $1x10^3$ (Pryor et al., 2002) and sprayed onto areial parts of each test plant, until run-off, with an pressure hand sprayer. Controls were sprayed with sterile distilled water. Four replicates were used for each isolates.

Two weeks after inoculation, pathogenicity were evaluated on 0 to 5 scale (Pryor and Gilbertson, 2002).

Disease assessment

Isolates of *Alternaria* spp. were assessed for their pathogenicity on carrot disc and 6-8 week old seedling test using 0 to 4 scale from Coles and Wicks (2003): 0= no discoloration, 1=slight discoloration, 2= slight discoloration with mycelial growth, 3= grey to black necrosis with the production of conidia, 4 = grey to black necrosis with abundant production of conidia.

Isolates of *A. radicina* and *A. dauci* were assessed for their pathogenicity on plant test using a 0 to 5 scale from Pryor and Gilbertson (2002):

0 = no disease, 1= 1% leaf necrosis, 2= 5% leaf necrosis, 3= 10% leaf necrosis, 4= 20% leaf necrosis, 5= more than 40% leaf necrosis

These scale values were converted to disease severity values (Xi et al., 1990) using the following formula:

$$\text{Disease sev.} = \frac{\Sigma(\text{no. of plant in category x category value}) \times 100}{\text{max. category value x total no.of plants}}$$

The isolates were classified according to disease severity values such as highly virulent (75-100%), moderately virulent (50-74,9 %) and weakly virulent 0-49,9%).

The data were subjected to ANOVA, and the means were separated by the least significant difference (LSD) test.

Molecular analysis

Approximately, 300 mg mycelium were harvested and ground with liquid nitrogen in a sterile mortar for DNA extraction from culture medium. Genomic DNA was extracted using a Qiagen DNeasy ®Plant Mini Kit, as specified by the manufacturer, and stored at 20 °C prior to use. PCR reaction mixtures and condition were modified from previous studies (Aroca and Raposo 2007; Cobos and Martin, 2008). The reaction mixtures of PCR, a final volume of 50 µl, contained 5µl of 10X buffer [75 mM Tris HCl, pH 9.0, 50 mM KCl, 20 mM (NH4)2SO4], 2 µl of 5 µM each primers, 5 µl of 1.5mM MgCl2, 2 µl of 10 mM deoxynucleoside triphosphates (dNTPs), 1 U Taq polymerase (Fermatas), 5 µl of DNA template for each reaction and 5 µl of bovine serum albumin (BSA: 10 mg/ml).

DNA amplifications were carried out in a Techne TC-5000 thermal cycler by the following program: 94 C for 2 min, followed by 34 cycles of (1) denaturation (94□C for 30 s), (2) annealing (60 □C for 30 s) and (3) extention (72 □C for 30 s), and a final extension step 10 min at 72 □C. The ITS region of the isolates was amplified using the universal primers ITS-1 (5’ TCC GTA GGTGAA CCT GCGG 3’) and ITS -4 (5’TCC TCC GCT TAT TGA TATGC3’). The PCR products were separated in 1.5 % agarose gels stained with ethidium bromide, and visualized

under UV light. They were sequenced by REFGEN (Gene Research and Biotechnology Company, Ankara, Turkey).

RESULTS AND DISCUSSIONS

Identification of Alternaria isolates and their pathogenicity

A total of 2,297 da carrot growing areas were surveyed in Ayaş and Beypazarı districts of Ankara province in 2008–2009. Sixty isolates of *Alternaria* were obtained from infected carrot root and foliage. *Alternaria radicina, A. alternata A. tenuissima, A. dauci,* were isolated from diseased plants collected in the survey. Of the identified isolates, 22,42% were *A. radicina*, 56,14% were *A. alternata*, 7,14 % were *A. tenuissima* and 14,28 % were *A. dauci*.
Our survey showed that *A. radicina* was associated with root and leaf of carrot and was widespread in carrot plantings in Ankara. The fungus was encountered most frequent from carrot rot and crown in summer and autumn. In our study *A. alternata* was obtained from dissected diseased tissue. *A. alternata* is one of the most common saprotrophs or facultative parasites associated with various parts of plants (Scheffer, 1992). Up to 68% of carrot root samples collected in several European countries were found to be contaminated with the fungus (Solfrizzo et al., 2005). As much as 70% of mature carrots can be rendered unmarketable if heavily infested or infected by *A. radicina* and *A. alternata* (Solfrizzo et al., 2005).
Fungal identification was confirmed by DNA sequencing.

Alternaria radicina

Alternaria radicina was isolated from roots and crown of young and mature carrots. Symptoms of the disease as the black rot was observed by dry, black, decay, sunken lesions on carrot roots. Lesions were quickly expand, and decay the entire root (Figure 4). Symptoms seen on the roots and crown of carrot seedlings were observed initially as small chlorotic spots and these spots were joined together by expanding. Lesioned tissues were significantly separated from the healthy tissue.

Figure 4. Black rot symptoms on the root and crown of carrot

During the survey it was observed that maturing carrots were often damaged around root regions. Our results showed that this was the effect of *Alternaria radicina* infection.
The colony color was dark green–blackish on PDA in 10-14 days. We shown that conidia were borne singly, or occasionally in chains of two, and were typically dark olive-brown to natal brown, broadly ellipsoid to ovoid, 12–17x19–37 µm, with one to four transepta and one to two longisepta in any or all segments, except basal and apical segments, which usually are free of septa (Figure 5).
Morphological features of our tested isolates on PDA were similar with descriptions of Ellis (1970, 1971), Rotem (1994) and Simmons (1995).

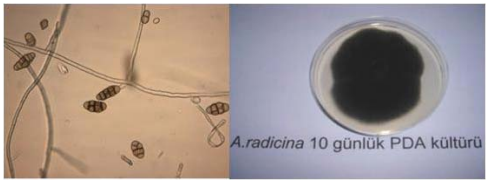

Figure 5. Morphology of conidia of *A. radicina* (x40)*:* colony appearance of *A.radicina* on PDA

The resulting sequences were compared to other *A.radicina* sequences in the GenBank and were 99 and 100 % identical.
Isolates of *A.radicina* were tested using all three pathogenicity tests. All isolates showed a large variation in virulence. In the results of carrot disc pathogenicity tests, disease severity values of isolates were found between 52,1 to 75,0 %. Disease values were determined as 41,7-85,0% and 45,0-98,6% in seedling and plant tests, respectively. *A.radicina* caused the

high and moderate disease ratio on carrot disc, seedling and plant in pathogenicity tests.

Alternaria alternata

Alternaria alternata was usually the fungus most frequently isolated from symptomatic root rot.

Colonies were usually black or olivaceous black on PDA. Conidiophores arising singly or in small groups, simple or branched, straight or flexuous, pale to mid olvaceous or brown, one or several conidial scars. Conidia were formed in long chains, obclavate, obpyriform, ovoid or elipsoidal, with up to 3-5 transverse and several longitudinal septa, overall length 9-11x20-32 µm and 5-16 chains (Figure 6).

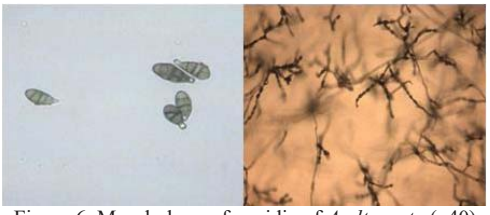

Figure 6. Morphology of conidia of *A.alternata* (x40) and chain structure (x20)

The resulting sequences were compared to other *A.alternaria* sequences and were 98-99% identical to other *A.alternata* sequences in the GenBank.

As a result of the pathogenicity test, we have found differences in virulence of tested isolates of *A. alternata.* Disease severity values of *A. alternata* were between 32,6 to 81,25% in carrot disc pathogenicity method.

Alternaria tenuissima

The fungus was isolated from symptomatic chlorotic leaf spot, discoloration and crown rot.

Colonies usually were pale black or olivaceous black on PDA. Conidiophores solitary or in groups, simple or branched, straight or flexuous, septate, pale brown, with one or several conidial scars.

Conidia formed 3-5 chains, obclavate, obpyriform or elipsoidal, generally with 3–5 transverse and several longitudinal, overall length 8–10x18–20 µm, beak measurment 5–9 µm (Figure 7).

Figure 7. Morphology of conidia (x40) and chain structure of *A. tenuissima* (x 20)

The resulting sequences were compared to other *A. tenuissima* sequences in the GenBank and were 97 and 99% identical.

As a result of the carrot disc pathogenicity test, *A. tenuissima* isolates were found to be weakly pathogenic (31,3-35,4% disease severity) on carrot plants.

Alternaria dauci

During survey, foliage infection by *Alternaria dauci* was observed on carrot plants growing in Ankara. Initial symptoms first appeared on older leaves as irregularly-shaped, minute, dark brown-to-black spots, with yellow borders on the edge of the leaflet blade. As the disease progressed the lesions expanded, causing the leaflets to turn brown and die (Figure 8).

The fungus was consistently isolated from the margins of these lesions.

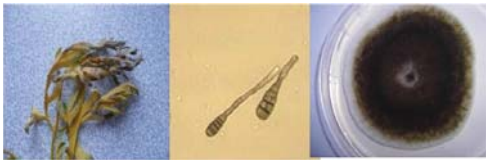

Figure 8. Alternaria leaf blight symptoms, morphology of conidia (x40) and colony appearance of *A.dauci* on PDA

The colony color was pale or dark gren-blackish on PDA in 10-14 days (Figure 8). Conidiophores were medium olivaceous brown, and either simple, with a single terminal conidiogenous site, or 1-2 geniculate and conidiogenous. Conidia were typically borne singly, but occasionall a sturdy terminal secondary conidiophore bearing a secondary spore is produced. Conidia were medium to dark olive-brown, long ellipsoid to obclavate, 10-22x45–70 µm (spore body), with 3 to 7 transepta and 1 to 2 longisepta in fewer than

half of the transverse segments (Figure, 8). Mature conidia are rostrate with a terminal filamentous beak 30–120 µm, conidia occasionally in chains of single or two.
Morphological features of isolates on PDA were similar with descriptions of Ellis (1970, 1971), Rotem (1994) and Simmons (1995).
As a result of the plant pathogenicity test, we have found that *A.dauci* isolates were highly virulent with 89,25 to 92,75 % disease severity values.

CONCLUSIONS

Detection and identification of *Alternaria* species pathogenic in carrot growing areas in Ankara is fundamental to guide the development of appropriate strategies for disease management. *Alternaria radicina, A. alternata A. tenuissima, A. dauci* were identified through classical and molecular methods among the 60 isolates obtained from carrot growing areas in Ankara province.
Isolates of *A. radicina* and *A. dauci* showed high virulence although *A. tenuissima* were found as low virulent. *A. alternata* isolates were determined as moderately virulent. It was found differences among the virulence of isolates of *A. alternata*.
These results will be useful in developing of integrated strategies for disease management and breeding programs to Alternaria leaf blight and black rot disease on carrot.

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