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THE CAUSATIVE OF LEAF SPOT ON CAROB- ISOLATION AND IDENTIFICATION

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ABSTRACT. *The main objective of the present study was to isolation and identification of the cause carob leaves spots. In this study, a total of 4 fungal isolates (referred as A1, A2 and A3) were obtained from symptomatic leaves collected from carob trees distribution in El-Beida city and its suburbs. The pathogen associated with the disease was identified as Alternaria alternata (Fr.) Keissl. All the isolates produced olive-brown to medium-brown conidia with obclavate shape, but the isolates varied in size (length) of conidia, the number of cells per conidium, rate of growth and sporulation on media. Koch´s postulate was verified by inoculating healthy leaves. This was the first report of leaf spot disease of carob caused by Alternaria alternata in Libya.*

KEYWORDS: Carob, leaf spot, *A. alternata*, pathogenicity, symptomatology, Libya.

INTRODUCTION

Carob (*Ceratonia siliqua* L.) is a perennial evergreen plant belonging to Fabaceae – Caesalpinioideae (Orwa et al. 2009). Its abundant in Al-Jabal Al-Akhadar forest (Libya) in Mediterranean region. The plant prefers light (sandy) and medium (loamy) soils, requires well-drained soil and can grow in nutritionally poor soil. The plant prefers acid, neutral and basic (alkaline) soils. and can grow in very alkaline soils. It cannot grow in the shade. It requires dry or moist soil and can tolerate drought, the highest temperatures and strong winds. Information regarding its applications in folk medicine in Arab countries is scanty. However, fruits, pods and leaves of this plant are sold in medicinal plant shops (Attars) all over the Arab counties. It is prescribed in Turkish folk medicine as anti-diarrhoeal and diuretic (Kivak and Mert, 2002). The gum of Carob tree is a galactomannan, a valuable natural food additive for products such as ice cream, sweets and soups. It is also used in the textile and cosmetics industries (Santos et al., 2005). The pods of the carob fruit has long been used as a feed for livestock and in human nutrition, including sweets, biscuits and processed drinks, because of its high sugar content and low price (Khair et al., 2001). The crude extract of pods was used as antimicrobial agents against some pathogens and spoilage bacteria (Hsouna, et al. 2012).

The genus *Alternaria* was first described in 1816 with *A. tenuis* as the type isolate (Nees, 1817). The genus is distributed worldwide as saprophytes, endophytes, plant pathogens and as emerging human pathogens, especially in immune compromised patients (Peever et al., 2004; Thomma, 2003).

Leaf spot is one of the common diseases in the agricultural production. Many studies showed that leaf spot is caused by fungus pathogens such as *Alternaria*, *Ceratocystis*, *Guignardia*, *Phoma*, etc. And among those pathogens, *Alternaria* account for the most. For instance, leaf spot on *Atractylodes* is caused by *A. tenuissima* (Wang et al., 2007), and *A. brassicae* caused leaf spot on Chinese cabbage (Michereff et al., 2012). Diseases caused by *Alternaria* species are common and these diseases occur worldwide. Both plant pathogenic and saprophytic species of *Alternaria* may cause leaf spot and blight on numerous plant taxa including

vegetables, fruit trees and ornamentals (Simmons, 1997; Mirkova and Konstantinova, 2003, El-Gali, 2014). Infection by *Alternaria* species typically causes the formation of necrotic lesions, which sometimes have a target-like appearance surrounded by an un-invaded chlorotic halo (Agarwal et al., 1997). For example, *A. alternata* (Fr.) Keissl. is an opportunistic pathogen on numerous host plants causing leaf spots, rots and blights (Droby et al., 1984; Pryor and Michailides, 2002; Maiti et al., 2007). More than 380 hosts including *Ceratonia siliqua* have been recorded to be infected with *Alternaria* species in the world. (Final rep., 2003; Mmbaga et al., 2011). The first report of *Alternaria* sp causing leaf spot disease of some forest trees reported in Libya (Final rep., 2003). *Alternaria alternata* has been reported as a leaf spot pathogen of Lemon (El-Gali., 2014).

Most species of *Alternaria* have been generally identified and classified based on cultural and conidial morphology (Yu, 2001; Zhang, 2003; Simmons, 2007). *Alternaria* is easily identifi ed by the shape of its conidia, which are large, ovoid to obclavate, dark-colored (melanized), multicellular with longitudinal and transverse septations (phaeodictyospores). Near the base, they are broader becoming taper to the apex, providing a club-like appearance. Conidia are produced in single or branched chains on short conidiophores (Barnett and Hunter, 1998). The main objective of this study was to identify the causal agent of leaf spot on *Ceratonia siliqua*. Morphological examinations and pathogenicity assays were carried out to characterize the fungi isolated from infected *Ceratonia siliqua* leaves and to verify the Koch´s postulate.

MATERIALS AND METHODS

Sampling

Carob trees were examined in the fields of the Al-Jabel Al-Akhder region. The primary site is 882 km above sea level. Maximum and minimum mean temperatures are 30°C and 10°C, respectively. Annual rainfall averages 650 mm and is most abundant during October to March. Digital photographs of leaf symptoms were recorded using a Sony (Cyber-shot) model digital camera. Naturally infected leaves were collected from fields in El-Beida and the surrounding areas. The symptoms different from Deep spots or lesions (Fig. 1-A), Spots surrounded by a yellow halo (Fig. 1-B) and Blotch (Fig. 1-C). Plant materials were placed in labeled plastic bags and transportation until the samples were processed in the laboratory.

Figure (1). Different symptoms on leaves carob. A) lesions, B) Spots with Halo and C) Blotch.

Fungal isolation, purification

Diseased leaves were surface sterilized by soaking in ethanol (75%, v/v) for 40 s, followed by 4 min in hypochlorite (1%, w/v) and subsequently soaking in ethanol (75%, v/v) for 30 s again to remove residual hypochlorite, finally rinsed in sterile distilled water three times and dried with sterile filter paper. Leaves were cut into 0.5 cm2 at the joint section of diseased and healthy tissues and transferred to a plate of malt extract agar (MEA) containing 2% malt extract and 2% agar (w/v) supplemented with streptomycin sulphate at 50 mg/L to prevent bacterial growth. Six pieces of leaf tissues were placed on three plates and incubated at 25°C in the dark. After incubation for 4 days, the fungal hyphae growing from the diseased leaf tissues were cut off from the edge of the colonies and sub-cultured on a plate of potato dextrose agar (PDA) for purification. For storage, the fungal isolates were maintained on PDA slant.

Identification

The fungal isolates were identified based on their colonial morphology. Microscopic examination of fungal isolates was carried at both vegetative and sporulating stages. A clean slide with a drop of lactophenol in cotton blue in its centre was used. A portion of the mycelium at the edge of the colony at the desired stage was picked and dropped in the lactophenol in the cotton blue. This was teased and covered carefully with a cover slip. The slide was mounted on the microscope, focused and observed.

Pathogenicity of the isolates

All isolates were conducted pathogenicity tests, and the tests were performed on detached healthy leaves of *Ceratonia siliqua* removed from the tree. Before inoculation, leaves were surface disinfested by immersion in 10% bleach solution (0.5% sodium hypochlorite) for 2 min, rinsed in SDW, and then air-dried in a laminar flow hood and maintained in Petri dish containing small glass beads and sterile distilled water. For each isolate, three fully expanded leaves were inoculated by placing a PDA plug (0.5 cm2) of the fungal mycelia on upper surfaces of the leaves, and each leaf was slightly wounded on both side with a sterile needle prior to inoculation. Another three leaves treated with sterile PDA plugs served as the uninoculated control. The Petri dishes were covered and placed under light of a 12 h photoperiod at 25°C and 95% relative humidity for 7 days. The pathogenicity test was repeated three times. The severe symptoms were observed on 7 days after inoculation and the disease intensity was recorded. The symptoms were observed and compared with the original symptoms. The fungus was reisolated from artificially inoculated carob leaves and compared with original culture isolate.

Morphological observations

The morphological characteristics of the causal organism were studied on culture in the laboratory *(in-vitro)*. Isolates of fungus were grown for 7 to 10 days on plates of PDA, in an incubator at 25°C. After incubation, cultures were examined for The important characters studied were as follows:

Colony: Colour, shape, margins and pigmentation; Mycelium: Colour, shape, septation, branching; Conidia: Colour, shape, size and septation.

Conidia produced from 7-day-old fungal cultures were mounted on slides and examined for morphological characteristics at magnification of 100× under a compound microscope (Olympus CX41, Tokyo, Japan). The size of conidial spores was based on measurements of 30 conidia. The genus and species of the fungal isolates were identified according to Zhang (2003) and Simmons (1997).

Results and Discussion

Isolation and identification of the pathogen

Leaf symptoms appear as circular to irregular-shaped brown to grey lesions surrounded by a dark brown border. Based on the symptoms the fungus was identified as *Alternaria alternata* (Ellis, 1971).

The process of isolation resulted in four isolates (referred as A1, A2 and A3) of pathogen were obtained from symptomatic leaves of carob. A1 was isolated from deep spots (lesions), A2 from halo and A3 was isolated from blotch. Most *Alternaria* species are divided into different groups based on the size of the conidia. The length and width are good taxonomic markers for the genus and stable within the different media and the generations in most cases.

Morphological observations

Isolates were cultured on PDA medium and incubated under 16 hours photoperiod at room temperature for 5-7 days. Typical cultural and morphological characteristics are shown in Figure (2). On PDA plates, most of the isolates at first produces a mycelium which is grayish black with very thin white margin and tints of olive or brown. Colonies are spreading hairy and grey brown to black, possessing a texture similar to cotton, felt or velvet. The colors became darker green as fungal age increased (Fig. 2).

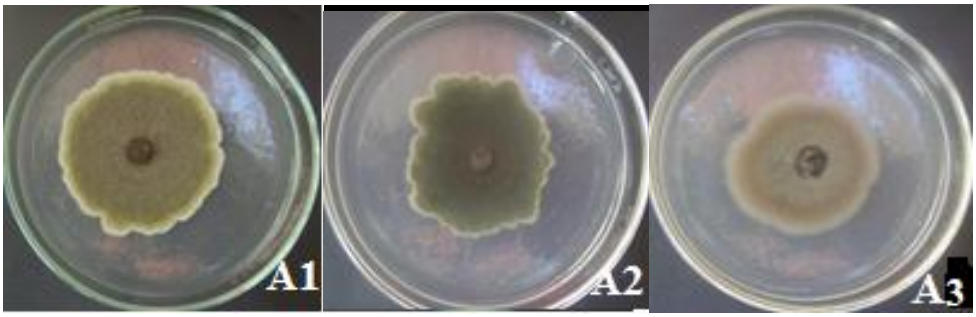

Figure (2). Deferent Colonies of *A. alternata* on PDA plate.

The fungus produced straight or curved primary conidiophores, short to long, simple or branched, with one or several apical conidiogenous loci. Conidia of the fungus either beaked and unbeaked. They are obclavate, long ellipsoid, small or moderate in size, septate, slightly constricted near some septa, with few longitudinal septa, in moderately long to long, simple or branched chains. The conidium body can narrow gradually into a tapered beak or secondary conidiophore. Secondary conidiophores can be formed apically or laterally with one or a few conidiogenous loci (Fig. 3-A1). Morphological characters are important tools in identification and classification of the fungus. The variation in shape dimension which may be either, due to host or environmental factors and hence considered to fall within limits for species (Abubaker and Ado, 2009; Ramjegathesh and Ebenezar, 2012). Based on the morphological description Ellis (1971), Simmons (1997), Zhang (2003) and Woudenberg *et al*. (2013) the fungus was identified as *A. alternata*.

Conidia of the isolates were catenated in long and sometimes branched chains of 2 to 6 spores. The spores were obclavate, medium-brown to dark olive, with 2 to 7 transverse and 0 to 2 longitudinal or oblique septa (Fig. 3-A1and A3). The length of their conidia was varied from15.1 to 29.1 µm. The comparisons between isolates of *A. alternata* was tabulated in Table (1). The longest conidia was in A2 (29.08 µm) followed by A3 (22.9 µm). The conidium was the shortest in A1(15.10 µm). The number of cells in each conidium varied from 2- 9. Among all the isolates, the conidia of A4 had 2-9 cells per conidium. The character agreed with the original description given by *A. alternata*. However, Nolla (1927) reported the length of

conidia 10.26- 77.52 µm in *A. alternata*. Neergaard (1945) reported 1-9 cells in *A. alternata*. Muthulakhsmi (1990 reported *A. alternata* produced both beaked and unbeaked conidia. Pandey and Vishwakarma (1999) reported that 4-6 septation observed in *A. alternata*.

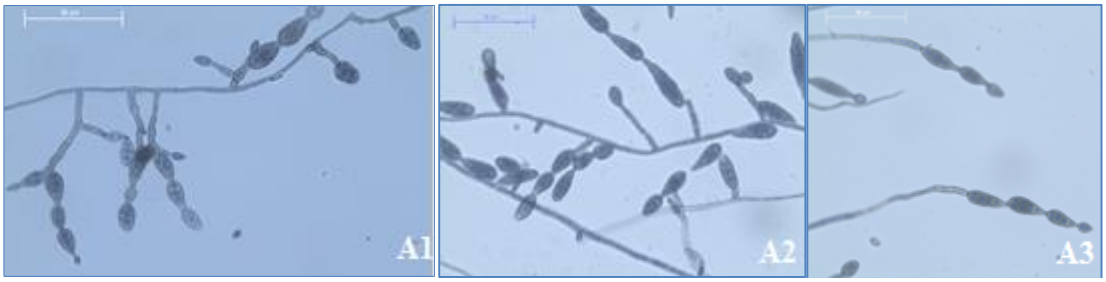

Figure (3). Conidiophores, Conidial and mycelial morphology of *Alternaria alternata*. (50µm).

Table 1: Fungal structures of *A. alternata* isolated from carob leaves.

Fungal structures	*A. alternata* A1	*A. alternata* A2	*A. alternata* A3
Culture color	Olive-green	Dark green	Medium-brown
Mycelium	Septate, velvet	Septate, velvet	Septate, Cotton
Conidiophore	Septate, olive-brown, single or branched	Septate, olive-brown, single or branched	Septate, medium-brown, single
Conidia	Produced singly or in chains of 2- 5 spores	Produced singly or in chains of 3- 6 spores	Produced singly or in chains of 3- 4 spores.
Spore length (µm)	15.10	29.08	22.9
Transverse septation	2- 4	2- 6	2- 5
Longitudinal septation	0- 2	0- 2	0- 2
Rate of growth and sporulation on media	Fast growth and abundant sporulation	Moderate growth and abundant sporulation	Fast growth and abundant sporulation
No. of cells	2- 6	2- 7	2-9

Pathogenicity test

The inoculated leaves with mycelial disks showed initially, five days after inoculation the symptoms first appeared in the form of small dark circular brown spots on the leaves. Seven days after inoculation, These increased in size and became blotch or sunken lesions (Fig. 3). The lesion gradually exhibited a grayish tint at the centre surrounded by a yellow halo. Ten days after inoculation, the estimated disease severity on leaves of *Ceratonia siliqua* was approximately 80% (Fig. 5). *A. alternata* produced conidia abundantly on the surface of the carob leaves. Pathogenicity tests, toxin assays, detection of toxin biosynthesis genes, or other genetic markers are required to distinguish fungi. The symptoms described earlier were the same as those observed on leaves of *Ceratonia siliqua* plants by natural infection in the field, whereas no symptoms developed on wounded leaves with sterile agar plugs applied. Then I repeated the isolation, and re-isolation of the fungus from symptomatic leaf tissues (inoculated

with A1, A2, A3and A4)) on PDA confirmed that the causal agent was *A. altrnata,* fulfilling Koch's postulates. This is the first report on carob leaf spot caused by *A. alternata*.

Figure (4). Appearance of leaf spot on *Ceratonia siliqua* caused by *A. alternata* (A1, A2 and A3) after 7 days postinfection with wounding versus non inoculated wound (*at arrow*).

Figure (5). Blotch symptoms on Carob leaves at 10 days after inoculation. No disease was observed on the side of leaf at non inoculated wound (*at arrow*).

Upon infection the isolate induced symptoms on leaves in 3-4 days, consisting of brown to black sunken necrotic lesions with typical concentric rings (Fig. 4), which increased regularly and covered almost 3/4 of the leaf area within 10 days (Fig. 5).These results confirmed the isolated pathogen as *Alternaria alternata*. According to Ellis (1971), it contains 44 species. *Alternaria* species are either parasites on living plants or saprophytes on organic substrate. The host range of pathogenic *Alternaria* is very broad. It is easy to recognize *Alternaria* sp. by the morphology of their large conidia. They are catenate, formed in chains or solitary, typically ovoid to obclavate, often beaked, pale brown to brown, multi-celled and muriform (Ellis, 1971). *A. alternata* is one of the major plant pathogens foliar which invade leaves and external parts of plant (Agrios, 2005). Most Alternaria species can survive on infected debris that remains on the ground after crops are harvested, or on alternate hosts (Jackson, 1959; Humphersonjones, 1989; Rotem, 1990). Conidia, hyphae and resting bodies like chlamydospores and microsclerotia, which are very resistant to unfavourable environmental conditions, have been reported to be involved in the survival of many Alternaria species (Tsuneda & Skoropad, 1978; Patterson, 1991; Vloutoglou, 1994). After contact between The spores of fungus and plant tissue, the spores find way into inside host tissue through natural opening such as stomata, wounds or direct penetration for cuticle tissue (Deverall, 1969; Takano *et al*., 1997; Gupta, 1998). Most *Alternaria* species produce general phytotoxins such as alternariol, macrosporin, alternaric acid, tentotoxin and tenuazonic acid. These toxins, which

are involved in the pathogenesis of these fungi, can cause chlorosis and necrosis of plant tissues when introduced into the plants. *A. alternata* is the only known *Alternaria* species that produces host-specific toxins with the same host-specificity as the pathogen. These toxins (AM-, AC-, AK-, AF-, AT-, and AL-toxin) are produced by six pathotypes of *A. alternata* (Kohmoto, et al., 1981; Lacey, 1992). The toxin is not required to kill the host cells but to suppress the general resistance mechanism in susceptible plants by causing a slight disruption of host metabolic activities. Therefore, based on the morphological characteristics and together with pathogenicity confirmation, I concluded that the pathogen responsible for leaf spot on carob in Al-Jabel Al-Akhder region of Libya was *A. alternata.*

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