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First record of *Pestalotiopsis* spp. from affected leaves of mastic shrubs (*Pestacia lentiscus* L.) in northeastern of Libya.

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Abstract: This study was carried out to identify the unknown different symptoms and their causes as plant pathogenic fungi from Al-Jabal Al-Akhdar District. Plant materials with fungal signs and symptoms were collected and examined. The main fungi consistently isolated from symptomatic leaves and twigs were *Pestalotiopsis* spp. Morphology, colony characteristics, and pathogenicity of the isolates were examined. My report the occurrence of *Pestalotiopsis* spp. on leaves of mastic (*Pistacia lentiscus*) for the first time in Libya.

Key words: Libya; *Pestacia lentiscus*; *Pestalotiopsis* spp.; leaves; spot; chlorosis; isolation.

Introduction

Mastic (Anacardiaceae) is a small evergreen tree or shrub, up to 4 min height, and distributed in the Mediterranean region up to 700 m above sea level. In Libya, it is an important medicinal plant grown in several regions geographical in Al-Jabal Al-Akhdar province (Figure 1).

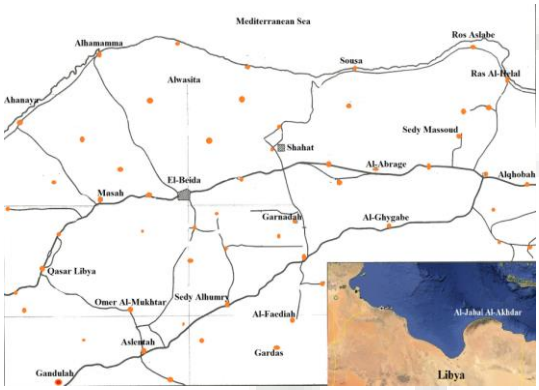

Figure 1: Distribution of the mastic tree (red circle) in Al-Jabal Al-Akhdar region of Libya according to final report at 2005.

Mastic gum is an essential oil obtained from leaves, fruits or trunk exudate, used for the relief of upper abdominal discomfort, stomach aches, dyspepsia and peptic ulcer. The aerial parts of the plant have been used as a popular cure for hypertension. The oil has been used in the perfumery, food and pharmaceutical industries (Mohd *et al.,* 2014), and is currently being evaluated as a flavouring in alcoholic beverages and chewing gum (Kıvc¸ak and Akay, 2005). Leaf extracts of *Pistacia* spp. used as antimicrobial (Rhouma *et al*, 2009**).** There are some published reports about disease on mastic: *Cylindrocldium pauciramosum* and *C. scoparium* (Vitale and Polizzi, 2008); *Pestalotiopsis guepinii* (Göre *et al.,* 2010); *Ulocladium* sp. on *P. atlantica* (Al-sharafa and Al-limoum, 2015). leaves of mastic (*P. lentiscus* L.) with the symptoms of discoloration, dieback, grey/light spots or necroses and twigs lesions were observed on plants from different regions in northeastern of Libya. The aim of this work was to identify the organisms occurring on affected mastic leaves in green areas of Al-Jabal Al-Akhdar and confirm its pathogenicity on healthy leaves.

Materials and Methods

Field observations and symptoms

Leaves and twigs samples (Figure 2) were collected from different sites in Al-Jabal Al-Akhdar region. 500 leaves from 30 plants were collected for mycological analysis.

Figure 2: Most common symptoms on leaves and twigs of mastic shrubs seen in the field. A, B and C Dieback and discoloration. D. Grey/light spot. E. Lesions on twigs at arrow. (*a. upper surface, b. lower surface*).

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The numbers of leaves taken from the top, central and lower part of the plant were approximately equal. The samples were placed in separate plastic bags lined with tissue paper and brought to the laboratory for study.

Isolation of pathogen

The surface of tissue samples was washed with running tap water and sterilized with 10% NaClO (2 minutes). Samples were washed three times with sterilized water, cut into about 0.5-0.6 cm segments and transferred to PSA medium in Petri-dishes. Plates were incubated at 25C for 5-10 days and checked regularly. When mycelial growth and spores were observed (Figure 3), further isolation were carried out into PSA plate then transferred to PSA slants and stored at 4 °C for further study. Mycelial growth rate was assessed by transferring 6 mm diameter PSA medium disks, derived from seven-day old colonies, into other PSA medium plates, including four replicates for each isolate and after the end of incubation period (7 days), the linear growth (mm) was determined in diametrically opposite directions. Mycelial Growth Rate (MGR) was calculated by the formula**:** [fungal linear growth (mm) /diameter plate (9mm) × 100]. Fungal mycelia and spores were observed under a Light microscope and photographed. Conidia were measured using a light microscope with a micrometer at 40X magnification. twenty conidia were measured for each isolate. The isolates were identified initially by comparing morphological and cultural characteristics (i.e., size of conidia, color, number of cells and number of apical appendages).

Figure 3: Initial colonies of *Pestalotiopsis* from infected leaves

Pathogenicity tests

Koch's postulates were performed on surface-sterilized healthy leaves *P. lentiscus*. For this purpose, leaves removed from the tree and 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. leaves were placed in humid chamber at room temperature (24°C). Leaves were inoculated with mycelial disks (3 mm diameter) of *Pestalotiopsis* spp. grown for 5 to 7 days at 25°C. were putt reversely on leaf surface directly. Non-inoculated leaves (only plugs of PSA culture), served as control. After symptom expression, isolation was carried out in order to confirm the genus identification with subsequent pathogen re-isolation in PSA medium to fulfill Koch's postulates and to identify the species morphologically. Concerning of twigs The shoots were wounded with a 5-mm punch. Mycelial plugs 5 mm in diameter from old culture were placed on the wounds and covered with Parafilm and aluminum foil. After 27 days, control wounds inoculated with sterile PSA plugs had healed.

Results and Discussion

Description of Symptoms

In the spring 2015-2016 the observations in the Al-Jabal forest revealed that discoloration with dieback (Fig. 2A, B) and usually continue to expand and large areas of the leaf was killed, grey spots in both leaf surface (Fig. 2C), as well as Symptoms on leaves began as small dark brown spots that expanded to become gray/light brown circles surrounded by a dark brown border (Fig. 2D-a) that associated with gnawed on lower leaf surface (Fig. 2D-b) and grey lesions on twigs (Fig. 2E). These were the first time that these symptoms were observed on mastic shrubs endemic species in Libya.

Description of Species

Cultural characteristics.

From the collected material 90 white colonies of fungus, belonging to five symptoms at rate 6 colonies/ plate in three replicates. Colonies on PSA reaching 7 cm diam. after 5 days at 25°C, with crenate edge, whitish, with aerial mycelium on surface; Some isolates showed faster daily mycelial growth rate (MGR), such as P_2 and P_5 following P_4, with MGR greater than 90% mm, whereas some isolates (P_1 and P_3) exhibited the lowest (inferior to 50%) MGR (Table 1). Although this character is influenced by culture media and environmental conditions. However, in this case, this is not the reason for these differences among isolates because all of them were cultivated in the same media and growing conditions; therefore, variation could be related to the genetic variability between different isolates or to intraspecific variability. All colonies showed similar pigmentation, reverse of culture whitish to pale yellow with acervuli were produced in the center at arrow in old culture (Figure 4).

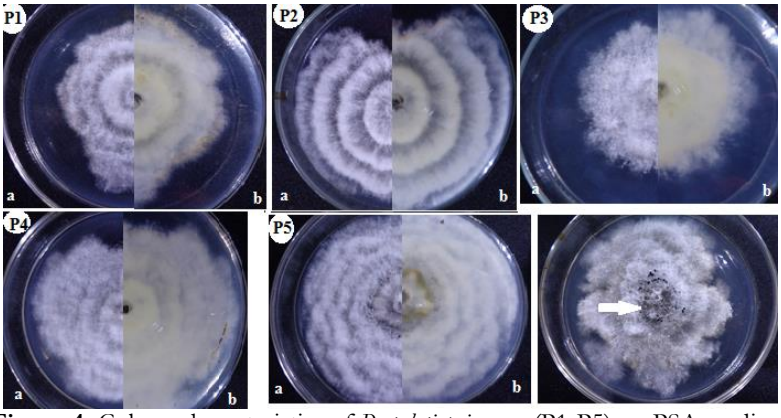

Figure 4: Colony characteristics of *Pestalotiopsis* spp. (P1-P5) on PSA medium after 7 to 10 days at 25c° *(a. from above, b. from below)*. Black droplets in the center (*at arrow*)

Description of conidia

Conidia were fusiform, straight or slightly curved and five celled. The darker median cells were three celled with a thick wall. Normally, the upper two cells were brown with a darker band at the septa between them, while the lowest cell was lighter colored The apical and basal cells were conical in shape, thin walled and colorless (red arrow). Appendages appeared at the apex and base. There were two to three appendages in apex. Basal appendage was single and centric (Figure 5).

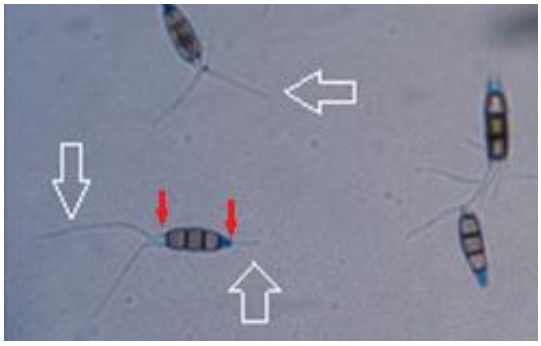

Figure 5: Conidia with apical and basal appendages

Conidia from all isolates were different in shape and size (Figure 6 and Table 1). Conidia dimensions ranged in length from 20.80 to 28.60 µm and in width from 5.46 to 7.28 µm. the terminal filiform appendage number ranged from two to three. Based on these morphological features described in the keys of Steyaert (1949), Guba (1961) and Sutton (1980) all isolates were identified as the members of the genus *Pestalotiopsis*.

Most *Pestalotiopsis* 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 (Hu *et al.,* 2007). Apical appendage number are also widely used characters for species identification (Maharachchikumbura *et al.,* 2011). Regarding the morphological characterization of *Pestalotiopsis* spp. isolates, Hu *et al.,* (2007) and Liu *et al.,* (2010) found that the morphology varies inter- and intra-species and should be interpreted with caution; therefore, it can be a way to differ isolates, but a complementary analysis is necessary. Wei and Xu (2004) identified *P. kunmingensis* as a *Podocarpus macrophyllus* endophyte organism. These authors highlighted that, despite the huge divergences to classify *Pestalotia* sp. or *Pestalotiopsis* sp., the latter usually has some specific morphological and physiological characteristics, such as: fusiform conidia formed within compact acervuli; conidia with usually five cells, with three colored median cells, and two colorless end cells; and conidia with two or more apical appendages arising from the apical cell. Liu *et al.,* (2007) used the following characters for the morphological description of *P.hainanensis* isolates: size, length, and width of conidia; median cell color; number, position, and length of apical appendage; apical appendage tip (branched or unbranched); basal appendage presence/absence of basal appendage; and fungi habit. Results found by these authors are an indicative that some morphological characters have major impact on isolate differentiation, and could be used for an initial division among them to identify different species and to conduct pathogenic studies.

Table 1: Characteristics of the colonies and conidia of pathogenic *Pestalotiopsis* spp. to mastic shrubs (*P. lentiscus*) grown in potato sucrose agar (PSA) media.

Isolates	MGR (%)	Length (µm)	Width (µm)	Cell number	Appendage number
P1	44.4	25.09	5.46	5	2-3
P2	94.4	24.31	7.28	5	2
P3	48.9	20.80	6.90	5	2-3
P4	92.2	28.60	5.59	5	2
P5	94.4	26.91	6.50	5	2

Maximum diameter of Petri plate is 90 mm.
MGR: mycelial growth rate calculated using the colony diameter at the final assessment day.

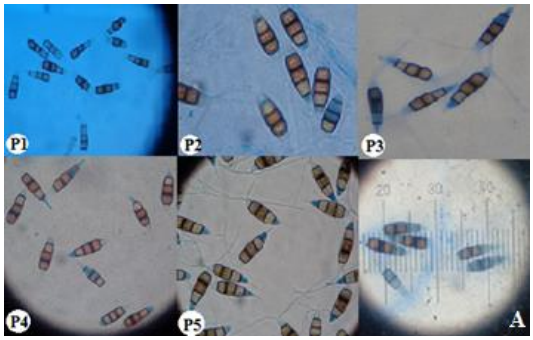

Figure 6: Immature conidia of *Pestalotiopsis* spp. (P1- P5), and (A) conidia observed in a microscope with 40X magnification

Pathogenicity tests

After 15 days of inoculation by a mycelial disc, a dark brown zone was developed in the surface of the inoculated leaves (Figure 7A). The lesions enlarged to eventually cover several centimeters and after 20 days the fungus began to form conidiomata as minute black dots, the acervuli scattered on the leaf spot (Figure 7A, 7F). The healthy leaves have not shown any symptoms at red rectangular (Figure 7A, 7F). Leaf spots are pale brown to brown, in the surface of the inoculated leaves surrounded by dark brown zone (chlorosis) was especially produced in leaves of mastic plants in Figures 7B, 7C, 7D and 7E. Black droplets over the surface were observed after 20 days (Figure 8). The *Pestaloiopsisi-* inoculated wounds showed lesions in the bark and wood discoloration.

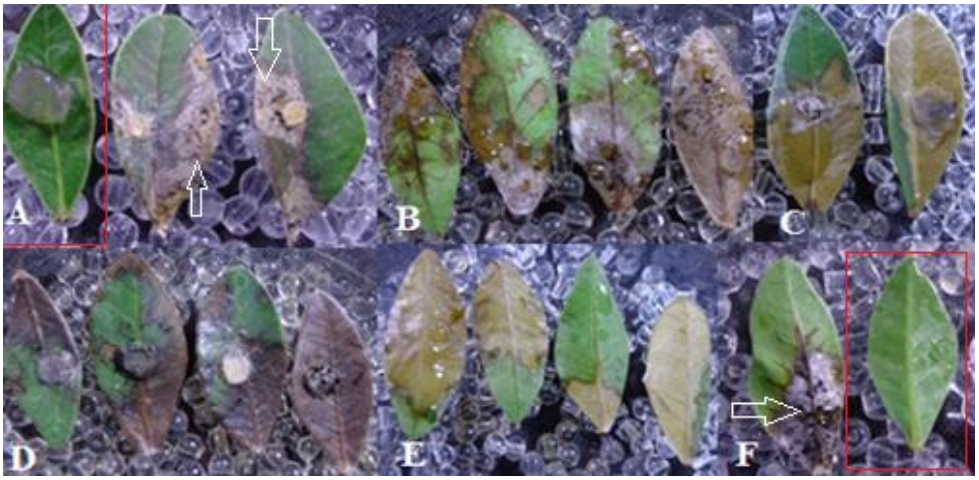

Figure 7: Effect of the inoculation with *Pestalotiopsis* spp. on the mastic leaves. None inoculated mastic leaf (red rectangular); inoculated mastic leaf show grey spot and acervuli (A and F at arrows) and chlorosis (B, C, D, E)

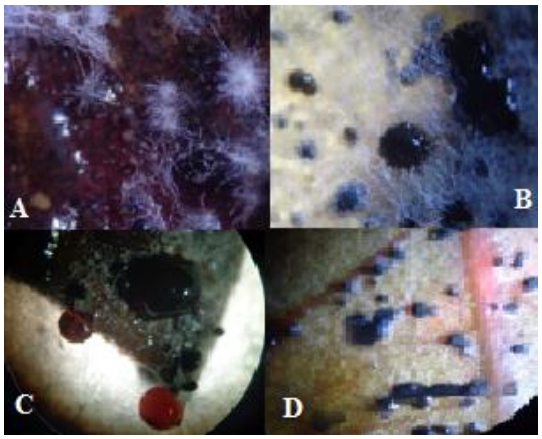

Figure 8: (A) (B) (C) and (D) Asurvuli of *Pestalotiopsis* on leaf

Pestalotiopsis is a species rich genus occurring as pathogens, endophytes and saprophytes (Jeewon *et al.,* 2004). However, most species of *Pestalotiopsis* are plant pathogens. They cause leaf blights in many plant species (Trapero *et al.,* 2003; Das *et al.,* 2010; Maharachchikumbura *et al.,* 2011). In Japan *Pestalotiopsis*, parasitic was recorded on conifers (Suto, and Kobayashi,1993), Symptoms of leaf blight on Japanese spicebush (*Lindera obtusiloba*) caused by *Pestalotiopsis microspore* (Jeon *et al.,* 2007). leaf spot disease of *Proteaceae* caused by *Pestalotia* sp. in Zimbabwe (Swart *et al.,* 1999).

In Morocco, several pathogenic species of the genus *Pestalotia* were also reported as *Pestalotiopsis cruenta* witch provokes brown lesions with clear black circle on the leaves of Chamaerops humilis (Khey *et al.,* 2013).

Pestalotia subcuticularis causing lesions on the leaves of *Pyrus mamorensis* (Yamni *et al.,* 2006), and *Pestalotia fici* the causal agent of the olive leaves chlorosis and olives rot (Chliyeh *et al.,* 2014). *Pestalotiopsis guepinii* causing twig blight on hazelnut and walnut (Karaca and Erper, 2001) and newly reported to cause dieback on *Pistacia lentiscus* var. *chia* (Göre *et al.,* 2010) and report of leaf blight of arborvitae (*Thuja orientalis*) caused by *Pestalotiopsis* sp. by Ozan *et al.,* (2012) in Turkey.

After re-isolating *Pestalotiopsis* spp. from leaves of the mastic shrubs, pathogenicity of this species

was demonstrated. However, this was the first report of *Pestalotiopsis* spp. causing leaf chlorosis grey blight and twig lesions in Libya.

Conclusion

Pestalotiopsis spp. are causal agents of mastic shrubs leaf spot, chlorosis and twig lesions. Colony diameter is an important character for species differentiation of *Pestalotiopsis* spp.; however, it is important to evaluate the most possible characters of conidia, as number of cells, color of medium cells, conidia dimensions, and position and length of apical appendage.

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