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Biological characteristics of *Diplodia sapinea* f. sp. *cupressi* infecting *Cupressus sempervirens* L. in Tunisia

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Abstract

Cupressus sempervirens L. (Italian cypress), is a Mediterranean evergreen coniferous tree. Due to its ecological values, this cypress has been used in forest protection against desertification and soil conservation. Last decades, cypress dieback has been commonly observed in Tunisian forests. Symptoms of shoot dieback, necrosis, twig blight, and trunk cankers have been observed on cypress trees in Bou Chrik (Nabeul) region. The causal agent was identified as *Diplodia pinea* f. sp. *cupressi*. The mycelial growth rate of the fungus was evaluated by using four different media culture at 25 °C and at seven temperatures ranging from (5 °C to 35 °C) on PDA medium. The results showed that the species was able to grow in a range of temperatures ranging from 20 to 25 °C and showed a higher growth rate on PDA medium. A pathogenicity test was conducted on *C. sempervirens* seedlings, and the aggressiveness of the fungus was approved.

Keywords – cypress – dieback – media – pathogenicity – temperature

Introduction

Mediterranean cypress (*Cupressus sempervirens* L.), is a valuable evergreen conifer due to its ecological and economical properties, almost common natural or introduced forest species disseminated around the Mediterranean basin (Caudullo & de Rigo 2016). Moreover, natural *C. sempervirens* trees are found in Cyprus, Cyrenaica, Lebanon, Palestine, South Anatolia, Syria and the islands of Southeast Greece (Pavari 1934, Strid & Tan 1997, Zohary 1973). According to Xenopoulos et al. (1990), a natural stand of *C. sempervirens* var. *numidica* was found in Tunisia and a spontaneous population of *C. sempervirens* var. *atlantica* in Morocco. A population of *C. dupreziana* A. Camus, 1926, a species related to *C. sempervirens*, was also reported in Algeria by Ducrey et al. (1999). In Tunisia, *C. sempervirens* was widely used in reforestation specially in the central stands on poor, dry soils, in which covering 1500 ha (Hlaiem 2006). It is commonly used also as a windbreak for vegetable or fruit tree crops, which covers 28% of the orchards in the Nort-East (Hlaiem 2006). Although, that *C. sempervirens* is adapted to the Mediterranean climate and resist to drought, it is suffered from the decay induced by different diseases with complex etiology (Panconesi et al. 1999).

Regrettably several diseases attack mainly Mediterranean cypress trees affected by biotic and abiotic factors (Ben Jamâa et al. 2005). Over the last 40 years in most of the Mediterranean countries, especially France, Italy and Greece, cypress is damaged by the canker disease, caused by

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Seiridium cardinale fungus and cause heavy damage in forests, nurseries and ornamental plantations (Panconesi et al. 1999). However, in Tunisia the decline phenomena of cypress have been noted since the 1960's in the Northeast of the country (Hafsia & Mrad 1966). Nevertheless, the cypress declining becoming serious in the late of 1990's due to the successive drought years which often predisposes trees to biotic attack (Ben Jamâa & Khaldi 1998). High damage caused by bark beetle of cypress (*Phloeosnus aubei*) has been reported in different forest plantations in the center of Tunisia and in windbreaks in the coast (Belhabib 2004, Belhabib et al. 2007). Besides insects, cankers symptoms have been also noted on declining trees (Ben Jamâa & Khaldi 1998). In spite of that, cypress disease has been less well-documented in Tunisia. In this context, the aim of this study was to characterize the cypress pathogen in terms (i) to evaluate the effects of culture media and temperature and (ii) to assess its pathogenicity.

Materials & Methods

Source of fungal isolate

Investigations were carried out in the northeastern Tunisia, Nabeul Region (Bou Chrik) characterized by semi-arid climate and citrus cultivation. In this region *Cupressus sempervirens* L. trees are used as windbreak. Dieback symptoms were observed on 30 years old trees of *C. sempervirens* (Fig. 1) (Ben Jamaa, personal observation). Sampling was done from diseased *C. sempervirens* and the fungal pathogen DF IMG86 (DAOM 234788) was identified as *Diplodia pinea* f. sp. *cupressi* (Intini et al. 2005).

Fig. 1 – Disease symptoms observed on *Cupressus sempervirens* trees.

Effect of media on mycelial growth

The growth rate of *D. sapinea* isolate on four different media were evaluated, namely Potato Dextrose Agar (PDA), Agar Potato Saccharose (APS), Agar Glucose (AG) and Malt-Agar. Mycelial plugs (3 mm) containing young mycelia 7 days old culture of the tested fungi were inoculated onto the tested media in 9 cm sterilized plastic Petri dishes. Five replicates were prepared for each used medium. All Petri dishes were incubated in the dark at room temperature 25 °C. The mycelial growth of the fungal colony was recorded every day, then the average colony diameter and growth rate were determined using the method described by Palmer et al. (1987) as following:

Mycelia radial growth = (maximal diameter + minimal diameter)/2.

Effect of temperature on mycelial growth

Mycelial plugs (3 mm) taken from seven days old culture were incubated on PDA, at 7 different temperatures (5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C and 35 °C) according to Milijašević (2006). Five replicates were performed for each temperature. The radial mycelium growth of the colony was recorded every day. The average colony diameter and growth rate were measured as the method mentioned above.

Pathogenicity test

The pathogenicity of *Diplodia sapinea* f. sp. *cupressi* isolate was assessed on two 4-year-old *C. sempervirens* seedlings (n = 5) according to the Lieutier et al. (1988) methodology. Briefly, a mycelial plug (3 mm) of the strain, taken from the margin of an actively growing 7-day-old colony on PDA was put in a shallow wound made with a scalpel on the stem foot of each seedling. Sterile PDA plugs were placed into similar wounds on control seedlings (n = 5). Inoculation points were sealed with Parafilm to retain moisture and monitored weekly for lesion development for one month at the nursery of the National Institute for Research on Rural Engineering, Water and Forestry in Ariana, Tunisia. The causal agents were then re-isolated at the end of the trial.

Statistics analysis

The results were evaluated statistically by analysis of variance (ANOVA) with SPSS v.20.

Results

Effect of media and temperature on mycelial growth

After 4 days the fungal isolate showed similar radial growth on PDA (3.6 cm), APS (3.3 cm) and AG (2.9 cm). The highest radial growth occurred on PDA medium and the least one observed on Malt-Agar (approximate 2 cm) (Fig. 2). However, no recognizable growth was exhibited on plates incubated on PDA at 5 °C and 35 °C. Seven days post incubation on PDA, the optimum growth was 7.4 cm appeared at 25 °C (Fig. 3).

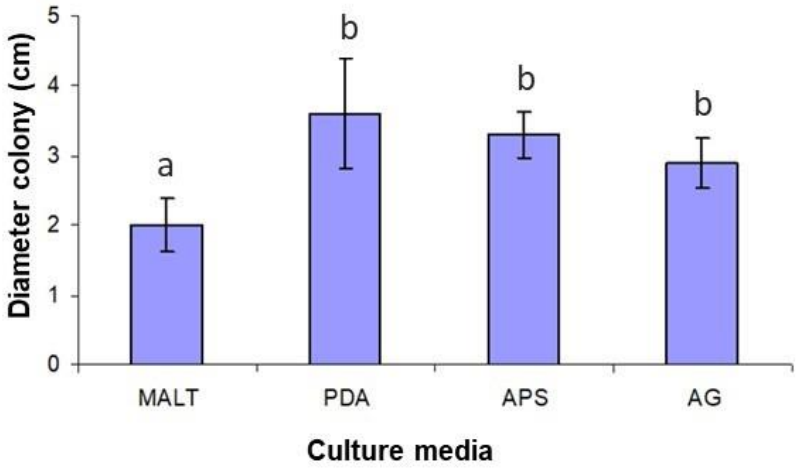

Fig. 2 – Mycelial growth of *D. sapinea* on 4 different media at 25 °C in the dark. MALT = Malt-Agar, PDA = Potato Dextrose Agar, APS = Agar Potato Saccharose, AG = Agar-Glucose. Values within histograms flowed by the same letter are not significantly different (*P* = 0.01).

Pathogenicity test

Artificial inoculation on healthy seedlings of cypress yielded to the same symptoms as observed in the field: brown lesion on wood tissues of the stem foot, and excessive resin flows from

infected twigs. The lower canopy turns brown and eventually leading to death. Thirty days post-inoculation, noticeable necroses extending from inoculation points were observed, confirming the aggressiveness of *Diplodia sapinea* isolate (Fig. 4). Stem lesions measured 64 ± 48 mm. In the meantime, control remained asymptomatic. The fungal pathogen was successfully re-isolated with a detection rate about 94%. Its identity was confirmed based on morphological features. Colonies on PDA medium at 25 °C were initially white with fast-growing mycelium, fluffy and highly dense (Fig. 5). Then, became gray to dark-gray and finally turned to black. Conidia were firstly hyaline, then turned brown as they matured aseptate and some of them developed one medial septum.

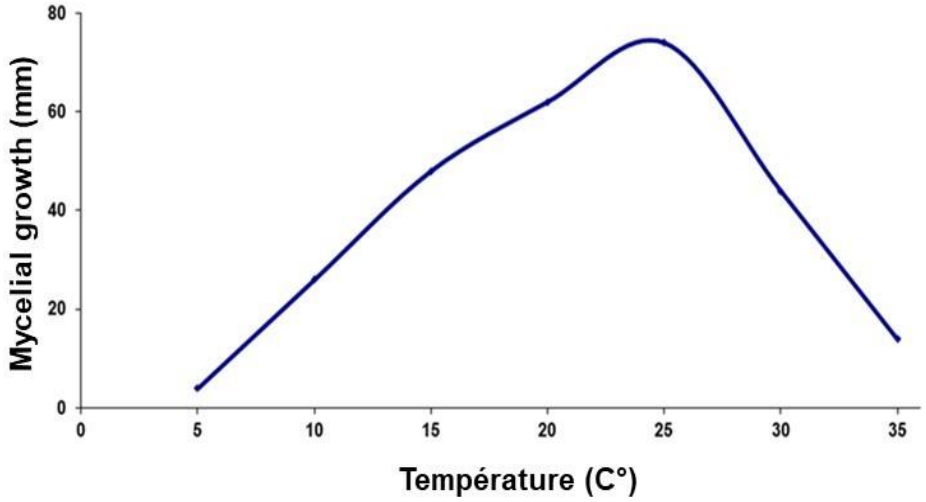

Fig. 3 – Mycelial growth of *D. sapinea* on PDA at 7 different temperatures in the dark.

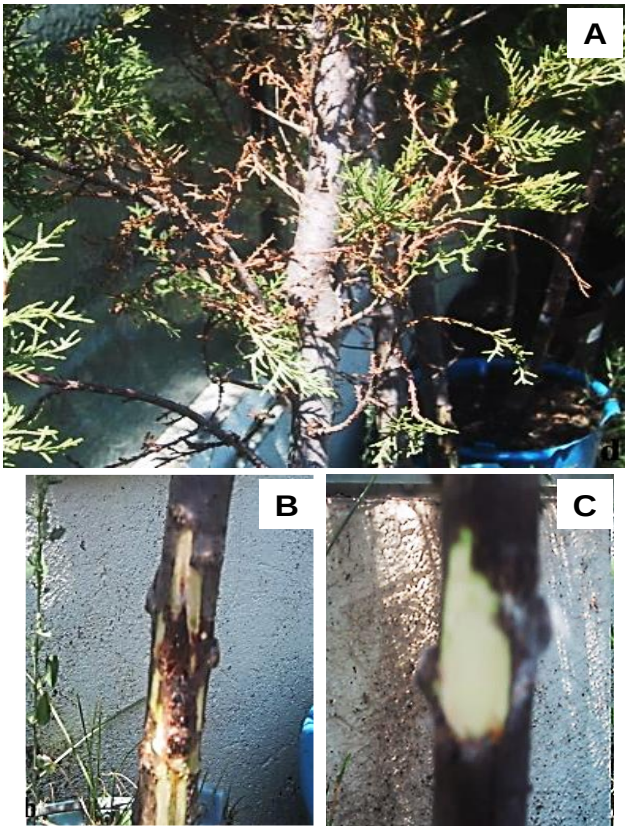

Fig. 4 – Pathogenicity assay. A Symptoms of disease observed in inoculated cypress seedling with brownish foliage four weeks post-inoculation. B Stem brown lesion caused by *D. sapinea* on cypress seedling 6 weeks after inoculation. C asymptomatic control.

Fig. 5 – Cultural characteristics of *Diplodia sapinea* colony incubated on PDA at 25 °C for 5 days.

Discussion

Plant fungal pathogens are an increasing emerging threat as climate change progresses (Ayres & Lombardero 2000, Anderson et al. 2004). Mainly, *Sphaeropsis sapinea* (Fr.) Dyko & Sutton [Botryosphaeriaceae, Botryosphaeriales; selected synonyms: *Diplodia sapinea* (Fr.) Fuckel, *D. pinea* (Desm.) J. Kickx f.] is responsible for *Diplodia* tip blight disease (or *Diplodia* shoot blight. Moreover, *Diplodia sapinea* f. sp. *cupressi* was first described on Italian cypress in the Middle East (Solel el al. 1987) and later has been reported from other countries including Morocco (Frisullo & Graniti 1990), Italy (Evidente et al. 1996), South Africa (Linde et al. 1997), Greece (Xenopoulos & Tsopelas 2000), and Tunisia (Intini et al. 2005). Given, climate change influences the dynamics of host-pathogen interactions (Pearson & Dawson 2003). It is presumed that damage caused by *D. sapinea* increase at high temperature (Bosso et al. 2017). Thus, to better understand the relationship between climatic factors and the fungal pathogen isolated from symptomatic *Cupressus sempervirens* trees, the estimated temperature at which fungus reaches its maximum radial growth on *in vitro* synthetic medium was studied. The results obtained showed that the temperature and the incubation period affected the *in vitro* mycelial growth of the pathogenic fungi. the optimal temperature for mycelial growth was 25 °C. The extremum temperatures tested (i.e., 5 and 35 °C), inhibited the mycelial growth. The optimal temperature for the growth of *D. sapinea in vitro* is about 20 to 25 °C (Palmer et al. 1987, Keen & Smits 1989, Milijašević 2006). In several studies, the association between temperature and disease incidence could be observed. Indeed, warm May and June temperatures were associated with higher damage in Sweden (Brodde et al. 2019) and low winter temperatures correlated negatively with its occurrence in France (Fabre et al. 2011). Temperature has been proved has an influenced on the rate of fungal growth (Sajilia et al. 2017). This was aligned to study by Slade et al. (1987), temperatures above 30 °C may have an inhibitory effect which may suggest their adaptation to the Mediterranean climate in the north of Tunisia.

Additionally, fungal radial growth changes not only with temperature but also with the media used. Shabana et al. (2015) reported that culture media and other factors play main role in growth of fungi. The findings of this study revealed that culture media influenced the growth of tested fungus. It shows that *D. sphaeropsis* exhibit greater colony growth on PDA (3.6 cm) 4 days post incubation. Several researches mentioned that PDA was the best media for mycelial growth (Maheshwari et al. 1999, Saha et al. 2008).

In the other hand, the pathogenicity assay proved the aggressiveness of *D. sapinea* isolate on *C. sempervirens* seedlings showing disease symptoms and necrotic lesions around the inoculation point. Several works assessing pathogenicity of *Diplodia* species causing canker and dieback, wounded trunks or branches have been inoculated by mycelial plugs of pathogen colony on PDA plates (Taylor et al. 2005, van Niekerk et al. 2004). Furthermore, it is important to consider that

wounding is a prerequisite for infection of *Diplodia* species on host tissue (Hartill & Everett 2002). In Southern and Central Europe, *Diplodia pinea* caused shoot dieback, stem canker and crown wilt on pines (Fabre et al. 2011, Luchi et al. 2014). In Tunisia, *D. pinea* has been recorded on *P. pinaster* and *P. radiata* (Linaldeddu et al. 2008).

Conclusion

Additional studies are therefore required to understand the occurrence of *Diplodia sapinea* on *Cupressus sempervirens* disease, which is arise due to climate changes. To our knowledge, this is the first report that describe the biological characteristic of *D. sapinea* f. sp. *cupressi* associated with Mediterranean (or Italian) cypress dieback in Tunisia.

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