Antifungal activity of *Acacia tortilis* subsp. *raddiana* tar on *Fusarium oxysporum* f.sp. *albedinis*, the cause of Bayoud Disease of the date palm in Southwest Algeria

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ABSTRACT

Bayoud caused by *Fusarium oxysporum* f.sp. *albedinis* (Foá), is the most destructive disease of the date palm (*Phoenix dactylifera* L.) in Morocco and Algeria and there is no effective control strategy. We found that although *Foá* isolates vary morphologically, *Foá* strains can be identified by species-specific primers. PCR analysis revealed that the strains that we isolated from infected date palm rachis were the Bayoud pathogen *Foá*. We used these strains to evaluate the antifungal activity of tar extracted from *Acacia tortilis* subsp. *raddiana*. The *A. raddiana* tar had a density of 1.15, a refraction index of 1.3850, a pH of 5.2 and a dried matter ratio of 48.75%. The *A. raddiana* tar effectively inhibited the growth of *Foá* in vitro with a minimum inhibitory concentration of 3 μg/ml.

Key words: Antifungal, Bayoud disease, Date palm.

INTRODUCTION

Although the economically important date palm (*Phoenix dactylifera* L.) is resistant to abiotic factors, it is threatened by Bayoud, which is an Algerian term to describe phytopathogenic fungi that infect many plants, especially the date palm (Benabbes et al., 2015; Mahadik and Mali., 2018). Bayoud is fatal to the date palm (Sghir et al., 2014) and the most pathogenic fungi belong to the genus *Fusarium* spp (Shabani et al., 2014; Zebboudj et al., 2014). Several control strategies have been considered, but they remain inefficient and difficult to apply. The most effective measure to reduce the incidence of this disease would be to select and plant 5-fluoroorotic acid-resistant cultivars (Karkachi et al., 2014). Unfortunately, date palm lines that are resistant to Bayoud disease usually produce low-quality fruits. Therefore, more integrated management strategies are needed to tackle Bayoud disease long term. Such strategies should combine various alternatives that accommodate quality requirements as well as environmental constraints (Dihazi et al., 2012). Biological control methods represent a promising disease control alternative (Fitrianingsih et al., 2019) and they are based on screening for plant extracts that confer protection against plant pathogens (Imazaki et al., 2015).

The World Health Organization estimates that 80% of people living in developing countries depend on traditional medicinal practices for their primary health care needs and plants are the main source of drug therapy in traditional medicine (Subba and Rai, 2018). Southern Algeria, with its rich floral resources and ethnobotanical history, is an ideal place to screen plants for biological activity. *Acacia* is a cosmopolitan genus containing more than 1,350 species and *Faidherbia* is a related African and Middle Eastern monotypic genus. *Acacia tortilis* (Forssk.) Haynesubsp *Raddiana* (Savi) Brenan, commonly known as *Acacia raddiana*, is a shrub legume in the *Fabaceae* family, an autochthonous taxon, which is particularly valuable in Algeria (Grouzis et al., 2003). To the best of our knowledge, there are no previous reports on the tar from *A. raddiana*. Thus, as a continuation of our ethnopharmacological, phytochemical, and antimicrobial studies of medicinal plants in the Algerian Sahara, we found that tar from *A. raddiana* had an inhibitory effect on *F. oxysporum* f.sp. *albedinis* (Foá), which causes Bayoud on the Algerian Saharan date palm.

MATERIALS AND METHODS

Plant material and extraction of tar: Aerial parts of *A. raddiana* (wood) were collected from the Bechar region in southwestern Algeria from September–May 2016. A voucher specimen was deposited at the Herbarium of VRVSA Laboratory, Faculty of Natural sciences and life (University Tahri Mohamed, Bechar, Algeria). Wood samples were air-

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dried and carefully cut before the extraction. The extraction was conducted according to the distillation per descensum method with some modifications. Trunks were cut in small pieces and placed in a special dispositive for 5 h. The distilled tar was stored at ambient temperature until analysis (Rageot, 2018).

Physicochemical properties of *A. raddiana* tar: The specific density at 20°C (AFNOR NFT 60-214), refractive index (RI; AFNOR NFT 60-212), acid index (AFNOR NFT 60-204), and pH (AFNOR NFT 04-408) of the tar were determined according to the AFNOR methods.

Fungal isolates and growth conditions: *Foa* strains were isolated from date palm rachis with symptomatic Bayoud, which were obtained in Igli in the southwest of Algeria. The rachis were cut into small -3-cm pieces, disinfected with 2% sodium hypochlorite for 3 min and rinsed several times with sterile distilled water. Three fragments were placed onto each potato dextrose agar (PDA) plate and plates were incubated at 25°C in the dark according to the method of Karkachi et al., (2014) with a few modifications. After isolation and purification, all of the cultures were single-spored and maintained as mycelia on PDA slants for short-term storage or in glycerol at -80°C for long-term storage (Rusli, 2012).

Pathogen characterization: *Fusarium*-like colonies were consistently isolated from symptomatic rachis tissue (Kettout, 2010). Single-conidial isolates were characterized morphologically by growing them on PDA (Yezli et al., 2015). Cultures were examined for the presence and characteristics of microconidia, mesoconidia, macroconidia, chlamydospores, sclerotia, coloration of medium and colony morphology (Elliott et al., 2010). Isolates were incubated at 25°C for 3–4 d without shaking for microscopic examination by the microculture method.

PCR assays for identification of *Foa* strains: Genomic DNA was extracted using the method described by Edel et al., (2000) with a few modifications. PCR primers for amplification of *Foa* included two primer pairs: FOA1 (CAGTTTATAGAAATGCCCAGC) coupled with BIO3 (GGCGATCTTTAGTTATTTGGTG) and FOA28 (ATCC CGTAAAGCCTGAAGC) coupled with TL3 (GGTCGGT CCGCAGATATACCGGC) (Fernandez et al., 1998). The PCR reactions were performed in a total volume of 25 μl, contained 1 μl of genomic DNA (~100 ng), 1.5 mM MgCl₂, 25 mM dNTP, 0.5 U Taq DNA polymerase (Biometric), 2.5 μl 10x reaction buffer, 1 μl DMSO, 17 μl ultra-pure water, and 0.5 μM of each primer. Amplification was performed using a GTC96S Thermal Cycler (Cleaver). The amplification program included one cycle for 4 min at 95°C followed by 30 cycles for 30 s at 92°C, 30 s at 60°C and 30 s at 72°C for the BIO3-FOA1 primer pair and 30 cycles for 30 s at 92°C, 30 s at 62°C and 45 s at 72°C for the TL3-FOA 28 primer pair. One cycle for 15 min at 72°C was conducted after the 30 cycles. After amplification, 5 μl of the PCR amplification products were electrophoresed in 2.0% agarose gels in TBE buffer at 80 V for 2 h. The gels were then stained with ethidium bromide and photographed under UV light (Rosado et al., 2014; Tantauoi et al., 1996).

*In vitro* antifungal activity and determination of the minimum inhibitory concentration (MIC): The antifungal activity of the *A. raddiana* tar extract was determined according to the method described by Kabore et al., (2001) with slight modifications. For hyphal growth inhibition experiments, fungal isolates were inoculated onto PDA and incubated at 25°C for 7 d (Dihazi et al., 2003). After the 7 d, a spore suspension was prepared for every isolate. Spores were harvested by adding sterile distilled water onto the cultures and the suspensions were adjusted to a concentration of 10⁶ spores/ml (Dihazi et al., 2012; Yezli et al., 2015). PDA plates containing various concentrations of the tar extract were inoculated with the spore suspensions and incubated at 25°C for 8 d (Bhutani et al., 2018). One control plate did not contain any tar extract. Radial growth was measured every 2 d for 8 d (El Hassan et al., 2004). The MIC of the tar extract was the lowest concentration that completely inhibited visible growth (Bhandari et al., 2000; Rosca et al., 2007). The diameter of a fungus colony was determined by averaging two perpendicular diameter measurements (Kaboré et al., 2001). For each isolate and concentration, three replicates were performed. When the mycelium filaments reached the periphery of the control dish containing *Foa*, the inhibition rate was calculated using the formula \% Inhibition = (D₀ – Dₓ)/ D₀ × 100 in which D₀ = diameter of the *Foa* on the control plate and Dₓ = diameter of the *Foa* on the plate containing extract. This technique was inspired by the culture technique recommended by Patel and Brown (1969).

Statistical analyses: All the measurements were made in triplicate and the results obtained were expressed as the mean ± standard deviation (SD). One-way ANOVA was carried out to test for any significant difference. Differences between means at *P* ≤ 0.05 level were considered significant.

RESULTS AND DISCUSSION

Physicochemical indices of *A. raddiana* tar: *A. raddiana* tar is a dark brown, viscous liquid with an acrid and smoky odor. It has a density of 1.15, a refraction index of 1.3850, a pH of 5.4 and a dried matter ratio of 48.75%. It is very slightly soluble in water, is soluble in chloroform, ether and ethyl acetate alcohol and is partially soluble in petroleum ether.

Isolation and morphology of the pathogen from infected rachis: We isolated *Foa* strains from the rachis of contaminated fagouse cultivar palms in the Igli palm grove. This cultivar is sensitive to vascular fusarium wilt. *Foa* grows 2-3 mm a day on PDA and after 7 d, *Foa* growth reaches its
maximum on PDA. The Foa isolates revealed minor macroscopic morphological variability, such as the color of the mycelium as observed by Benabbes et al., (2015). Foa is characterized by a thin, curly, salmon pink mycelium. According to Tivoli (1988) and Assigbeste (1989), variability in the mycelium appearance is common for special forms of F. oxysporum.

Molecular identification of Foa: Morphological criteria are insufficient for the identification of Foa from other special forms of Fusarium and for determining the cause of Bayoud disease in the date palm. We found that Foa-specific primers were able to differentiate Foa DNA from DNA of other pathogenic Fusarium species as previously shown by Freeman et al., (2000) and Fernandez et al., (1998). To identify our pathogenic date palm isolates, we used the specific PCR assay primer pairs FOA28-TL3 and FOA1-BIO3; if the strain is Foa, 400 bp (from FOA28-TL3) and 204 bp (from FOA1-BIO3) PCR products would be detected. Using this specific PCR assay, we detected the 400 bp and 204 bp fragments and determined that the strains we isolated from the infected date palms were the Foa Bayoud pathogen (data not shown). Kistler (1997) previously reported that isolates with a shared host range are likely derived from a single, particularly successful pathogenic genotype, and this appears to be the case here. Further, this method will be implemented in future plant protection services.

Antifungal activity and MIC determination: We demonstrated anti-Foa activity by the A. raddiana tar extract (Table 1). The mycelial growth of pathogens on PDA were reduced in the presence of various concentrations of A. raddiana tar. The diameters of fungal colonies were reduced by 2–40 mm with the highest reduction observed for Foa at 43.51 mm. The MIC of the A. raddiana tar extract against Foa was 3 µg/ml and the inhibition rate of this concentration was 93.05%. The C1, C2, C3 and C4 A. raddiana tar concentrations were somewhat able to inhibit fungal growth, but the C5, C6, C7 and C8 concentrations inhibited > 60%.

Foa is a telluric fungus able of maintaining itself in the soil and in palm debris for long periods of time due to the thick and resistant membranes of its chlamydospores. It is important to note that Foa can survive in the soil in spore or mycelial form, thus can infect a plant directly from the soil. We found that our A. raddiana tar extract inhibited Foa growth on PDA medium indicating the presence of potent antifungal compounds in our extract. Similar anti-fungal activity has been observed for Olea europea and Juniperus oxycedrus tars by Belarbi et al., (2014), Terfaya et al., (2017), and Rageot (2015).
CONCLUSION

This study observed that the Tar used showed good antifungal activity against phytopathogen Fusarium oxysporum f.sp. albedinis. We have made it clear that Acacia tortilis subsp. raddiana tar induces a reduction of Fusarium oxysporum f.sp. albedinis. Further studies in vivo should be carried out, aiming at elaborating a biopesticide, is necessary, on one hand, to confirm the results we have obtained and, on an other hand, to study their potential efficacy under different conditions of the environmental conditions and also to clarify the cellular mechanism and molecular antagonistic plant-pathoge interactions involved in the protection.

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