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Antagonistic activity of *Sinorhizobium meliloti* strains against pathogenic fungi isolated from alfalfa in Algerian Sahara

Salim Azib[1*], Abdelhak Idder[2], Sara Attab[1], Sofiane Bensalem[3], Ouiza Djeroudi[1]

[1]*Laboratoire des Bioressources Sahariennes: Préservation et Valorisation, Université de Ouargla, BP 511, Ouargla 30000, Algeria*

[2]*Laboratoire de Recherche en Phœniciculture, Université de Ouargla, BP 511, Ouargla 30000, Algeria*

[3]*Laboratory of Conservation and Valorization of Marine Resources, National School of Marine Science and Coastal Management (ENSSMAL), University Campus of Dely Ibrahim, Bois des Cars, 16320 Cheraga, Algiers, Algeria*

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Abstract

The purpose of this study was to identify the fungal species attacking roots and foliage of alfalfa in Ouargla and assess the antagonistic potential, *in vitro*, of three *S. meliloti* strains (E543, E141 and O211) isolated from alfalfa root nodules on the growth of these fungi. Results showed that alfalfa plants could be attacked by several fungal pathogens. Based on the macroscopic and microscopic characters, three fungal species were identified as *Alternaria sp., Fusarium sp.* and *Curvulria sp.* The antagonistic test have shown that *S. meliloti* strains significantly reduced the growth of *Curvularia sp.* at 31.27%, *Alternaria sp.* at 40.12% and *Fusarium sp.* at 52.11%, respectively, thus confirming their role in controlling the growth of many soil-borne plant pathogenic fungi belonging to different genera.

* **Corresponding Author:** Salim Azib ✉ s_azib@hotmail.com

Introduction

Alfalfa (*Medicago sativa* L.) is the most important perennial forage legumes in the arid and semi-arid areas of North Africa (Elboutahiri *et al.*, 2010), with a global production area of over 32 million hectares across the world (Chen *et al.,* 2020). In Algeria, its growing area is still limited compared to other crops (Chedjerat *et al.* 2016). Paradoxically, in the Algerian Sahara, alfalfa is by far the main forage species cultivated (Chaabena *et al.*, 2012). This species is a perennial forage legume that has excellent nutritive value, high digestibility and a high biomass yield and contributes to the incorporation of large quantities of nitrogen (up to 250 kg/ha/y), which results in the economic benefit of reducing the need to apply synthetic fertilizers (Carlsson and Huss-Danell, 2003; Jensen and Hauggaard-Nielsen, 2003).

Many pests and diseases affect alfalfa in Algerian Sahara, but the damage caused by such attacks remains undocumented. Like all farm crops, alfalfa diseases can reduce forage yield and quality and decrease stand persistence (Vincelli and Smith, 2014). Excellent knowledge of the fungal agents is an essential step in guiding control interventions (Qin *et al.,* 2016).

The uses of disease-resistant varieties, seed treatment with fungicide and crop control are used to minimize disease damage to alfalfa seedlings. However, these measures do not always allow satisfactory control (Jones and Samac, 1996) and the application of rhizobia has been shown to be effective, not only to play a major role in biological nitrogen fixation but also to improve plant growth and reduce the incidence of diseases (Deshwal *et al.*, 2003; Das *et al.*, 2017). The practice of artificial inoculation of plants with rhizobia has been shown to make significant contributions to the inhibition of *Fusarium oxysporum* (Kumar *et al.,* 2011; Attab *et al.,* 2019) and many other pathogens.

The present work aimed to identify the fungal diseases attacking roots and foliage of alfalfa in Ouargla and ii) to assess the antagonistic potential of three *Sinorhizobium meliloti* strains (E543, E141 and O211) isolated from alfalfa root nodules on the growth, *in vitro*, of these fungi.

Material and methods

Bacterial strains

Three *Sinorhizobium meliloti* strains were used (E543, E141 and O211). In our previous study, they were isolated from root nodules of alfalfa cultivated in southern Algerian regions (Ouargla and El Oued) and selected from about forty-eight other strains on the basis of their ability to resist the predominant edaphoclimatic factors (pH, temperature and salinity) and antibiotics. They were genetically characterized by the housekeeping genes using *recA* (a recombinase) and *glnII* (a glutamine synthetase) to define their taxonomic position (Fig.1) (Azib, 2020).

Bacterial strains were grown for 3 days at 28°C on Yeast Mannitol Agar (YMA) of the following composition (g/l): mannitol, 10; yeast extract, 1; K_2HPO_4, 0.5; $MgSO_4$ $7H_2O$, 0.2; NaCl, 0.1; $CaCl_2$ $2H_2O$, 0.05; Agar, 15 (Vincent, 1970).

Plant material

In December 2019, alfalfa plants, which showed symptoms of fungal diseases, were randomly collected. Subsequently, they were placed in hermetically sealed bags and transported to the Microbiology Laboratory of the Biology Department of the University of Ouargla, where they were stored at 4°C until further use.

Fungal isolation and identification

Leaf, stem and root samples were cut separately with a sterile scalpel into small fragments of 2-5 cm, washed with sterile water and immersed in sodium hypochlorite solution (5%) for 2 min. The excess disinfectant was removed with sterile distilled water three times (Quintana-Obregón *et al.*, 2013). Disinfected samples were placed on Petri dishes containing potato dextrose agar medium (PDA) and incubated at 25°C for up to 10 days (Lin *et al.,* 2018). Pure cultures of each isolate were obtained by transferring hyphal tips from the margin of the fungal

colonies onto new PDA plates.

The isolates were identified according to the methods described by Botton *et al.* (1990) and Leslie and Summerell (2006), based on the macroscopic and microscopic characters.

In vitro screening for antagonism

The antagonistic activity of the *Sinorhizobium* strains against the radial mycelial growth of fungi was determined in PDA according to the methodology described by Gupta *et al.* (2006). In the center of a Petri dish (90 mm diameter) containing the PDA medium, a 5mm disc of fungal mycelium was deposited. The bacterium, from a suspension of 10^8 CFU/mL concentration, was seeded by straight striation parallel to each side of the fungus (approximately 2 cm away). The control was a Petri dish with inoculation of the fungus in the medium, in the absence of *Sinorhizobium*. The cultures were incubated at 28°C for 8 days and the percentage growth inhibition (R) was calculated by using the following formula of Whipps (1987):

(%) Inhibition = (R control - r test) / R control ×100

Where (R) is the radial growth of fungus in control, and (r) is the radial growth of fungus in dual culture after 8 days of incubation.

Statistical analysis

To determine differences in radial growth between samples and controls, data were subjected to analysis of variance (ANOVA) using the XLSTAT Version 2016.02.28451 (www.statsoft.com) and means of parameters were compared using Tukey's test (P=0.05).

Results and discussion

Phylogenetic position of the rhizobial strains

The phylogenetic tree, corresponding to concatenated housekeeping genes *glnII* and *recA,* showed that the strains were clustered into the same groups with high bootstrap support of 99, closely related to reference strain *S. meliloti* USDA 1002^T (Fig. 1.).

This finding is in agreement with Del Papa *et al.* (1999), Silva *et al.* (2007), Zribi *et al.* (2014), Kang *et al.* (2018) and Tabares-da Rosa *et al.* (2019), who reported that *S. meliloti* is the predominant symbiont nodulating perennial alfalfa.

Table 1. Inhibition of fungal species growth by endophytic bacteria associated with roots of *Medicago sativa.*

	Relative inhibition (%)		
	E543	O211	E141
Alternaria sp.	62,963 a	0,000 c	57,407 a
Fusarium sp.	45,519 b	55,185 a	55,625 a
Curvularia sp.	23,989 c	24,826 b	44,981 a
Pr > F	0,000	0,000	0,224
Significatif	Yes	Yes	No

Identification of fungi

This study showed that alfalfa plants could be attacked by several fungal pathogens despite the dry climatic conditions of the study area. In total, three fungal diseases of different species have been identified and represented by: *Alternaria sp., Fusarium sp.* and *Curvulria sp.*

Fusarium sp.

Symptoms on plants begin with yellowing and wilting, until the whole plant dries up, due to root and crown rot. On roots, wilt can be identified by the dark brown-reddish coloration in the center of the taproot (Fig. 2A.) (Orloff *et al.*, 1997). The isolates produced, on PDA medium, yellow to light brown pigments mainly in the center of the colony covered with white cotton rich in mycelium (Fig.2B.) as indicated by Ignjatov *et al.* (2018). Morphological analysis of the microscopic structure of the isolates showed fine partitioned hyphae with numerous ramifications. Micro and macroconidia are elliptical to cylindrical in shape, round, smooth and isolated chlamydospores or in pairs at the ends of hyphae or with them (Fig.2C.) (Teixeira *et al.*, 2017).

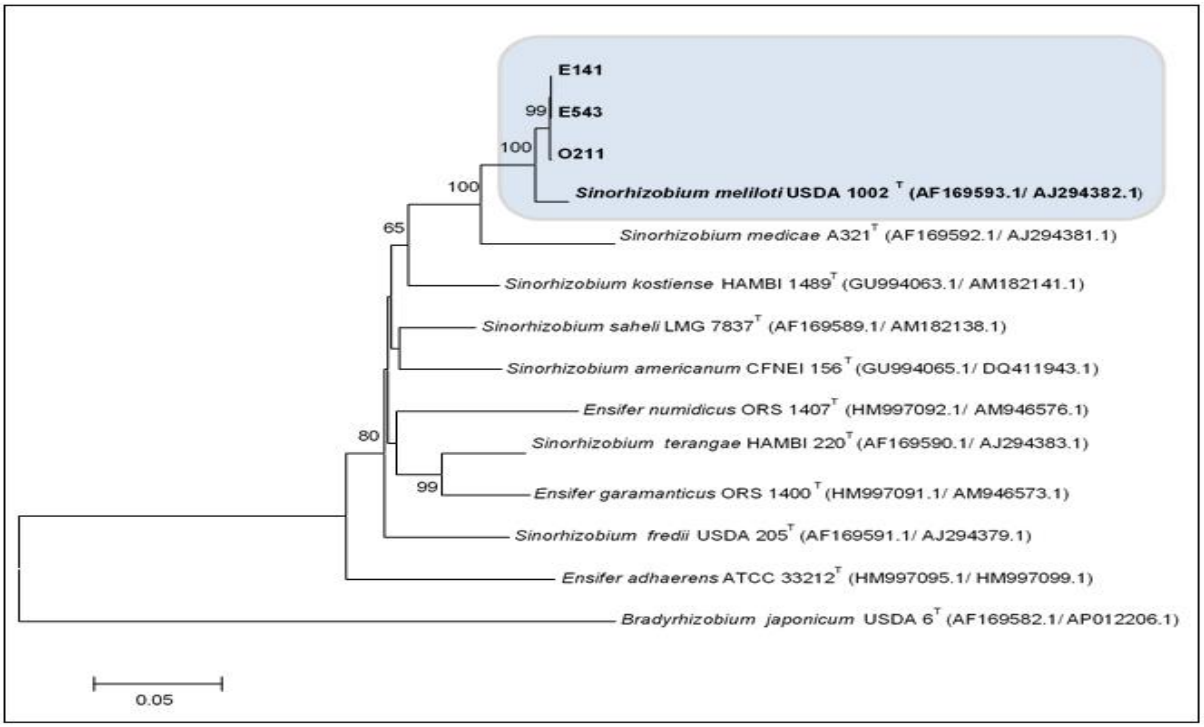

Fig. 1. Neighbor-joining tree constructed from concatenated housekeeping genes *glnII* and *recA* (985 bp) showing phylogenetic relationships of the studied strains and related species of the *Sinorhizobium–Ensifer* group. Bootstrap values (1000 replicates; only values over 50 % are given) are indicated above the branches. *Bradyrhizobium japonicum* USDA6^T was used as an outgroup. Type strains are indicated with a superscript ^{T}.

Alternaria sp.

The symptoms seen on plants are characteristic of *Alternaria* blight (Fig. 3A.). Necrotic lesions have a greyish-brown center surrounded by a halo on the leaves and stem, as described by Kgatle *et al.* (2020).

The isolate on PDA medium shows colonies that are light gray in color and then turn dark green to black with a white margin (Fig. 3B.). Their lapels are black and their growth is rapid (Yu *et al.*, 2015). Under the microscope, the fungus presents a partitioned mycelium associated with the presence of multicellular conidia in irregular brown chains, often club-shaped, partitioned longitudinally and transversely, with simple or branched dark sympodial growing conidiophores (Fig. 3C.). These morphological characteristics agree with that cited by Woudenberg *et al.* (2013).

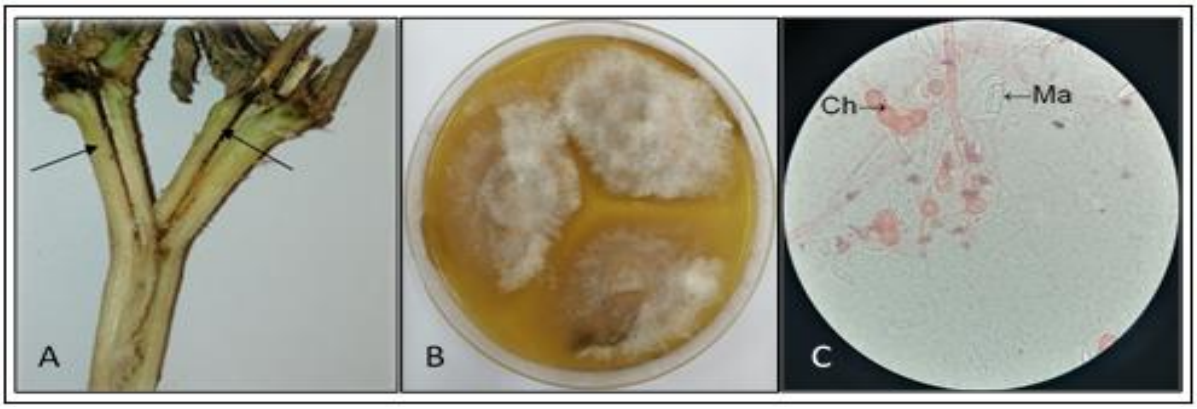

Fig. 2. Symptoms of *Fusarium sp.* on alfalfa roots indicated by arrows (A), colony morphology on PDA medium (B) and microscopic characteristics (G x100) showing macrospores (Ma) and Chlamydospores (Ch) after staining with Congo red (C).

Curvularia sp.

The symptoms on the plant are those characteristics of *Curvularia* (Fig. 4A.). As described by Zhang *et al.* (2017), we observed dark brown circular or V-shaped spots surrounded by yellow margins. On Petri dish, the colonies are circular with a slightly wavy margin, greenish-brown with a colorless edge, paler with age, abundant aerial mycelium, very flaky with age and underside dark brown to greenish-black, blacker with age (Fig. 4B.).

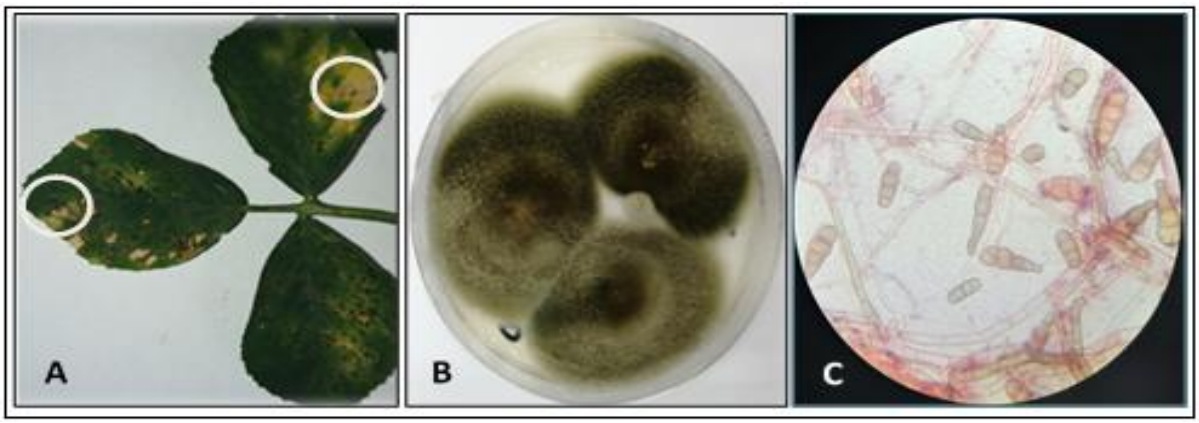

Fig. 3. Symptoms of *Alternaria sp.* on alfalfa leave shown in circles (**A**), colony morphology on PDA medium (B) and microscopic morphology (G x100) showing characteristic conidia (**C**).

This description agrees with those of Mehrabi-Koushki *et al.* (2018), Kiss *et al.* (2019) and Kee *et al.* (2020). Morphological characteristics show septate and smooth hyphae.

The conidiophores are erect, branched and the conidiogenous cells are integrated, terminal with sympodial proliferation (Fig. 4C.). In general, central cells were broader and darker than the end cells.

In vitro antagonistic activity

Our results revealed that sinorhizobial strains, E543, O211 and E141, were able to inhibit radial growth of all fungal species on PDA medium at 28°C when compared to the negative control without rhizobia, except in the case of O211 strain with *Alternaria sp.* which showed no inhibition of fungal growth (Table 1), so they may have the potential to act as biocontrol agents.

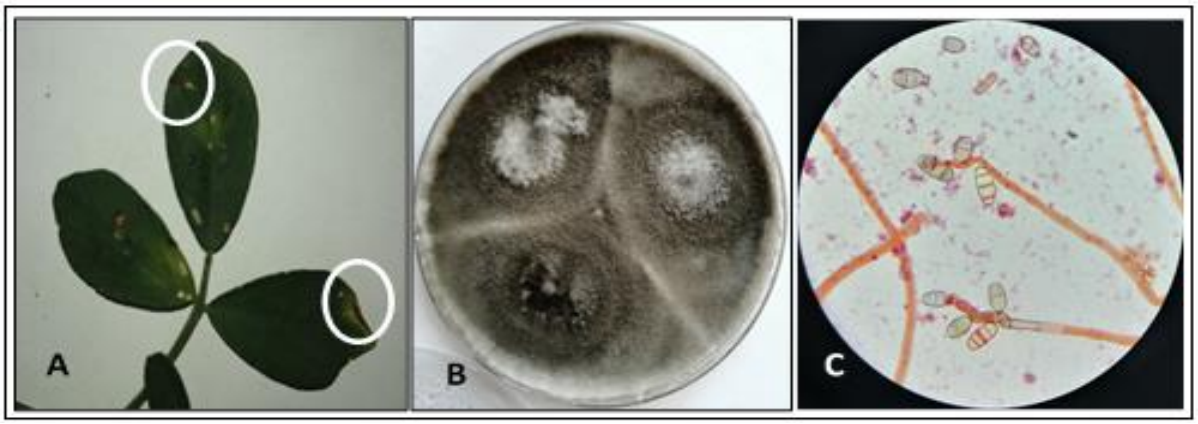

Fig. 4. Symptoms of *Curvularia sp.* on alfalfa leave shown in circles (**A**), colony morphology on PDA medium (B) and microscopic morphology (G x100) showing characteristic conidia (**C**).

Results revealed inhibition of *Curvularia sp.* at 31.27%, *Alternaria sp.* at 40.12% and *Fusarium sp.* at 52.11%, respectively. In several studies, *S. meliloti* was indicated as a common species that inhibits 50% of *F. oxysporum* growth *in vitro* (Antoun *et al.* 1978), 60 % of *P. medicaginis (*Mrabet *et al.*, 2011), and can

also hinder the growth of pathogenic fungi through the production of toxic metabolites (Sharif *et al.* 2003), such as chitinase, b-1,3- glucanase, siderophores, phosphate solubilization, indole acetic acid, amino cyclopropane carboxylate (ACC) deaminase enzymes and hydrocyanic acid (HCN).

These metabolites play a significant role in the rhizosphere and influence plant health and growth (Chandra *et al.,* 2007).

Conclusion

The symbiosis between rhizobia and legumes can reduce the dependence of host plants on nitrogen fertilizers and can decrease the amount of fungicide needed by their antagonistic action. The present study indicated that strains of *S. meliloti* significantly reduced the growth of *Curvularia sp.*, *Alternaria sp.* and *Fusarium sp.* In the field, biological control by rhizobia seems very rare. It is essential to continue the screening process to obtain rhizobia strains with strong antagonistic potential and to improve their survival and competitiveness once introduced as a biological control agent.

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