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Optimizing growth and conidia production of *Cercospora medicaginis*

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Summary. The fungus *Cercospora medicaginis* is pathogenic to annual and perennial *Medicago* species. It grows slowly and produces only few conidia. To test the pathogenicity or virulence of *C. medicaginis* and to breed resistant lines of *Medicago truncatula* we optimized *in vitro* the growth and the conidium production of four isolates of *C. medicaginis* derived from *M. truncatula* and *M. polymorpha*. Of the eight media tested, that with wheat bran juice (WBJ) yielded optimal growth and conidium production with most strains. The optimum growth temperature on WBJ medium was 25–30°C. Growth and conidia production were better in conditions of alternating light and darkness than with constant darkness. The best growth in the liquid WBJ medium occurred at pH 6–7, but the greatest number of conidia in that medium was obtained at pH 8–9.

Key words: culture medium, pH, photoperiod, temperature.

Introduction

Medicago truncatula and *M. polymorpha* are widespread in Tunisia; the former is present from north to south (Badri *et al.*, 2007), the latter only in the north (Abdelkefi *et al*. 1996). These species represent important genetic resources (Thoquet *et al.*, 2002) that can be exploited for improving other, related legumes (Aubert *et al*., 2006).

In Tunisia, annual *Medicago* species are subject to several diseases, including summer black stem, and leaf spot (Djébali and Aouani, 2004) caused by *Cercospora medicaginis* Ellis & Everh.

Many studies reported that several *Cercospora* species exhibit slow growth and low conidia production when they are cultured on synthetic media. For pathogenicity or virulence studies, a supply of homogeneous, concentrated inoculum is important. In this study, we tried to optimize *in vitro* the conditions for the growth and conidium production of local *C. medicaginis* strains to produce inoculum for plant infection tests and for the selective breeding of *M. truncatula* lines resistant to this pathogen.

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Materials and methods

Fungal material

Four monospore cultures of *C. medicaginis*, BrMp, SoMt, AmMt and Jr13Mt, were recovered from leaves and stems of *M. truncatula* and *M. polymorpha* plants exhibiting summer black stem and leaf spot under natural conditions. The fungal strains obtained were maintained by monthly subculture on PDA (Pronadisa, Madrid, Spain) medium.

Growth conditions and parameters measured

The effect of culture medium, temperature,

Table 1. The composition of the culture media used.

Medium	Abbreviation	Composition (per litre of distilled water)
Potato dextrose agar	PDA	24 g of Potato Dextrose Broth (PDB, Pronadisa) and 15 g of Agar (Pronadisa).
	½ PDA	12 g of PDB and 15 g of agar.
V8 juice	V8J	200 ml of V8 juice, 3 g of $CaCO_3$ and 15 g of agar
Synthetic nutrient-poor agar	SNA	1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose and 15 g of agar.
Carrot juice agar	CJA	20 g chopped carrots soaked in 1 L distilled water for 1 h, and boiled for 5 min; 15 g of agar added.
Wheat bran juice	WBJ	24 g of wheat bran were boiled for 1 h in 0.5 L distilled water. Filtrate adjusted to 1 L and 15 g of agar added.
Medicago crushed seed	MCS	24 g of finely crushed *Medicago sativa* seeds and 15 g of agar in 1 L water.
Medicago leaf juice	MLJ	300 g fresh leaves of *Medicago* boiled for 1 h in 0.5 L distilled water. Filtrate was adjusted to 1 L and 15 g of agar added.

photoperiod and pH on mycelium growth and conidia production of the four *C. medicaginis* strains was studied. The fungus was grown on eight media (Table 1). The media were brought to pH 7 and then autoclaved at 121°C and 1.5 bars for 24 min. The fungus was sub-cultured in 90 mm Petri dishes containing solid media and grown at 25°C in constant darkness, or with a 16 h day produced by neon tubes that gave an overall intensity of 8000 lux. Cultures were grown on solid WBJ medium (pH 7) in constant darkness at 5, 15, 20, 25, 30 and 35°C. The colonies grown at 25°C were compared with colonies grown in constant darkness and with colonies grown with a 16 h day. Each of the four strains was also grown in 50 mL liquid WBJ medium at the pH ranges 4–5, 6–7 and 8–9. The pH was adjusted according to Papavizas and Davey (1960) using sterile 0.1 N HCl or NaOH solutions. The cultures were grown in a shaker (120 rpm) at 25°C with a 16 h day.

Growth and conidium production were assessed after 4 weeks of culture. The colony diameter of the culture was measured on the solid media, and the dry weight of the mycelium was measured on the liquid media. Conidia produced on the solid media were counted using a Thoma haematocymeter after scraping the culture in 10 ml of distilled water, and conidia in liquid media were counted directly by sampling the medium.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using Statistica software Version 5.1

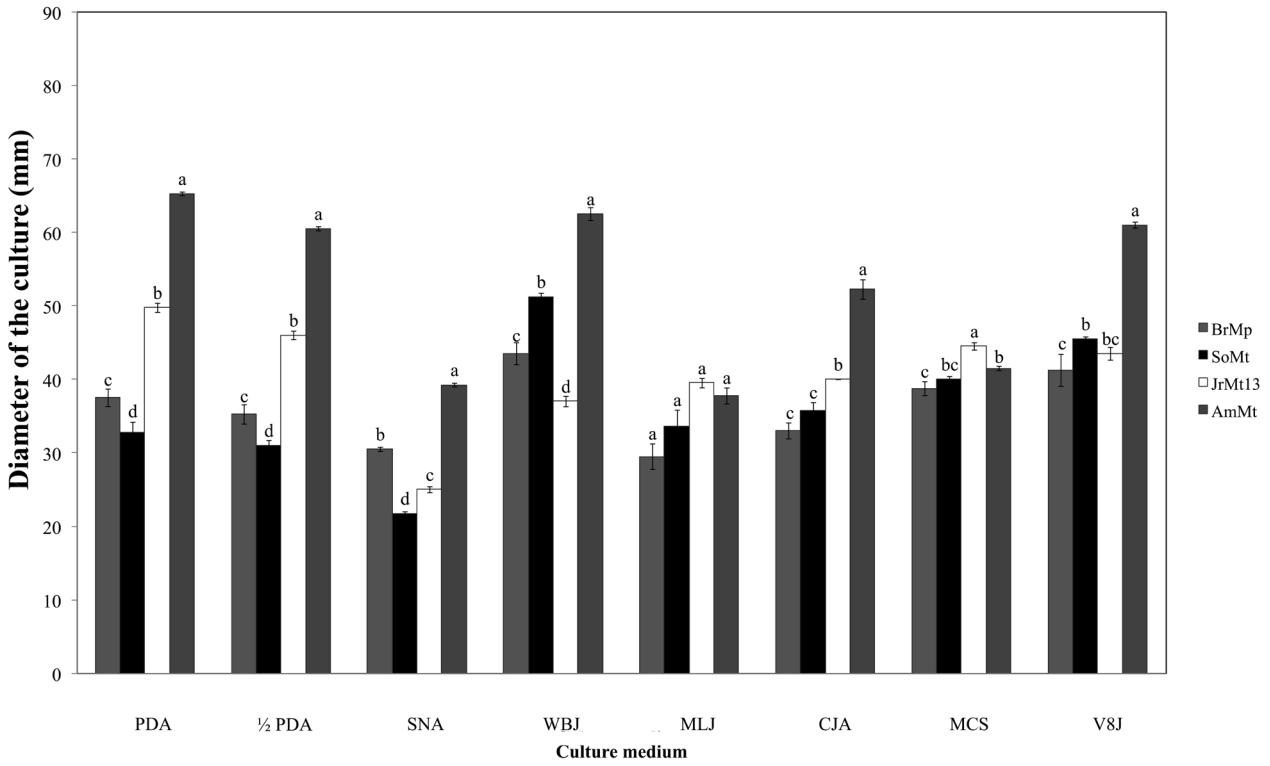

Figure 1. Growth of four strains of *Cercospora medicaginis* on different solid media. Potato dextrose agar (PDA and ½ PDA), synthetic nutrient-poor agar (SNA), wheat bran juice (WBJ), *Medicago* leaf juice (MLJ), carrot juice agar (CJA), *Medicago* crushed seed (MCS) and V8 juice (V8J).

(www.statsoft.com) and the means were compared with the Duncan multiple range test ($P \leq 0.05$). Correlations between the parameters were determined by computing the Pearson correlation coefficient (r). Four replicates were used to study the effect of the medium, the temperature, the photoperiod and the pH on fungal growth and conidia production.

Results

The type of medium, temperature, photoperiod and pH all significantly affected both the growth and the sporulation of *C. medicaginis* (Figure 1–3). Strains BrMp and SoMt grew most rapidly on WBJ medium (Figure 1). Strain Jr13Mt grew best on the PDA-based media. AmMt grew well on PDA, V8J and WBJ. The lowest growth for the four strains was recorded on SNA. Media containing potato (PDA and ½PDA) and *Medicago* leaf juice (MLJ) did not cause production of conidia. SNA, CJA, MCS, WBJ and V8J caused at least one strain to sporulate. The solid WBJ medium was the only medium that caused sporulation of two strains, BrMp and SoMt.

Strains SoMt and BrMp from northern Tunisia had similar growth at 25°C and at 30°C on solid WBJ medium, while Jr13Mt and AmMt from southern Tunisia grew best at 30°C on this medium (Figure 2A). With the SoMt, Jr13Mt and AmMt strains, growth was higher with a 16 h day than under constant darkness (Figure 2B). Under constant darkness no strain produced conidia on solid WBJ medium, but with a 16 h day BrMp and SoMt did so (data not shown).

In liquid WBJ medium, all *C. medicaginis* strains grew best at pH 6–7 and worst at pH 4–5 (Figure 3A). The pH range 8–9 was best for spor-

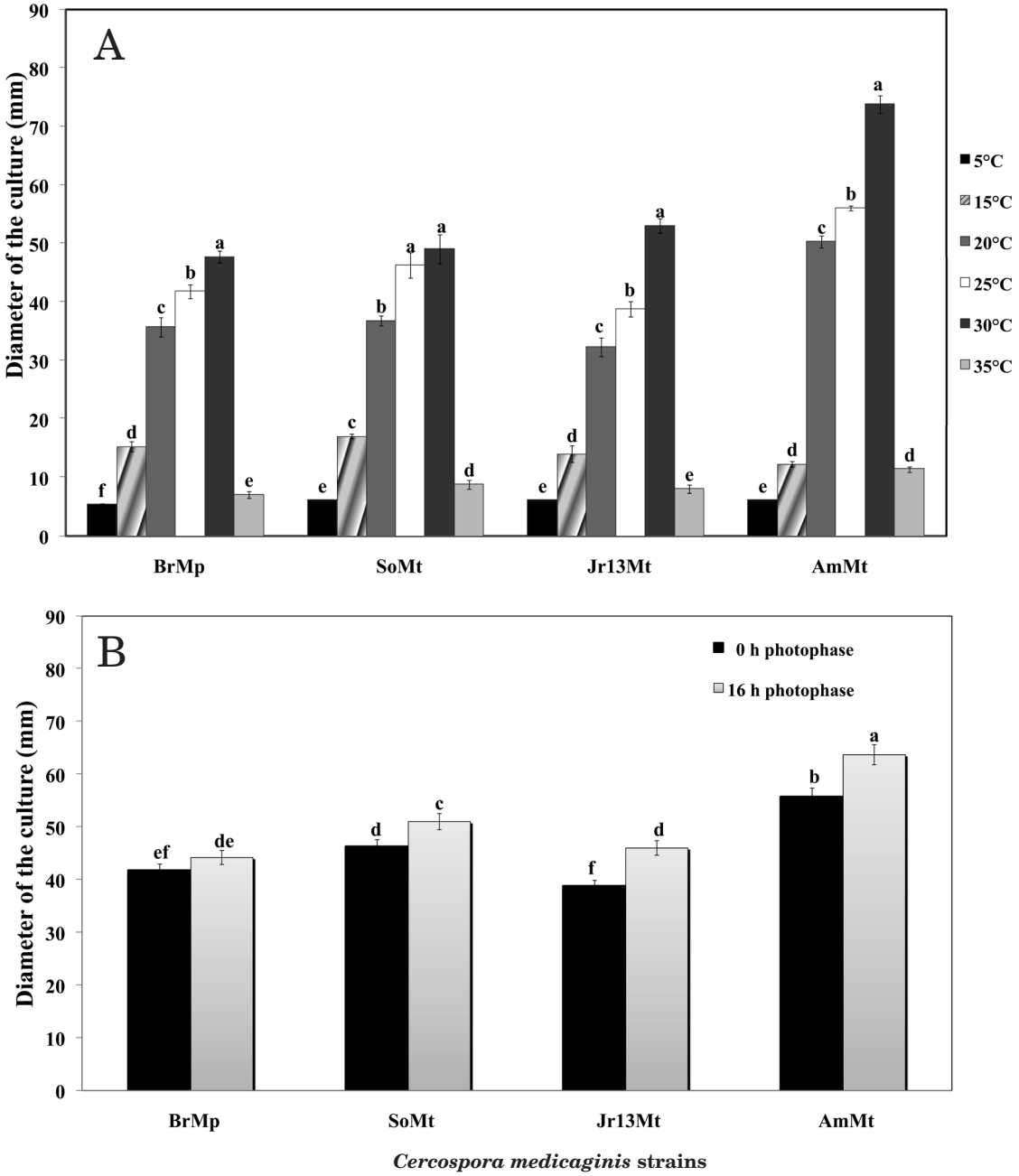

Figure 2. Growth of four strains of *Cercospora medicaginis* at different temperatures (A) and with different lighting (B).

ulation of conidia in BrMp, Jr13Mt and AmMt, while for SoMt it was better at pH 4–5. For all four strains, production of conidia was lowest at pH 6–7 (Figure 3B). No significant correlation was found between growth and conidium production of any of the four *C. medicaginis* strains and the medium, the photoperiod or the pH.

Discussion

The media PDA, SNA, CJA, WBJ and VJ8 were chosen because previous studies had found that they positively influenced conidia production of the genus *Cercospora* (Nagel, 1934; Stavely and Nimmo, 1969; Dhingra and Sinclair, 1995; Adese-

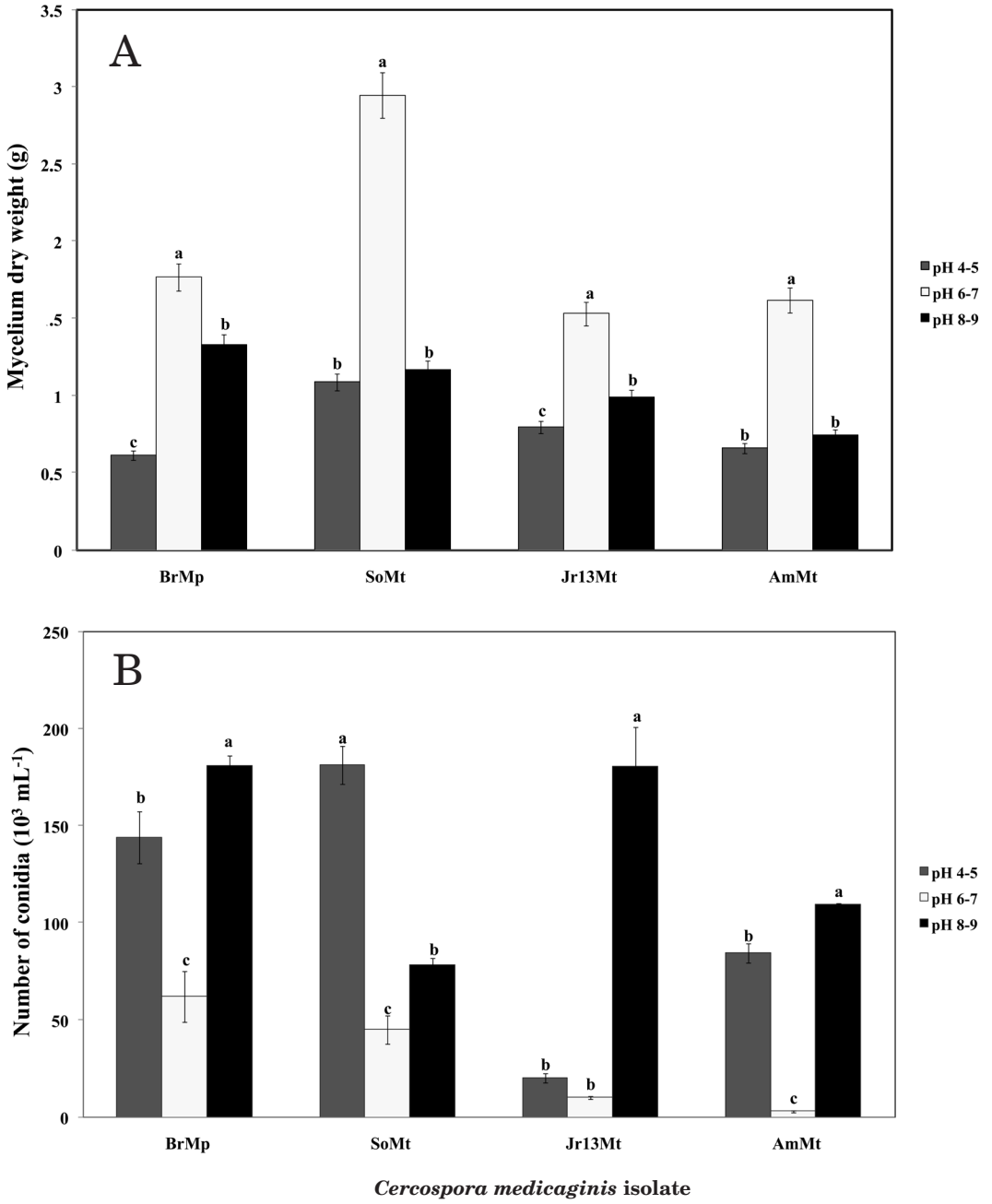

Figure 3. Mycelium dry weight (A) and conidia production (B) of four *Cercospora medicaginis* strains after 4 weeks of culture on a wheat bran-juice based medium with different pH ranges.

moye and Adedire, 2005; Hassan and Bullerman, 2009), while the MLJ and MCS media are reported in this study for the first time. The fungus grew well on most media, especially PDA and WBJ. PDA is known to favour the growth of a number of *Cercospora* species, such as *C. cerasella* (Nagel, 1934), *C. zeae-maydis* (Beckman and Payne, 1983) and *C. beticola* (Gargouri, 2004). On PDA no conidia were produced by any *C. medicaginis* strain, but on WBJ sporulation was substantial. This last medium is reported to cause high levels of macroconidia in a number of *Fusarium* species (Hassan

and Bullerman, 2009). V8J, SNA and MCS also enabled some strains of *C. medicaginis* to produce conidia. V8J is reported to favour sporulation of several *Cercospora* species (Stavely and Nimmo, 1968; Beckman and Payne, 1983). MLJ did not cause sporulation of any *C. medicaginis* strain. This is consistent with Nagel (1934) and Wyss *et al*. (2001), who reported that adding the plant host leaves to the medium did not cause sporulation in *C. cerasella*.

With most *C. medicaginis* strains, growth and conidia production were greater with a 16 h day than when the strains were grown under constant darkness. This is consistent with Beckman and Payne (1983), who found that the alternation of light and darkness was more conducive to conidia production of *C. zeae-maydis* than either constant light or constant darkness.

Cercospora medicaginis grew best at pH 6–7, but the number of conidia was greatest at pH 8–9 in liquid WBJ medium. A similar discrepancy between the optimum pH for growth and for sporulation was also found by Gargouri (2004) with *C. tripolitana, C. beticola* and *C. bizzozeriana*. Conidia production on solid WBJ medium at pH 6–7 was lower than in liquid WBJ medium at that pH range. Similarly, Gargouri (2004) obtained more conidia when he grew *C. beticola* and *C. bizzozeriana* on liquid V8J medium than when he grew these species in V8J supplemented with 2% agar. Nagel (1934) suggested that higher levels of compounds secreted by the fungus on the solid medium caused the degradation of the conidial cell walls.

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