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Temperature Effect on Mycelial Growth and on Disease Incidence of *Fusarium oxysporum* f.sp. *radicis-lycopersici*

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Abstract: Fusarium crown and root rot of tomato (*Lycopersicon esculentum*) caused by *Fusarium oxysporum* f.sp. *radicis-lycopersici* is a new damaging disease of greenhouse crops in Tunisia. This pathogen was detected in Southern Tunisia, where temperature is usually high. Thus, temperature effect on its development was studied *in vitro*, on mycelial growth and *in vivo*, on disease incidence. Temperature effect on mycelial growth of *Fusarium oxysporum*. sp. *radicis-lycopersici,* evaluated on Potato Dextrose Agar (PDA) media, revealed that this pathogen grows well at temperatures ranged from 20 to 30°C. However, the optimum of mycelial growth was recorded at 25°C (growth speed exceeded 13 mm D^{-1}). On disease incidence, temperature effect was evaluated by breeding inoculated plants under two thermal conditions: 19 and 29°C. Damages observed, after an incubation period ranged between 30 and 50 days, were more important at 19°C than that at 29°C. Indeed, at 19°C, disease incidence exceeded 96% and it was always more than 70%. At 29°C, however, this value didn't exceed 60% and this by using two inoculation methods.

Key words: *Fusarium*, tomato, temperature, disease incidence

INTRODUCTION

In Tunisia, tomato culture is an important crop vegetable; indeed, the surface currently reserved for this culture is about 19,1 thousand ha, representing 13,7% of the total surface reserved for vegetable cultures. However this culture is always confronted to parasitical attacks, especially in protected cultures. During 2000-2001 crop season, a new disease on tomato was observed in "5ème saison" exploitation located in Hammet Gabès in Southern Tunisia, affecting more than 90% of plants in some greenhouses.

Caused by *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL), (Hajlaoui *et al.,* 2001; Hibar, 2002), this disease is characterized by wilt at fruit ripening, cortical rot at the soil level, vascular discoloration of the lower stem and conspicuous pinkish masses of conidia along the stem (Jarvis, 1988). It constitutes thus a menace for tomato production despite the use of cultivars known for their resistance to several diseases (Larkin and Fravel, 2002).

Although it's relatively recent apparition, this pathogen was the subject of several studies concerning its epidemiology and its geographical distribution (Chérif and Benhamou, 1990). Climatic conditions which favour its development are different from those required by *Fusarium oxysporum* f.sp. *lycopersici* (FOL) which prefer high temperatures (Blancard, 1991). Known to grow well at fresh temperature raging from 18 to 20°C (Veschambre, 1995), FORL was detected in tomato greenhouses heated with geothermal water in Southern Tunisia where temperature is usually high. The main question that can be proposed: Are FORL isolates, obtained from these greenhouses, adapted to high temperatures characterizing this type of culture?

The objective of this research is to study the effect of incubation temperature on mycelial growth of four local FORL isolates as well as on the disease incidence.

MATERIALS AND METHODS

Fungal isolates: FORL isolates used in this study were recovered from tomato plants showing typical crown and root rot symptoms at "5ème saison" exploitation in Hammet Gabès in Southern Tunisia where tomato culture heated with geothermal water is practised.

Fungal pathogen was isolated by planting plant tissues (surface-disinfected with 1% sodium hypochlorite for 2 min) on PDA (Potato Dextrose Agar) and incubating them at 25°C for 5 days (Katan *et al.*, 1991). Isolates were

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Table 1: FORL isolates used for study

Isolates	Host plant (Cultivar)	Date of isolation
Fo1.01	Durintha	2001
Fo2.01	Durintha	2001
Fo3.02	Elena	2002
Fo4.02	Bochra	2002

Table 2: Tomato cultivars used for pathogenicity tests

Cultivars	Abbreviation	Characteristics[y]
RIOGRANDE	CvRi	R to FOL race1 and S to FORL
ROXANE	CvRo	R to FOL races 1, 2 and S to FORL
MARIA	CvM	R to FOL races 1, 2 and S to FORL.
ELKO	CvE	R to FOL races 1, 2 and S to FORL.

[y]R : resistant, [y]S : sensitive, [y]FOL: *Fusarium oxysporum* f.sp. *lycopersici* [y]FORL *Fusarium oxysporum* f.sp. *radicis-lycopersici*

identified as *F. oxysporum* morphologically based on characteristics of the macroconidia, phialids, microconidia, chlamydospores and colony growth traits (Messiaen and Cassini, 1968). The *forma specialis* of this pathogen was identified using pathogenicity tests (Hibar, 2002). Based on these tests, the more aggressive isolates were selected for this study. The four isolates used in *in vitro* and *in vivo* tests are presented in Table 1.

Tomato cultivars: Tomato cultivars (*Lycopersicon esculentum* Mill.) used in this study, are selected basing on their sensitivity to FORL (Benhamou *et al*., 1997).

Selected cultivars and their characteristics are presented in Table 2.

Temperature effect on mycelial growth: Temperature effect on mycelial growth of this pathogen was evaluated *in vitro* at various incubation temperatures (10, 15, 20, 25, 30, 35 and 40°C) on PDA (Potato Dextrose Agar), the best media for mycelial growth of FORL (Hibar, 2002). For all the experiment, agar discs of 6 mm diameter, taken on a young FORL culture, were deposited in the center of Petri plates. The speed of mycelial growth was calculated by measuring the average diameter of each colony (two perpendicular diameters) from which we subtract the initial inoculum's diameter.

In this essay, colonies diameters were measured on 5 Petri plates and the experiment was repeated twice. The experimental device used in this essay is the one of a factorial test with two variables: factor A represents incubation temperatures and the factor B corresponds to FORL isolates.

Temperature effect on disease incidence: Basing on the biology of the two *forma specialis* (FORL and FOL), a pathogenicity test was conduced under two different temperatures: 19°C, temperature more favourable for FORL and 29°C which is preferred by FOL (Blancard, 1991).

Seeds of each cultivar were sterilised by immersion in absolute ethanol for 7 min, followed by extensive rinsing in sterile distilled water (Benhamou *et al*., 1997). After drying, seeds were put aseptically in Petri plates, containing filter papers soaked with sterile distilled water. Seed pre-germination was ensured by incubating them at 20°C during 4 to 5 days.

Once pre-germinate, seedlings were treated differently depending on the used inoculation method.

Root-dip inoculation: Root-dip inoculation requires a seedlings breeding. Pre-germinate seeds were sown in alveolus plates filled with previously sterilised peat. Seedlings were grown in a growth chamber at 24 to 26°C with 12 h photoperiod and 70% humidity. They were watered daily and fertilized twice a week with a standard nutrient solution according to Pharand *et al.* (2002). Experiments were performed with 5-week-old tomato plants carrying five or six fully expanded leaves (Benhamou and Bélanger, 1998).

Seedlings were uprooted from the substrate; their roots washed in tap water, rinsed with sterile distilled water and dipped in fungal suspension containing 10^7 spores mL^{-1} for 30 min. Seedlings of each cultivar, dipped in sterile distilled water, served as un-inoculated control. Once inoculated, seedlings were transplanted to plastic pots (7.5 cm diameter) filled with previously sterilized perlite. Transplanted Seedlings were grown in two growth chamber at 19 and 29°C with 12 h photoperiod. Seedlings were watered once every two days using the same nutritive solution (Pharand *et al*., 2002).

The disease severity was recorded on 0 to 3 visual scale, in which 0 = no symptoms; 1 = light yellowing of leaves, light or moderate rot on taproot and secondary roots and crown rot; 2 = moderate or severe yellowing of leaves with or without wilting, stunting, severe rot on taproot and secondary roots, crown rot with or without hypocotyls rot and vascular discoloration in the stem; and 3 = dead seedlings (Vakalounakis and Fragkiadakis, 1999).

Disease incidence percentage was determined using the following formula (Song *et al*., 2004):

$$\text{Disease incidence}(\%) = \left[\frac{(\sum \text{scale} \times \text{No. of plants inf ected})}{(\text{highest scale} \times \text{total No. of plants})}\right] \times 100$$

"Soft" or non traumatising plant inoculation: In order not to stress seedlings when inoculating, we have proceeded with another inoculation method which requires the following steps:

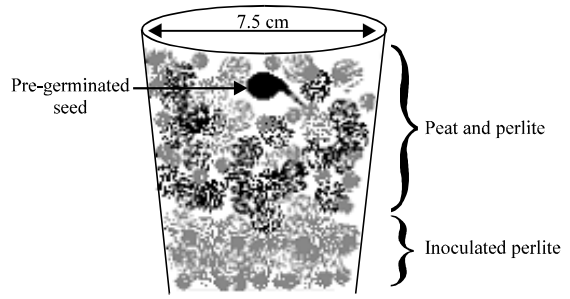

Fig. 1: Schematic representation of the experimental material used in " Soft inoculation"

Inoculum preparation: After the pathogen was cultured in the PDB (Potato Dextrose Broth), a spore suspension of 10^7 spores mL^{-1}, determined using a Malassez Blade, was obtained. 10 mL of this conidial suspension was served to inoculate 600 cm^3 of perlite (enriched with 200 mL of PDB) prepared in Roux boxes. Infested perlite, prepared in these boxes and incubated for four weeks at 25°C was served to inoculate tomato plants.

Seedlings inoculation: Pre-germinate seeds were transplanted to plastic pots (7.5 cm in diameter) containing inoculated perlite in the bottom level and a mixture (50; 50%) of peat and perlite, previously sterilized, in the remaining area (Fig. 1). After transplanting, plants were incubated under two thermal conditions: 19 and 29°C.

Symptoms were evaluated 50 days after transplanting using the same visual scale described above.

Statistical analysis: Experimental design used to determine the effect of temperature on disease incidence was a complete plan with three factors. Factor A corresponds to incubation temperatures, factor B represents tomato cultivars and the factor C corresponds to the four FORL isolates and an un-inoculated control. The number of repetitions was 10 seedlings by elementary treatment. Statistical analyses for data from all assays were conduced using the general linear models procedures (GLM) of the Statistical Package for the Social Science (SPSS 10.0). Experiments were analysed using the standard analysis of variance (ANOVA) with factorial treatment structure and interactions. Significance was evaluated at $p<0.05$ for all tests. Means separation was accomplished using Student-Newman-Keuls (SNK) test.

RESULTS

Effect of incubation temperature on mycelial growth: In order to know the best temperature for mycelial growth of FORL, seven temperatures, varying from 10 to 40°C with an interval of 5°C were tested.

Variance analysis revealed a significant interaction ($p<0.05$) between incubation temperatures and FORL isolates. Indeed; results obtained show that the highest growth speed was recorded at 25°C (>13 mm D^{-1}); whereas, this value is nil for extreme temperatures (10 and 40°C) (Fig. 2).

At 20 and 30°C, mycelial growth was considerable and it was about 10 mm D^{-1} for the two temperatures and the four isolates. However, mycelial growth was relatively low at 15 and 35°C and it ranged between 2 and 4 mm D^{-1}.

It results from this that low temperatures and those very high slow down FORL development. This proves that climatic conditions, particularly the temperature, could have an effect on disease expression.

Temperature effect on disease incidence: In order to know the best temperature for FORL development, inoculated plants were grown under 2 thermal conditions (19 and 29°C).

Variance analysis for the first test (root-dip inoculation) revealed a high significant effect only for the temperature factor. Indeed, results obtained show that damages caused by this pathogen were more important at 19°C than that at 29°C (Table 3). At 29°C, disease incidence did not exceed 60% for the four used cultivars however, at 19°C this percentage was, for the most cases, more than 80%.

The same test repeated for the same FORL isolates and the same tomato cultivars by varying the inoculation method ("soft" inoculation) and similar results were obtained (Table 4). Indeed, variance analysis revealed a significant effect only for the temperature factor ($p<0.05$).

Results obtained show that tomato cultivars were more damaged at 19°C than that at 29°C (Table 4). At 19°C, disease incidence ranged between 76.67 and 96.67% and for the most cases it was more than 80%. At 29°C however, disease incidence didn't exceed 57% and it ranged between 43.33 and 56.67%.

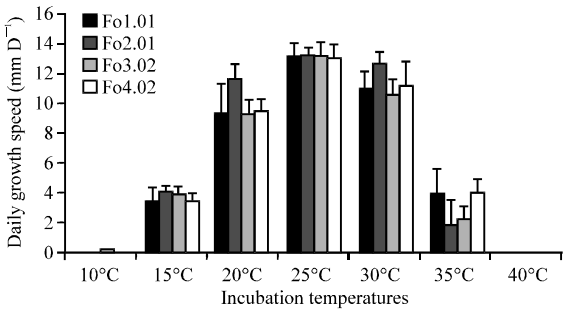

Fig. 2: Daily growth speed of four FORL isolates incubated at various temperature for 6 days.

Table 3: Disease incidence on tomato cultivars at 19°C and 29°C, 30 days after root-dip inoculation

	Disease incidence (%)*							
	CvRi		Cv Ro		CvM		CvE	
	19°C	29°C	19°C	29°C	19°C	29°C	19°C	29°C
Fo1.01	80	43.33	76.67	46.67	80	40	70	46.67
Fo2.01	76.67	50	80	50	76.667	46.67	76.67	50
Fo3.02	83.33	50	90	53.33	83.33	50	70	43.33
Fo4.02	86.67	53.33	86.67	50	86.67	53.33	86.67	56.67
Un-inoculated plants	0	0	0	0	0	0	0	0

Table 4: Disease incidence on tomato cultivars at 19°C and 29°C, 50 days after "soft" inoculation

	Disease incidence (%)*							
	CvRi		Cv Ro		CvM		CvE	
	19°C	29°C	19°C	29°C	19°C	29°C	19°C	29°C
Fo1.01	86.67	43.33	80	46.67	86.67	43.33	76.67	46.67
Fo2.01	90	46.67	83.33	50	83.33	46.67	86.67	53.33
Fo3.02	93.33	50	86.67	43.33	93.33	53.33	80	56.67
Fo4.02	96.67	53.33	90	53.33	90	50	83.33	50
Un-inoculated plants	0	0	0	0	0	0	0	0

* Disease incidence percentage was determined using the formula presented in materials and methods basing on 0 to 3 visual scale, in which 0 = no symptoms; 1 = light yellowing of leaves, light or moderate rot on taproot and secondary roots and crown rot; 2 = moderate or severe yellowing of leaves with or without wilting, stunting, severe rot on taproot and secondary roots, crown rot with or without hypocotyls rot and vascular discoloration in the stem and 3 = dead seedlings. Disease incidence was calculated on 10 plants per elementary treatment

These results show that FORL was more virulent at fresh temperatures than that at high ones. However, at high temperatures (29°C) damages were also important and they were close to 50%.

DISCUSSION

In Tunisia, a new disease was detected during 2000-01 crop season in the "5ème saison" exploitation in Southern Tunisia practicing tomato soilless culture heated with geothermal water. Caused by FORL (Hajlaoui *et al*., 2001; Hibar, 2002), this pathogen caused serious damages reaching 90% in certain greenhouses. This pathogen, known to grow well in fresh temperature (Blancard, 1991), was detected in Southern Tunisia where temperature can easily rich 50°C. In order to have an explanation to the occurrence of FORL in these regions, the biology of this pathogen, especially temperature effect was studied *in vitro* and *in vivo*.

Results obtained showed that an optimum of mycelial growth was obtained at 25°C. However, FORL was able to grow at 30°C and even at 35°C. These results showed that FORL was able to develop at high temperatures and this may explain its appearance in Southern Tunisia where temperature is always more than 30°C.

The effect of temperature on mycelial growth and disease incidence of two species of *Verticillium* (*V. albo-atrum* and *V. dahliae*) was studied by Jbnoun-Khiareddine *et al.* (2006). Authors showed that the two *Verticillium* species were able to grow from 10 to 30°C. Tested *in vivo*, the index of leaf damage differs significantly depending on *Verticillium* species and temperature ranges. Indeed, *V. albo-atrum* was more virulent at 17-21°C; however, *V. dahliae* was more virulent at 21-30°C. Similarly, Boughalleb (2001) concluded that *Fusarium oxysporum* f.sp. *niveum* has the highest mycelial growth at 25°C but also develops well at 20 and 30°C. Several other studies have been conducted on the effect of incubation temperatures on mycelial growth of *Fusarium* species. For example Daami-Remadi (1996) showed that 10 and 15°C block mycelial growth of 4 *Fusarium* varieties causing potato dry rot. Whereas, temperatures ranging between 20 and 30°C favour their development. She noted also that the optimum of mycelial growth ranged between 20 and 25°C for *F. roseum* and from 25 to 30°C for *F. solani var coeruleum*. Similar studies have been conducted for other pathogens to try to know the biology of some pathogens. Chase and Poole (1987) showed that temperature has an effect on mycelial growth of the fungus *Cylindrocladium spathiphylli*, the causal organism of root rot on various *Spathiphyllum* genus species. Indeed, this pathogen presented an optimum of mycelial growth at 27°C, but a significant growth was also recorded at 17 and 22°C.

By testing temperature effect on mycelial growth of 3 races of *F. oxysporum* f.sp. *tracheiphilum*, Swanson and Van Gundy (1985) indicated that this pathogen grows well at temperatures ranged between 24 and 27°C. The same authors noted that relatively high temperatures (27°C) required by *F. oxysporum* f.sp. *tracheiphilum*, were also preferred by *F. oxysporum* f.sp. *lycopersici*, the causal agent of tomato Fusarium wilt.

Several studies demonstrated that temperature has an effect on disease development. Data obtained in this study showed that relatively fresh temperatures (19°C) were more suitable for FORL development. Similarly, by testing influence of dew period and temperature on infection of onion leaves by dry conidia of *Botrytis squamosa*, Alderman and Lacy (1983) showed that lesion production was optimal at 20°C, lower at 15°C and greatly reduced at 25°C. Similar results have been reported by Tivoli *et al.* (1983) showing that at lower temperature and humidity, *F. solani* var. *coerulum*, *F. roseum* var. *sambucinum* and *F. roseum* var. *arthrosporioides*, causing dry rot on tomato tubers, generally continued to form conidia and mycelial filaments, whereas at higher

temperature and humidity, *F. solani* var. *coerulum* and *F. roseum* var. *sambucinum* persisted by forming resting spores. At 30°C, however, the three taxa disappeared because conidia were lysed and because the formation of resting spores was inhibited. In contrast, Bhatia and Munkvold (2002) showed that a temperature of 22 to 30°C was optimal for germination and growth of *Cercospora zeae-maydis* the causal organism of Gray leaf spot of maize.

Fresh temperatures preferred by FORL, were also required by many other pathogens such us *Botrytis cinera* which produce the greatest number of conidia at 21°C (Thomas *et al*., 1988). Similar results were obtained by Wainshilbaum and Lipps (1991) showing that Septoria leaf blotch on wheat caused by *Septoria tritici* was more important at 19 and 24°C than that at 29°C. However, *Septoria nodorum* causing the same disease was not affected by temperature and disease levels were important at 19, 24 and 29°C.

Several other studies demonstrated that temperature has an effect on disease incidence. For example Hershman *et al.* (1986) demonstrated that parsley damping-off caused by *Rhisoctonia solani*, *Pythium ultimum* and *Pythium irregulare*, was influenced by temperature. Indeed, the two *Pythium* species were highly pathogenic at 15, 23 and 30°C; *R. solani,* however, was much less pathogenic at 15°C than at either 23 or 30°C. Bosland *et al.* (1988) showed also that soil temperature has greatly influenced the virulence of the *F. oxysporum* f.sp. *conglutinans*. This pathogen was moderately virulent on sensitive cabbage seedlings at 18°C, whereas it was extremely virulent at 24°C. It was the same for *F. oxysporum* f.sp. *matthioli* which was extremely virulent on *Matthiola* seedlings at 24°C as at 18°C.

Temperature has an effect not only on disease development but also on biocontrol organisms. In fact, by testing effects of varying environmental conditions on biological control of Fusarium wilt of tomato by non-pathogenic *Fusarium* spp., Larkin and Fravel (2002) observed that the two isolates (CS-24 and CS-1) of non-pathogenic *Fusarium* spp. reduced disease incidence in the greenhouse at high temperatures (32°C), but were less effective at the optimum temperature for disease development (27°C). Landa *et al.*, (2001) showed also that Fusarium wilt of chickpea caused by *F. oxysporum* f.sp. *ciceris* was suppressed by rhizosphere bacteria only at 20 or 30°C and not at 25°C the optimal temperature for disease development.

CONCLUSIONS

During 2000- 2001 crop season a serious disease on tomato was detected in "5ème Saison" exploitation located in Hammet Gabès in Southern Tunisia causing damages reaching more than 90% in some greenhouses.

Isolations carried out from these plants and identifications of the causal organism revealed that FORL was at the origin of this disease. This pathogen, detected in Southern Tunisia, where temperature is usually high, was the subject to this study. In order to know its thermal requirements, temperature effect was studied on mycelial growth and on disease development.

Temperature effect on mycelial growth of four FORL isolates revealed that an optimum of mycelial growth was recorded at 25°C. Temperatures ranging from 20 to30°C were also favourable to FORL development. Low and high temperatures (10 and 40°C) however block its mycelial growth.

Temperature effect on disease incidence, evaluated by breeding inoculated seedlings under 2 different thermal conditions (19 and 29°C), revealed that damages were more important at 19°C than that at 29°C. These results were obtained using two different inoculation methods (root-dip inoculation and "soft" inoculation).

These results showed that relatively fresh temperatures (19°C) were more favourable to disease development. However, damages obtained at 29°C were also important showing that this pathogen can easily develop at high temperatures. The ability of this pathogen to grow at relatively high temperatures (29°C) may explain its appearance in Southern Tunisia.

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