Plant Pathology Journal

ISSN 1812-5387

science alert

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Plant Pathology Journal 5 (1): 1-6, 2006
ISSN 1812-5387

Effect of Temperature on *Verticillium* Wilts of Tomato in Tunisia

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Abstract: *Verticillium dahliae* and *Verticillium albo-atrum* have been isolated from tomato cultivars possessing the *Ve* gene in many greenhouses in the Chott-Mariem region. Two isolates of *Verticillium*, one from each species, were examined for their mycelial growth at different incubation temperatures and their pathogenicity to three tomato cultivars. Seedlings at the three leaf stage were root-dipped in a conidial suspension (10^7 conidia mL^{-1}) and maintained in two glasshouse cells at 17-21°C and 21-30°C. Disease severity was assessed through the index of leaf damage calculated 60 days after inoculation, at each temperature range. Growth test showed that *V. albo-trum* isolate as well as *V. dahliae*, were able to grow from 10 to 30°C. The three tomato cultivars exhibited typical *Verticillium* symptoms. The index of leaf damage differs significantly depending on cultivars, isolates and temperature ranges. *V. albo-atrum* was most virulent at 17-21°C; however, *V. dahliae* was more virulent at 21-30°C, despite the presence of the *Ve*-gene. These results confirmed the first report in Tunisia of a new pathotype of *V. albo-atrum* able to grow at 30°C and to infect cultivars having the *Ve* gene.

Key words: *Verticillium* sp., *Lycopersicon esculentum* MILL, inoculation, temperature, wilt

INTRODUCTION

Verticillium dahliae Klebahn, *Verticillium albo-atrum* Reinke and Berthold and to a lesser extent, *Verticillium tricorpus* I. Isaac, are of major economic importance because of the vascular wilt diseases they cause on a broad range of host plants[1,2]. These soilborne plant pathogens have a worldwide distribution[3,4] and occur especially in spring and autumn[5,6]. However, *V. albo-atrum* prefers cooler climates and *V. dahliae* the warmer latitudes, reflecting their individual temperature preferences, while *V. tricorpus* occasionally produce mild symptoms in very specializes niches[3,5-7]. All of these *Verticillium* species form characteristic resting structure: *Verticillium dahliae* has microsclerotia, *Verticillium albo-atrum* has dark resting mycelium, while *V. tricorpus* produces three resting structures: microsclerotia, dark resting mycelium and chlamydospores[2,7-9] .

Several strategies are currently used to control *Verticillium* wilt[4,10]; however, disease control through the cultivation of *Verticillium*-resistant cultivars has proven to be the most durable. These *Verticillium*-resistant tomato cultivars that carry the *Ve* resistance gene are widely used by commercial growers[4,11]. However, the reliability of this strategy has been questioned since several new virulent pathotypes of *Verticillium* sp. have appeared and were able to overcome the *Ve* resistance[4,10,11]. These new pathotypes, first described in the USA and now designated race 2, have since been isolated in California, Canada and Europe and present a chronic problem for tomato production[4,10,11].

In Tunisia, especially during spring 2002, wilt suddenly appeared in the early tomato cultivar, cv. Colibri (possessing *Ve* gene) grown under protected cultivation located in Chott Mariem region (Jabnoun-Khiareddine *et al.*, unpublished data). Isolations made from wilted plants revealed the presence of three *Verticillium* species: *V. dahliae, V. tricorpus* and *V. albo-atrum*. The two later species have been recently reported in Tunisia[12-14]. Jabnoun-khiareddine *et al.*[14] signalled that these new Tunisian isolates of *V. tricorpus* were able to attack tomato, eggplant and potato plants and cause moderately to relatively severe wilt[14]. The objective of the present research was, therefore, to study the virulence of the two other *Verticillium* species: *V. dahliae* and *V. albo-atrum*, by means of several pathogenicity tests conducted against three tomato cultivars. We used two temperature ranges in this study to explain the appearance of *V. albo-atrum* at relatively high temperature and to distinguish the thermal requirements of the two *Verticillium* species.

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MATERIALS AND METHODS

Tomato cultivars: Two cultivars (Colibri and Naya) resistant to race 1 (with *Ve* gene) and one susceptible cultivar (cv. Chico III, without *Ve* gene) were used in the experiments during the autumn and spring 2003.

After their germination at 25°C, seeds of each cultivar were placed in black polyethylene bags containing sterilized peat and perlite, mixed in equal proportions. Seedlings were kept in a glasshouse under ambient temperature (about 20°C).

Seedlings were irrigated regularly and were fertilized once a week with a nutrient solution to ensure optimal growth (N, 150 ppm; P, 50 ppm; K, 150 ppm; Ca, 150 ppm; Mg, 30 ppm; Fe, 3 ppm; Mn, 1.5 ppm; Zn, 0.2 ppm; B, 0.4 ppm; Cu, 0.1 ppm; Mo, 0.05 ppm and 1L of distilled water).

Fungal cultures and inoculum preparation: *Verticillium* isolates were obtained in 2002 from tomato plants taken from fifteen tomato greenhouses located in Chott Mariem region (two plants per greenhouse). The plants showed wilt and brown discoloration of the vascular tissue. Isolations were made at several levels: roots, collars and stems.

Sections (5 to 7 cm long) of tomato plant tissue exhibiting vascular discoloration were rinsed thoroughly in tap water and cut into 0.5-1 cm pieces. After surface-disinfesting in sodium hypochlorite (10%) for 3 min, the plant pieces were rinsed three times in sterile distilled water, dried on sterile filter paper and plated on PDA (Potato Dextrose Agar) medium amended with streptomycin sulphate (300 mg L^{-1}). Fungal cultures were incubated for two weeks at 20°C. The fungal isolates were cleaned up by subculturing successively on antibiotic plates from the edge of actively growing colonies and single spores were then isolated. *Verticillium* species were identified by their resting structure morphology. *V. albo-atrum* produces only dark-resting-mycelium as its resting structure and *V. dahliae* only microsclerotia[7,15-21].

One isolate from each species was retained for study, *Vd* and *Va*, respectively for *V. dahliae* and *V. albo-atrum*. Inoculum was prepared from one month old cultures on Potato Dextrose Broth and the density of each isolate was adjusted to approximately 10^7 conidia mL^{-1}.

To compare optimal temperature requirements of *V. dahliae* and *V. albo-atrum*, the mycelial growth of each species isolate was evaluated at seven temperatures between 5 to 35°C. A six mm plug was taken from an actively growing colony, transferred to PDA dish and incubated at each temperature. Cultures were incubated in the dark for 21 days and the diameters of all colonies were then measured. There were five replicate plates per treatment and each experiment was repeated twice. Analysis of variance was used to compare the growth of the two *Verticillium* isolates at each temperature.

Pathogenicity tests: Pathogenicity of *V. dahliae* and *V. albo-atrum* was determined by the root-dip method, using three tomato cultivars (Colibri, Naya and Chico III). Seedlings at the three leaf stage were uprooted from the substrate; their roots washed in tap water, rinsed by sterile distilled water and dipped for 30 min in fungal suspension containing 10^7spores mL^{-1}[22]. Seedlings of each cultivar, dipped in sterile distilled water, served as noninoculated control. All the seedlings were then transplanted to pots filled with peat and perlite, mixed in equal proportions and maintained in two glasshouse cells at 17-21°C and 21-30°C. They were irrigated regularly and fertilized using a nutrient solution (N, 150 ppm; P, 50 ppm; K, 150 ppm; Ca, 150 ppm; Mg, 30 ppm; Fe, 3 ppm; Mn, 1.5 ppm; Zn, 0.2 ppm; B, 0.4 ppm; Cu, 0.1 ppm; Mo, 0.05 ppm and 1L of distilled water).

Plants were observed daily and the appearance of the first foliar symptoms was noted. Symptom included chlorosis, necrosis and wilt. The criteria used to assess pathogenicity were leaf damage. A scale of 0 to 4 was used to assess disease severity 60 days post-inoculation, in which: 0 = leaf of healthy aspect; 1 = epinasty or wilted leaf without chlorosis; 2 = one or several bands slightly chlorotic on one or several leaflets; 3 = Chlorotic bands on all the surface of one or several leaflets or chlorotic band with a necrotic centre on one or several leaflets and 4 = complete necrosis of several leaflets or dead leaf. An index of leaf damage was then calculated, for every plant, according to the following formula: ILD = sum notes/possible maximum. Possible maximum is equal to four times the number of well developed leaves carried by the plant[23].

Statistical analysis: Four batches of thirty seedlings, having a comparable vigour, were used for each cultivar tested and arranged according to an experimental device in a complete plan with two factors and repeated once. Factor A corresponds to cultivars tested and factor B to *Verticillium* isolates. The number of replications is ten plants by elementary treatment. Analyses of variance, associated to the test of means comparison of Newman-Keuls, were conducted to distinguish groups according the values of the tested variables means. All statistical analyses were performed using SPSS (Statistical Package for the Social Sciences).

RESULTS

Effect of temperature on the mycelial growth of *V. dahliae* and *V. albo-atrum*: Temperature had a significant influence on the growth of *Verticillium* isolates. Analyses of variance indicated that the interaction between the temperature of incubation and the *Verticillium* isolates significantly affected the mycelial colony diameter. The two *Verticillium* species showed similar growth at temperatures between 10 to 35°C, but *V. albo-atrum* was fast growing (Fig. 1); in fact, the diameter of *V. albo-atrum* isolate was greater than that of *V. dahliae* isolate, at each incubation temperature. The optimal growth temperatures of *V. dahliae* and *V. albo-atrum* were 20 and 25°C. Although the two isolates were tolerant to 30°C, their mycelium was thin and irregular. Neither *Verticillium* species grew at 35°C. Contrary to *V. albo-atrum*, *V. dahliae* isolate wasn't able to grow at 5°C.

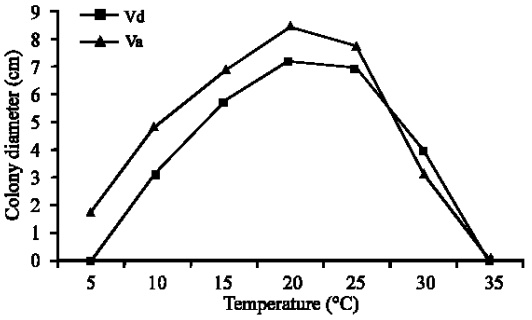

Fig. 1: Effect of temperature on mycelial growth of isolates of *V. dahliae* and *V. albo-atrum* from tomato. Colony diameter of each isolate was measured 21 days after subculturing the isolates on PDA, LSD = 0.15 cm

Fig. 2: Comparison between a non-inoculated control (on the left) with a susceptible tomato plant inoculated with *Verticillium albo-atrum* 45 days post inoculation (on the right) (Cv. Colibri, T<21°C). Stunting, wilting and withering are noted on the inoculated plant

Table 1: Damage of *Verticillium dahliae* and *Verticillium albo-atrum* on tomato cultivars, evaluated 60 days after inoculation under temperatures ranged between 17 to 21°C

	Index of leaf damage[a]				
Treatments	Va[b]		Vd[c]		Control[d]
Tomato cultivars	Mean	Range	Mean	Range	Mean
Chico III[e]	3.5	1.5-6.3	3	1.75-5.3	0
Colibri[f]	3.37	0.75-5.55	2	0.75-6.95	0
Naya[f]	0.75	0.25-2.3	0.5	0.15-1.5	0

[a]Index of Leaf Damage (ILD) is the index of leaf damage caused by *Verticillium* sp. and evaluated for each plant on a scale of 0 to 4, where, 0 = leaf of healthy aspect; 1 = epinasty or wilted leaf without chlorosis; 2 = one or several bands slightly chlorotic on one or several leaflets; 3 = Chlorotic bands on all the surface of one or several leaflets or chlorotic band with a necrotic centre on one or several leaflets and 4 = complete necrosis of several leaflets or dead leaf, [b]Va: *V. albo-atrum*, [c]Vd: *V. dahliae*, [d]control: Noninoculated tomato plants, [e]Chico III is a tomato cultivar lacking the *Ve* gene, [f]Colibri and Houda are tomato cultivars possessing the *Ve* gene

Pathogenicity of *Verticillium* sp. at 17-21°C: The index of leaf damage noted for inoculated and control plants, during eight weeks period, depends on the tomato cultivar and *Verticillium* isolate. Typical *Verticillium* wilt symptoms were observed in all tomato cultivars. These symptoms consist of a general decline of plant growth, chlorosis of the leaves, stunting and vascular discoloration followed by wilting (Fig. 2)[24]. These symptoms were first observed from 30 to 45 days post inoculation depending on cultivar and isolate. Inoculated tomato plants had significantly ($p \leq 0.05$) more dead and yellow leaves than the non-inoculated control plants which remain symptomless.

Cv. Colibri developed more severe symptoms than those developed on cv. Naya, when infected by both *Verticillium* sp. (Table 1). Although, these two tomato cultivars possess the *Ve* gene of resistance, the Index of Leaf Damage (ILD) noted at the end of the experiment, particularly with the cv. Colibri, was important (ILD = 3.37).

The pathogenicity of the *V. dahliae* and *V. albo-atrum* isolates on all tomato cultivars was clearly different (Table 1); The ILD caused by *V. albo-atrum* was more important than that of *V. dahliae* at 17-21°C. At these conditions, this *V. albo-atrum* isolate was highly pathogenic to the cultivar Chico III as well as to both tomato cultivars Colibri and Naya, possessing the *Ve* gene.

The re-isolation of the pathogen from infected tomato plants was successful.

Pathogenicity of *Verticillium* sp. at 21-30°C: Both *Verticillium* isolates caused typical symptoms on all inoculated tomato plants: a general decline of plant growth, chlorosis of the leaves, stunting and vascular

Table 2: Damage of *Verticillium dahliae* and *Verticillium albo-atrum* on tomato cultivars, evaluated 60 days after inoculation under temperatures ranged between 21 to 30°C

	Index of leaf damage[a]				
Treatments	Va[b]		Vd[c]		control[d]
Tomato cultivars	Mean	Range	Mean	Range	Mean
Chico III[e]	3.39	2.75-4.25	5.19	4.25-7.15	0
Colibri[f]	1.25	0.75-2.25	2.75	1.75-3.95	0
Naya[f]	0.75	0.15-1.95	3.37	3.0-3.75	0

[a]Index of Leaf Damage (ILD) is the index of leaf damage caused by *Verticillium* sp. and evaluated for each plant on a scale of 0 to 4, where, 0 = leaf of healthy aspect; 1 = epinasty or wilted leaf without chlorosis; 2 = one or several bands slightly chlorotic on one or several leaflets; 3 = Chlorotic bands on all the surface of one or several leaflets or chlorotic band with a necrotic centre on one or several leaflets and 4 = complete necrosis of several leaflets or dead leaf, [b]Va: *V. albo-atrum*, [c]Vd: *V. dahliae*, [d]control: Noninoculated tomato plants, [e]Chico III is a tomato cultivar lacking the *Ve* gene, [f]Colibri and Houda are tomato cultivars possessing the *Ve* gene

discoloration followed by wilting[24]. Plants infected by *V. dahliae* were the first to develop disease symptoms, about 23 days postinoculation. A significant interaction ($p = 0.05$) between *Verticillium* isolates and tomato cultivars was noted, showing a differential response of inoculated cultivars toward *Verticillium* isolates. Both resistant (cv. Colibri and cv. Naya) and susceptible (cv. Chico III) tomato cultivars developed *Verticillium* symptoms; however, for the plants infected by *V. dahliae*, the index of leaf damage was greater than those of the plants infected by *V. albo-atrum*, comparatively to the non-inoculated control plants which remain symptomless (Table 2). The ILD noted on the cv. Naya was less important than those noted on cv. Chico III and cv. Colibri.

These thermal conditions of plant breeding seem to favour the expression of *V. dahliae* more than *V. albo-atrum*.

The re-isolation of the pathogen from infected tomato plants was successful.

DISCUSSION

Two surprising elements were noted in this study: isolation of *V. dahliae*, from tomato cultivars with the *Ve*-gene, which means a break of resistance to *V. dahliae* race 1, or the possible appearance of a new race and the contemporary presence in the diseased plants of *V. albo-atrum*. These results join partially those obtained by Hajlaoui *et al.*[12] that indicated emergence of a new pathotype of *V. albo-atrum* in Tunisia able to attack cultivars possessing the *Ve*-gene of resistance.

Verticillium albo-atrum is commonly found in northern latitudes where cool wet weather prevails[6,25]. However, in our study, the growth test confirmed that *V. albo-atrum* isolate was able to grow well at 25°C and even at 30°C, which is unusual[17,19].

In order to explain the sudden occurrence of *V. albo-atrum* at relatively high temperatures and its coexistence with *V. dahliae*, different pathogenicity tests were conducted. The progress of these pathogenicity tests at two temperature ranges, allowed to distinguish thermal requirements necessary for *Verticillium* isolates to express their optimal aggressiveness.

The pathogenicity tests conducted in this study confirm well that *V. dahliae* is more adapted to higher temperatures than *V. albo-atrum*; however this last specie was also able to infect tomato plants at relatively high temperature.

In fact, at 17-21°C, the severity of foliar damage caused by *V. albo-atrum* isolate was grater than that caused by *V. dahliae* (Table 1). Latunde Dada and Lucas[26] showed that Lucerne isolates of *V. albo-atrum* are more virulent between 17 and 21°C.

However, at 21°to 30°C, symptom expression severity caused by *V. albo-atrum* isolate was even grate, but less than that of *V. dahliae* isolate (Table 2). It should be indicate that beyond 15 weeks, an ultimate stage of the infection translated by a total plant withering is observed.

In Morocco, in plastic tunnels, temperature is the limiting factor for tomato wilt, 90% occurring between November and April[27]. These conditions are in fact close to Tunisian conditions and more exactly to those of Chott Mariem region. Moreover, symptom expression of *V. dahliae* is often detected during this period. However, the incidence of *V. albo-atrum* in these regions as well as the appearance of symptoms at high temperatures is not usual.

In fact, it is possible that in these conditions, such specific interactions could be at the origin of a selective pressure on the pathogen populations. This pressure, if it is constantly directed in the same sense by the continuous use of the same host cultivar, could lead to changes in the structure of the pathogen populations: the favoured genotypes would eventually prevail[28].

Verticillium albo-atrum isolates, introduced into Chott Mariem region or existing already in the *Verticillium* population in the soil, were able to be adapted to the local climate and to express their pathogenic capabilities.

In fact, the risk of emergence of a new virulent race in the pathogen population, is conditioned by three essential factors: the probability of mutation at the level of genes implicated in pathogen capabilities, the number of genes conditioning this pathogen capabilities and the incidence of the mutation or rather the new character of virulence on the environment adaptation of the pathogen, or its ability to competition[29].

In this sense, Bhat *et al.*[23] mentioned that the intensive production of pepper in the same region may have increased selection pressure on strains of *V. dahliae* that colonize and reproduce on pepper plants more effectively, resulting in an increase of inoculum levels causing significant yield losses.

Another hypothesis is possible, that presented by Besri[30] who claimed that high salt levels in irrigation water led to a 100% breakdown of resistance of tomato cultivars resistant to race 1 of *V. dahliae*. He also mentioned that susceptibility to race 2 resistant cultivars also increased with increasing soil salinity. This could explain the incidence of *Verticillium* wilt in Chott Mariem region further to an unusual period of dryness (about three years of dryness).

Bender and Shoemaker[10] signalled that the slow decline of microsclerotia numbers in soil and the infection of weed hosts and other host crops may increase the incidence of *V. dahliae* race 1 on tomato race 1 resistant cultivars. They added that if the use of tomato cultivars with the *Ve* gene for resistance to race 1 continues, the prevalence of race 2 will probably increase and serious yield losses may occur. In fact, race 2 pathotypes may be a natural component of the *V. dahliae* population in Chott Mariem region and could have arisen as a mutation from tomato race 1 isolates or evolved from *V. dahliae* isolates pathogenic on other hosts. Further molecular analysis needs to be done to compare these new emergences of *Verticillium* pathotypes to *V. dahliae* race 2 and *V. albo-atrum* isolates from other countries.

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