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Plant phenology influences the effect of mycorrhizal fungi on the development of *Verticillium*-induced wilt in pepper

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Abstract

Verticillium dahliae alters water status and consequently, growth and production of pepper plants. On the other hand, arbuscular mycorrhizal fungi (AMF) can reduce damage caused by specific soil-borne plant pathogens and improve drought resistance of pepper. Therefore, one objective of this research was to assess if AMF can modify the development of *Verticillium*-wilt in pepper plants. A second objective was to study the influence of plant phenology at the moment when *V. dahliae* was inoculated on the possible biocontrol of the disease by AMF. Results suggested that AMF reduce the deleterious effect of *V. dahliae* on pepper growth and yield. However, bioprotection against *Verticillium*-wilt was conditioned by plant phenology at the moment of pathogen attack. The highest efficacy of AMF occurred when *V. dahliae* was inoculated during the vegetative stage of plants. AMF allowed leaf relative water content to be maintained for longer and delayed both the appearance of disease symptoms and the decrease of photosynthesis in *Verticillium*-inoculated plants. These benefits on plant physiology increased pepper yield.

Abbreviations: AMF – arbuscular mycorrhizal fungi; CER – CO_2 exchange rate; DM – dry matter; g_w – leaf conductance; RWC – relative water content; T – transpiration; Ψ – water potential.

Introduction

Verticillium dahliae is a systemic pathogen that causes vascular wilt disease in several plant species (Pegg, 1989). The fungus enters the root through wounds that expose the vascular system or grows between the cells of the apical meristem to gain access to immature xylem elements. Previous studies (Goicoechea et al., 2000; 2001) demonstrated that some negative effects caused by *V. dahliae* on the physiology and metabolism of pepper plants were related to yield reduction (García-Mina et al., 1996).

Arbuscular mycorrhizal symbioses play a key role in nutrient cycling in the ecosystem and also protect plants against environmental and cultural stress. In fact, several mechanisms can be involved in bioprotection by arbuscular mycorrhizal fungi (AMF) against soil-borne pathogens (Azcón-Aguilar et al., 2002). Moreover, it is well known that AMF affect the water balance of both well-watered and drought-stressed plants. Mycorrhizal association has been proposed to increase the drought resistance of plants by means of several mechanisms related or not to plant size (Augé, 2001). In pepper, AMF can improve drought resistance by enhancing the development of extraradical hyphae and thus promoting soil water uptake (Davies et al., 1993).

As *V. dahliae* alters water status and, consequently, growth and production of pepper plants (Goicoechea et al., 2000; 2001), the first objective of this research was to assess whether AMF can modify the development of *Verticillium*-wilt in pepper. However, as many different factors affect the efficacy of AMF as disease control agents (Singh et al., 2000), the second aim was to study the influence of plant phenology at the time of *V. dahliae*-inoculation on the possible bioprotective effect by AMF.

Materials and methods

Biological material, growth conditions and experimental design

Capsicum annuum cv. Piquillo seeds were germinated on washed sand. When one-month-old, 80 seedlings were transplanted to 1 l capacity pots (one per pot) containing a mixture of vermiculite–sand–soil (2.5 : 2.5 : 1 v/v/v). The soil had a pH (H_2O) of 8.9, 0.3% organic matter, 0.08% nitrogen, 2.0 mg kg^{-1} phosphorus, 58.8 mg kg^{-1} potassium and 41.98% $CaCO_3$. Before preparing the potting mix, the soil (2-mm sieved) was steam-sterilised at 100 °C for 1 h on three consecutive days. Plants were initially divided into two groups: (a) non-mycorrhizal plants (40 plants) and (b) plants inoculated with *Glomus deserticola* (Trappe, Bloss and Menge) (40 plants). AMF was applied as a soil-based inoculum including root fragments, spores and hyphae from a three-months-old culture of leek and alfalfa. Non-mycorrhizal and mycorrhizal plants were fertilised weekly with 200 ml Long Ashton Nutrient Solution (LANS; Hewitt, 1966) containing 11 mg P l^{-1} instead of the standard 44 mg P l^{-1} until the symbiosis was well established in the mycorrhizal treatment. In addition, plants received water twice a week to prevent wilting. Once *G. deserticola* was established (50 days after plant transplanting to pots), non-mycorrhizal peppers received complete LANS (44 mg P l^{-1}), and mycorrhizal ones were fertilised with 11 mg P l^{-1} in order to equalise size between mycorrhizal and non-mycorrhizal plants (Davies et al., 1993). Forty plants (20 non-mycorrhizal and 20 mycorrhizal) were kept as uninoculated healthy controls. Other 40 plants (20 mycorrhizal and 20 non-mycorrhizal) were divided into two groups before inoculation with *V. dahliae*. The first group (10 plants per treatment) was inoculated with the pathogen during the vegetative phase (three-months-old plants) and the second group, at flowering. As flowering did not occur synchronously, inoculation with *V. dahliae* was carried out when the totality of plants showed the first flower bud and the 50% of these flower buds had changed from green to white colour. Inoculation with the pathogen was performed by adding a suspension of 3.6×10^7 conidia to the substrate of each pot (Hoyos et al., 1993). *V. dahliae* was isolated from diseased pepper grown in field and maintained on Messiaen culture medium prior to inoculation.

Plants were grown in a greenhouse at 25/15 °C day/night and received natural daylight supplemented with irradiation from fluorescent lamps Son-T-Agro (Philips Nederland B.V., Eindhoven) that provided a minimum photosynthetic photon flux of 300 μmol m^{-2} s^{-1} during a 14 h photoperiod.

Two plant harvests were performed: (i) on the day when plants were inoculated with *V. dahliae* (at the vegetative period or at flowering) and (ii) at the end of their life cycle.

Water status measurement and plant growth parameters

Midday leaf water potential (Ψ) was determined with a pressure chamber (Scholander et al., 1965), and relative water content (RWC) was estimated by a modification of Weatherley's method (1950), both parameters being measured on the youngest fully-mature leaves. Plant dry matter (DM) was determined after drying at 80 °C for 2 days. Fruit DM was calculated after drying at 60 °C for 45 days. Fruit set was calculated as the number of fruits related to the sum of flowers (on plant and fallen flowers) and fruits.

Gas exchange measurements

Midday CO_2 exchange rates (CER), total leaf conductance (g_w) to water vapour and transpiration (T) measurements were made in the greenhouse on the youngest fully-mature symptomless leaves using an infrared gas analyser (LCA-2 model; Analytical Development Co., Hoddesdon, Herts., UK). Measurements were made on comparable leaves from all treatments. Air of known CO_2 concentration (350–400 μmol mol^{-1}) was supplied to the leaf chamber at a constant flow of 600 ml min^{-1}. Leaf temperature was 25 ± 1 °C. Irradiation was 400 μmol m^{-2} s^{-1} as measured with a Li-Cor meter, LI-188B (Li-Cor, Lincoln, NE, US) equipped with a quantum sensor.

Disease assessment and estimation of AMF colonisation

In order to assess whether *V. dahliae* had progressed from root to shoot, surface-disinfected cross-stem sections were cut and plated on Messiaen culture medium for the fungus isolation and identification. When plants showed activation of axillary buds, the presence of *V. dahliae* in lateral branches was also assessed. Plates were incubated in the dark at 25 °C for 10–15 days. The disease severity was non-destructively estimated

and a disease index was calculated as the sum of wilted, chlorotic and necrotic leaves related to the total leaves per plant, expressed as a percentage (Goicoechea et al., 2000).

Root samples were cleared and stained (Phillips and Hayman, 1970), and mycorrhizal colonisation was determined by examining 50–80 1-cm root segments under the microscope. Results are expressed as percentage of root colonisation (Hayman et al., 1976).

Statistics

The defoliated to total leaf DM ratio (Figure 1c), water status (Figures 4 and 5) and gas exchange parameters (Figures 6 and 7) were analysed with one-way analysis of variance (ANOVA); means ± SD were calculated and, when the F-ratio was significant, least significant differences were evaluated by the Tukey-b test. Plant growth parameters (Table 2) as well as fruit set, DM, length, diameter and yield (Table 3) at final harvest were analysed with two-way ANOVA, with mycorrhizal colonisation and *Verticillium*-inoculation as the main effects (Sokal and Rohlf, 1979). Means ± SD were calculated and, when the F-ratio was significant, least significant differences were evaluated by the Tukey-b test. When only two treatments were compared, means ± SD were calculated and their differences tested for significance by using Student's t-test. This statistical analysis was applied to growth

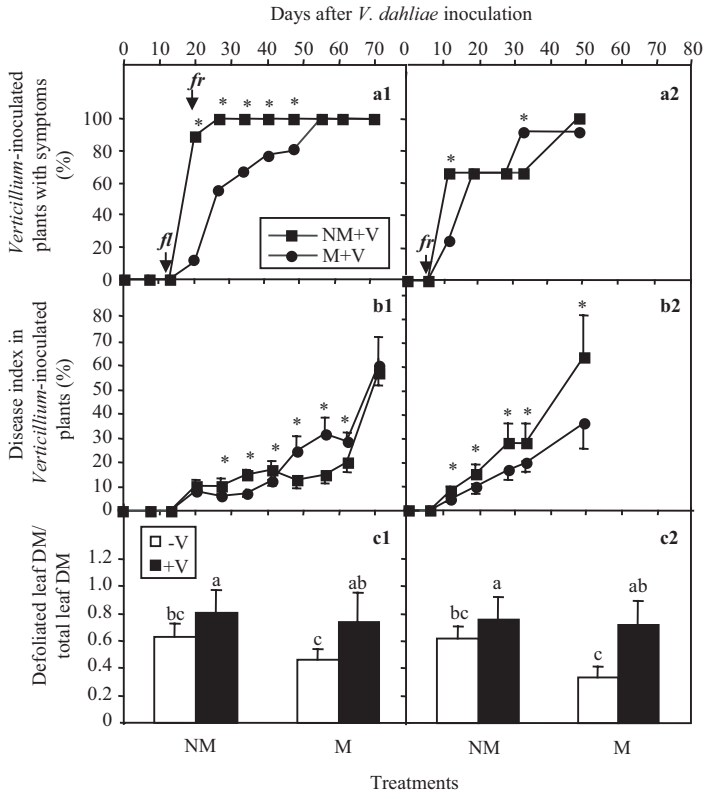

Figure 1. Percentage of plants with visible symptoms of disease (a) and disease index (%) (b) in non-mycorrhizal (NM, ■) and mycorrhizal (M, ●) treatments inoculated with *V. dahliae* (+V) during the vegetative growth (a1, b1) or at flowering (a2, b2), as well as the defoliated to total leaf DM ratio (c) in NM and M plants, inoculated (+V) or not (−V) with *V. dahliae* during the vegetative growth (c1) or at flowering (c2). Data on *Verticillium*-inoculated plants showing disease symptoms (Figure 1a) were subjected to arc-sin transformation before applying χ^2-test ($P \leq 0.05$). Each point in Figure 1b represents the mean ± SD of 8–10 plants. Means were compared with the Student's t-test. Within each graph, asterisks indicate significant differences ($P \leq 0.05$). The defoliated to total leaf DM ratios were analysed with one-way ANOVA; means ± SD (n = 8–10 plants) were calculated and, when the F-ratio was significant, least significant differences were evaluated by the Tukey-b test. Within each graph, histograms with the same letter do not differ significantly ($P \leq 0.05$). fl = flowering; fr = beginning of fruit set.

Table 1. Leaves, stem and root DM (g plant^{-1}) and mycorrhizal colonisation (%) of non-mycorrhizal (NM) and mycorrhizal (M) plants the day of inoculation with *V. dahliae*. Inoculation took place during the vegetative growth or at flowering

Time of *V. dahliae* inoculation	Treatment	Leaves DM* (g plant $^{-1}$)	Stem DM* (g plant^{-1})	Root DM* (g plant^{-1})	Mycorrhizal colonisation (%)
Vegetative growth	NM	0.56 a	0.15 a	0.28 b	0
(n = 3–4 plants)	M	0.69 a	0.16 a	0.38 a	27.15
Flowering	NM	1.73 a	1.38 a	0.87 a	0
(n = 4–6 plants)	M	1.46 a	0.78 b	0.74 a	34.16

*Comparison between means were made with the Student's t-test within each column and time of pathogen inoculation. Values followed by a common letter are not significantly different ($P \leq 0.05$).

Table 2. Leaves, stem and root DM (g plant^{-1}) and percentage of mycorrhizal colonisation (%) of non-mycorrhizal healthy controls (NM − V), non-mycorrhizal *Verticillium*-inoculated (NM + V), mycorrhizal healthy controls (M − V) and mycorrhizal *Verticillium*-inoculated (M + V) pepper plants at the end of their life cycle

Time of *V.dahliae* inoculation	Treatment	Leaves DM[1] (g plant^{-1})	Stem DM[1] (g plant^{-1})	Root DM[1] (g plant^{-1})	Mycorrhizal colonisation[3] (%)
Vegetative growth	NM − V	1.10 a	3.39 a	1.93 a	0
(n = 9–10 plants)	NM + V	0.56 b	1.40 b	1.30 b	0
	M − V	1.40 a	3.01 a	1.59 b	58.36 a
	M + V	0.62 b	1.52 b	1.36 b	55.77 a
	AMF[2]	ns	ns	ns	
	V. dahliae [2]	***	***	***	
	Interaction[2]	ns	ns	*	
Flowering	NM − V	1.11 b	3.74 a	1.27 b	0
(n = 8–10 plants)	NM + V	0.59 c	2.00 b	0.92 b	0
	M − V	2.24 a	3.54 a	1.95 a	47.89 a
	M + V	0.68 c	2.03 b	1.02 b	32.36 b
	AMF[2]	***	ns	***	
	V. dahliae[2]	***	***	**	
	Interaction[2]	***	ns	**	

[1]Within each column and time of pathogen inoculation values were analysed with two-way ANOVA with mycorrhizal colonisation and *Verticillium*-inoculation as the main effects. Means ± SD were calculated and, when the F-ratio was significant, least significant differences were evaluated by the Tukey-b test. Values followed by the same letter are not significantly different ($P \leq 0.05$).
[2]ns, *, ** and *** indicated non-significant or significant at 5%, 1% and 0.1% levels.
[3]Comparison between means were made with the Student's t-test within each time of pathogen inoculation.

parameters of non-mycorrhizal and mycorrhizal plants the day when they were inoculated with *V. dahliae* (Table 1), percentage of mycorrhizal colonisation in control and diseased pepper at final harvest (Table 2) as well as to disease index (Figure 1b), shoot height (Figure 2) and lateral branches to total shoot DM ratio (Figure 3b) in non-mycorrhizal and mycorrhizal plants inoculated with the pathogen. Frequencies of *Verticillium*-inoculated plants showing disease symptoms (Figure 1a) and activation of axillary buds (Figure 3a), as well as frequencies of green pepper, veraison and red pepper in non-mycorrhizal and mycorrhizal plants inoculated or not with *V. dahliae* (Figure 8) were analysed by Chi-square (χ^2) test. Data on percentages of *Verticillium*-inoculated plants with disease symptoms and activation of axillary branches were subjected to arc-sin transformation before applying χ^2-test. Significance levels were set at 5% level of significance.

Results

The biomass of the aerial part was similar in non-mycorrhizal and mycorrhizal pepper when *V. dahliae* was inoculated during the vegetative period, but the

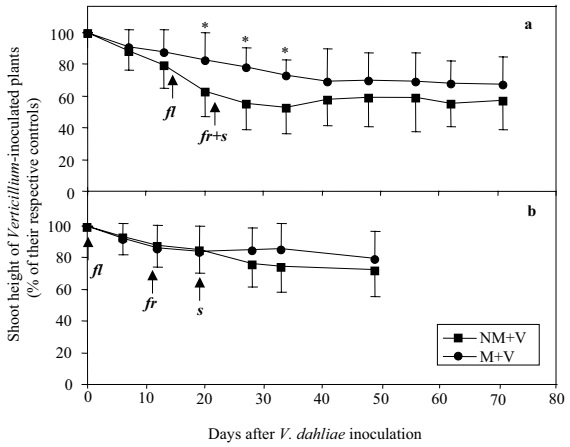

Figure 2. Shoot height of non-mycorrhizal (NM, ■) and mycorrhizal (M, ●) plants inoculated with *V. dahliae* (+V) during the vegetative growth (a) or at flowering (b). Values are expressed as percentages of their respective healthy controls (100%). Means ± SD (n = 8–10 plants) were compared with the Student's t-test. Within each graph, asterisks indicate significant differences between *Verticillium*-inoculated non-mycorrhizal and mycorrhizal plants ($P \leq 0.05$). s = first foliar disease symptoms. Otherwise as for Figure 1.

greatest root DM corresponded to plants colonised by *G. deserticola* (Table 1). In contrast, root biomass from non-mycorrhizal and mycorrhizal plants did not differ significantly when they were inoculated with *V. dahliae* at flowering (Table 1). In this case, non-mycorrhizal plants had higher stem DM than mycorrhizal ones may be due to the higher P fertilisation applied to non-mycorrhizal pepper. At both moments of pathogen inoculation mycorrhizal colonisation reached the 30%.

When *V. dahliae* was inoculated during the vegetative period, foliar disease symptoms appeared 3 weeks after inoculation in 100% of non-mycorrhizal plants, while only 15% of mycorrhizal ones had symptoms (Figure 1a1). Although the disease index remained unchanged in the mycorrhizal treatment between the third and the sixth week after inoculation (coinciding with the fruit set period), at the end of the experiment, the severity of the disease was similar in non-mycorrhizal and mycorrhizal *Verticillium*-inoculated plants (Figure 1b1). When the pathogen inoculation was done at flowering, foliar symptoms appeared at the same rate in both non-mycorrhizal and mycorrhizal pepper

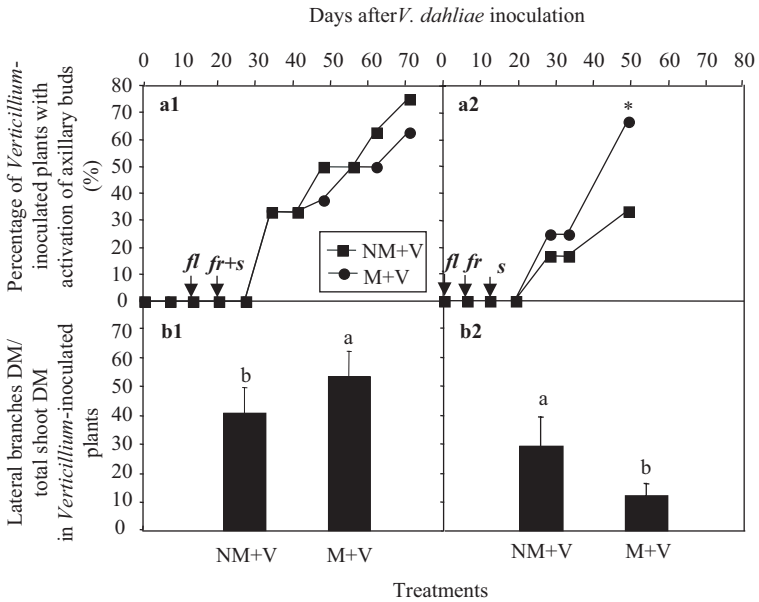

Figure 3. Percentage of plants showing activation of axillary buds (%) (a) and lateral branches to total shoot DM ratio (b) in *Verticillium*-inoculated (+V) non-mycorrhizal (NM, ■) and mycorrhizal (M, ●) treatments. Inoculation with *V. dahliae* occurred during the vegetative growth (a1, b1) or at flowering (a2, b2). Data on *Verticillium*-inoculated plants with activation of axillary buds (Figure 3a) were subjected to arc-sin transformation before applying χ^2-test ($P \leq 0.05$). Means ± SD (n = 8–10 plants) of lateral branches to total shoot DM ratio (Figure 3b) were compared with the Student's t-test. Within each graph, different letters indicate significant differences ($P \leq 0.05$).

(Figure 1a2), although the disease index was always significantly higher in non-mycorrhizal than in mycorrhizal plants, even at the end of the experiment (Figure 1b2). Non-inoculated controls remained symptomless. *V. dahliae* caused a significant defoliation in both non-mycorrhizal and mycorrhizal plants independently of pathogen inoculation time (Figure 1c1,c2).

Verticillium dahliae reduced plant height in both non-mycorrhizal and mycorrhizal plants (Figure 2). As plant height of non-mycorrhizal and mycorrhizal controls was not similar (data not shown), shoot height of diseased plants has been expressed as percentage of their respective healthy controls (100%). At the end of the experiment, shoot height of *Verticillium*-inoculated plants was 30–40% (Figure 2a) and 20–30% (Figure 2b) smaller than that of controls when the pathogen inoculation was done, respectively, during the vegetative period or at flowering. However, the negative effect of *V. dahliae* on shoot elongation when it was inoculated during the vegetative growth (Figure 2a), was less pronounced in mycorrhizal than in non-mycorrhizal plants between the third and sixth week after inoculation, coinciding with the fruit set and the appearance of first foliar symptoms.

Verticillium dahliae always decreased shoot biomass (Table 2), in part due to defoliation (Figure 1c1,c2). In addition, the pathogen reduced root DM of non-mycorrhizal plants inoculated during the vegetative period and mycorrhizal plants inoculated at flowering (Table 2). When *V. dahliae* was inoculated during the vegetative growth, the percentage of root cortex colonised by *G. deserticola* achieved similar values in control (58%) and diseased (56%) pepper (Table 2). In contrast, pathogen inoculation at flowering reduced mycorrhizal colonisation in diseased plants (32%) compared to control ones (48%).

While healthy control plants never showed lateral branches, *V. dahliae* always caused activation of axillary buds (Figure 3). At the end of the experiment, around 65% of non-mycorrhizal and mycorrhizal plants showed axillary buds when the pathogen was inoculated during the vegetative period (Figure 3a1). In this case, the biomass of lateral branches represented a higher percentage of total shoot DM in mycorrhizal (50%) than in non-mycorrhizal plants (40%) (Figure 3b1). Pathogen inoculation at flowering (Figure 3a2) produced more axillary buds in mycorrhizal (70% of plants with lateral branches) than in non-mycorrhizal pepper (35%). However, in this case, the lateral branches to the total shoot DM ratio was greater in non-mycorrhizal (30%) than in mycorrhizal plants (10%) (Figure 3b2).

Leaf Ψ was more influenced by *V. dahliae* than RWC (Figures 4 and 5). In fact, while the pathogen caused a decrease of Ψ in all inoculated plants (Figures 4a1,a2 and 5a1,a2), RWC remained unchanged in mycorrhizal *Verticillium*-inoculated pepper (Figures 4b2 and 5b2). In addition, the reduction in leaf Ψ of non-mycorrhizal diseased plants (Figures 4a1 and 5a1, days 45 and 22, respectively, after pathogen inoculation) occurred before any decrease in RWC was still observed (Figures 4b1 and 5b1).

Inoculation with *V. dahliae* significantly decreased CER, g_w and T rate in both non-mycorrhizal and mycorrhizal plants (Figures 6 and 7), being these reductions more evident when the pathogen was inoculated at flowering (Figure 7) than during the vegetative growth (Figure 6). When *V. dahliae* inoculation was done during the vegetative period, we found some differences between non-mycorrhizal and mycorrhizal diseased plants. First, mycorrhizal plants exhibited greater CER, g_w and T (Figure 6a2,b2,c2) than non-mycorrhizal ones (Figure 6a1,b1,c1) at final stages of the disease. Second, the declines in photosynthesis (Figure 6a1,a2) and g_w (Figure 6b1,b2) were first detected in non-mycorrhizal pepper. Third, while the reduction in g_w of non-mycorrhizal plants was concomitant with the development of the first visible symptoms of the disease (Figure 6b1), the decrease in g_w of mycorrhizal pepper occurred 15 days after symptoms were first observed (Figure 6b2).

When studying fruit characteristics and yield in healthy controls (Table 3), we observed a decrease in fruit set, DM and yield in mycorrhizal plants compared to non-mycorrhizal ones. However, such differences were only found in the experiment in which *V. dahliae* was inoculated at the vegetative stage. Moreover, the pathogen inoculated during the vegetative growth reduced both the percentage of fruit set and pepper yield, being such decreases more evident in non-mycorrhizal than in mycorrhizal plants. Also when comparing *Verticillium*-inoculated plants during the vegetative period, only non-mycorrhizal pepper showed a reduction in fruit length and DM compared to non-mycorrhizal controls. On the other hand, when *V. dahliae* was inoculated at flowering, neither the percentage of fruit set nor the fruit length was affected, but pepper yield, fruit DM and diameter in all diseased plants were smaller than those measured in their respective healthy controls.

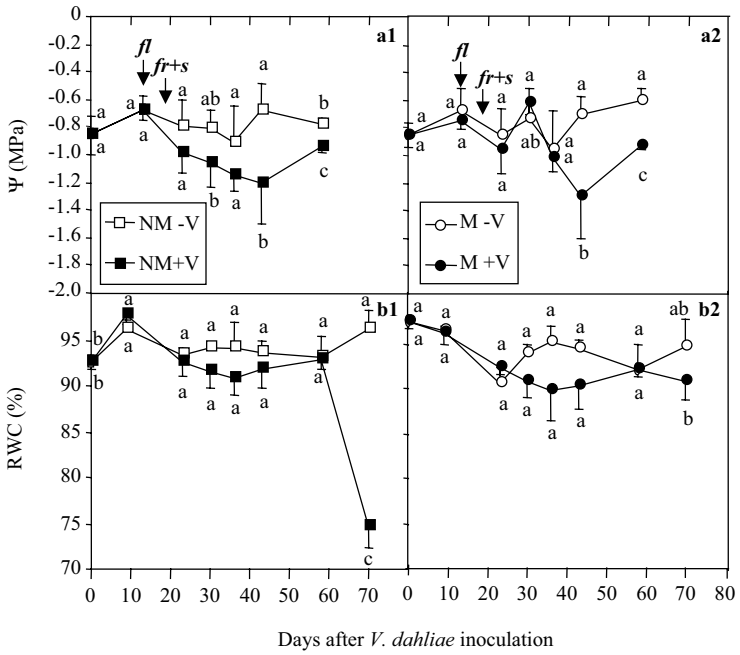

Figure 4. Water potential (MPa) (a) and RWC (%) (b) in leaves of non-mycorrhizal (NM) plants inoculated (+V, ■) or not (−V, □) with *V. dahliae* (a1, b1) and in mycorrhizal (M) plants inoculated (+V, ●) or not (−V, ◯) with *V. dahliae* (a2, b2) during the vegetative growth. On each day after pathogen inoculation, Ψ and RWC values from the four treatments (non-mycorrhizal and mycorrhizal plants, inoculated or not with *V. dahliae*) were analysed with one-way ANOVA. Means ± SD (n = 8–11 data) were calculated and, when the F-ratio was significant, least significant differences were evaluated by the Tukey-b test. Within each parameter and day after pathogen inoculation, values followed by the same letter are not significantly different ($P \leq 0.05$). s = first foliar disease symptoms; fl = flowering; fr = beginning of fruit set.

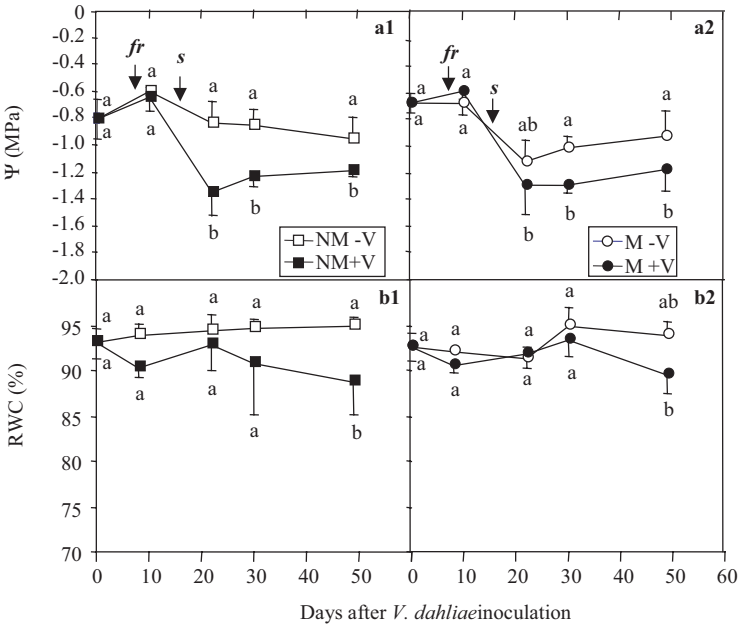

Figure 5. Water potential (MPa) (a) and RWC (%) (b) in leaves of non-mycorrhizal (NM) plants inoculated (+V, ■) or not (−V, □) with *V. dahliae* (a1, b1) and in mycorrhizal (M) plants inoculated (+V, ●) or not (−V, ◯) with *V. dahliae* (a2, b2) at flowering. Otherwise as for Figure 4.

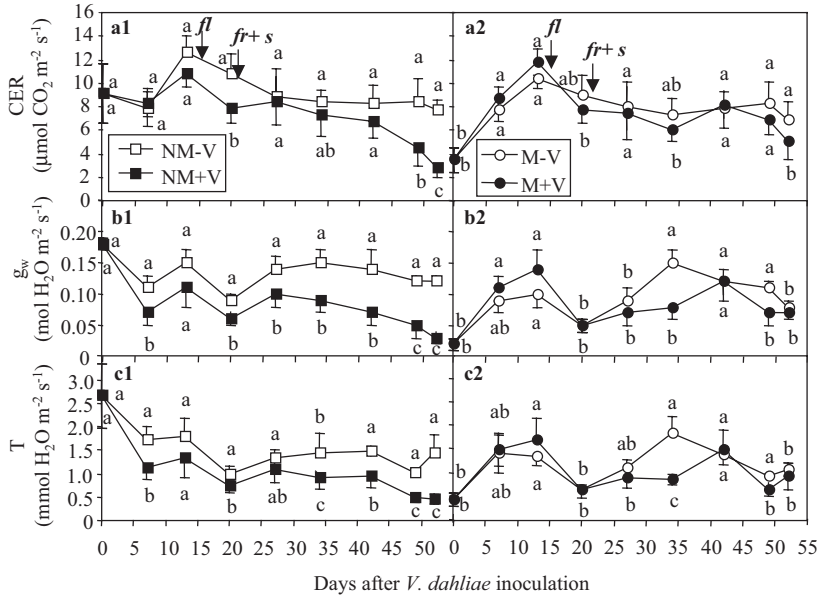

Figure 6. CO_2 exchange rate (μmol CO_2 m^{-2} s^{-1}) (a), g_w (mol H_2O m^{-2} s^{-1}) (b) and T rates (mmol H_2O m^{-2} s^{-1}) (c) in non-mycorrhizal (NM) plants inoculated (+V, ■) or not (−V, □) with *V. dahliae* (a1, b1, c1) and in mycorrhizal (M) plants inoculated (+V, ●) or not (−V, ◯) with *V. dahliae* (a2, b2, c2). Inoculation with *V. dahliae* was done during the vegetative growth. Values are means (n = 8–11 data). Otherwise as for Figure 4.

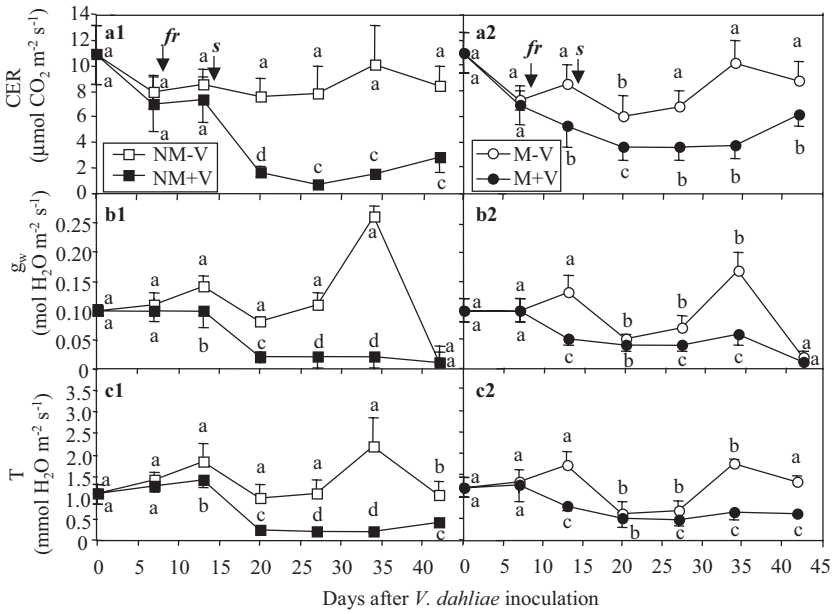

Figure 7. CO_2 exchange rate (μmol CO_2 m^{-2} s^{-1}) (a), g_w (mol H_2O m^{-2} s^{-1}) (b) and T rates (mmol H_2O m^{-2} s^{-1}) (c) in non-mycorrhizal (NM) plants inoculated (+V, ■) or not (−V, □) with *V. dahliae* (a1, b1, c1) and in mycorrhizal (M) plants inoculated (+V, ●) or not (−V, ◯) with *V. dahliae* (a2, b2, c2). Inoculation with *V. dahliae* was done at flowering. Values are means (n = 11 data). Otherwise as for Figure 4.

Table 3. Fruit set (%), fruit DM (g fruit^{-1}), length (cm) and diameter (cm) of peppers, and fruit yield (g plant^{-1}), in non-mycorrhizal healthy controls (NM − V), non-mycorrhizal *Verticillium*-inoculated (NM + V), mycorrhizal healthy controls (M − V) and mycorrhizal *Verticillium*-inoculated (M + V) plants

Time of *V. dahliae* inoculation	Treatment	Fruit set* (%)	Fruit DM* (g fruit^{-1})	Fruit length* (cm)	Fruit diameter* (cm)	Pepper yield* (g DM plant^{-1})
Vegetative growth	NM − V	34.65 a	2.90 a	6.46 a	2.72 a	4.82 a
(n = 9–10 plants)	NM + V	8.46 c	1.05 b	3.99 b	2.16 a	0.73 d
	M − V	22.68 b	1.67 b	6.56 a	2.63 a	3.31 b
	M + V	11.40 c	1.95 ab	5.99 a	2.75 a	1.45 c
	AMF	ns	ns	*	ns	***
	V. dahliae	***	*	**	ns	***
	Interaction	**	**	*	ns	***
Flowering	NM − V	19.57 a	2.43 a	6.79 a	3.07 a	3.06 b
(n = 8–10 plants)	NM + V	21.61 a	1.40 b	5.64 ab	2.38 b	1.29 c
	M − V	24.16 a	2.51 a	6.11 ab	2.94 a	4.46 a
	M + V	23.17 a	1.34 b	5.00 b	2.07 b	1.82 c
	AMF	ns	ns	ns	ns	***
	V. dahliae	ns	***	**	***	***
	Interaction	ns	ns	ns	ns	ns

*Within each column and time of pathogen inoculation values were analysed with two-way ANOVA with mycorrhizal colonisation and *Verticillium*-inoculation as the main effects. Otherwise as for Table 2.

Fruit ripening was delayed by AMF in healthy controls (Figure 8). The inoculation with *V. dahliae* during the vegetative period (Figure 8a) retarded fruit maturation in non-mycorrhizal pepper, while it had no effect on fruit ripening in mycorrhizal plants. In contrast, pathogen inoculation at flowering accelerated pepper maturation in both non-mycorrhizal and mycorrhizal treatments (Figure 8b).

Discussion

Time of plant inoculation with mycorrhizal fungi (before, simultaneously or after inoculation with pathogens) greatly influenced the efficacy of symbiotic fungi in the control of pathogens (Singh et al., 2000). We inoculated *V. dahliae* once AMF was established because pre-inoculation with AMF can nullify or reduce the detrimental effects of root pathogens on plant growth (Jaizme-Vega et al., 1997). Only when inoculated at flowering, *V. dahliae* negatively affected the colonisation of roots by AMF, which suggests a competition between *Glomus* and *Verticillium* for host resources (Borowicz, 2001) at the reproductive stage of pepper plants.

The increased capacity for nutrient uptake by the mycorrhizal association may allow host plants to be more vigorous and, consequently, more resistant or tolerant of pathogen attack (Azcón-Aguilar et al., 2002). In our study, non-mycorrhizal and mycorrhizal pepper plants had similar shoot biomass the day when they were inoculated with *V. dahliae* at the vegetative period. However, the higher root development in plants associated with AMF compared to non-mycorrhizal ones could be related to the reduction in the deleterious effect of *V. dahliae* on pepper growth and yield observed in the mycorrhizal treatment (Azcón-Aguilar et al., 2002). Similarly to the observations of Tzeng et al. (1985) in cotton, *V. dahliae* reduced plant height. However, when the pathogen was inoculated during the vegetative period, shoot growth was delayed at an earlier date in non-mycorrhizal than in mycorrhizal plants.

Inoculation with *V. dahliae* caused activation of axillary buds in both non-mycorrhizal and mycorrhizal pepper. Sadras et al. (2000) suggested that apical dominance of infected plants could be affected by some changes in hormone-like signals involved in the interaction between host plant and pathogen.

Independently of phenology at pathogen inoculation time, diseased non-mycorrhizal and mycorrhizal plants showed lower leaf Ψ than their respective controls, while RWC decreased later or remained unchanged. Goicoechea et al. (2000) found that leaf Ψ in pepper appeared to be more sensitive to infection with *V. dahliae* than RWC. When comparing *Verticillium*-inoculated plants at the vegetative stage, we found that leaf RWC in mycorrhizal plants at the end of the experiment was higher than that measured in

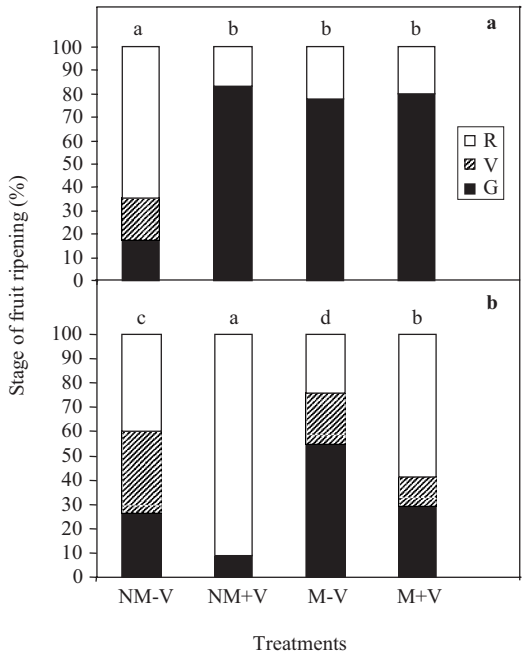

Figure 8. Stages of fruit ripening in non-mycorrhizal (NM) and mycorrhizal (M) plants inoculated (+V) or not (−V) with *V. dahliae* during the vegetative growth (a) or at flowering (b). G = green pepper, V = veraison and R = red pepper. Values are expressed as percentages. Within each graph, frequencies of green pepper, veraison and red pepper in the four treatments (non-mycorrhizal and mycorrhizal plants, inoculated or not with *V. dahliae*) were analysed by χ^2-test. Values followed by the same letter indicate similar stage of fruit ripening among treatments ($P \leq 0.05$).

non-mycorrhizal ones despite their similar leaf Ψ, suggesting an improved hydraulic conductance in plants associated with AMF (Mushin and Zwiazek, 2002).

Verticillium dahliae reduced CER, g_w and T in inoculated plants. Similar results have been observed in potato (Bowden and Rouse, 1991), tomato (Lorenzini et al., 1997) and pepper (Goicoechea et al., 2001). However, such decreases were more evident in non-mycorrhizal than in mycorrhizal plants, especially when the pathogen inoculation was done during the vegetative growth. This reinforces the idea that AMF may be especially important for plants subjected to adverse conditions (Goicoechea et al., 1997).

Previous studies showed that *V. dahliae* reduced the quantity and the quality of pepper fruits (García-Mina et al., 1996), may be due to the important decrease of photosynthesis combined with the premature fall of flowers caused by the pathogen (Goicoechea et al., 2001). Likewise, results of the present work show that *V. dahliae* negatively affected fruit DM with the exception of mycorrhizal plants inoculated at the vegetative period. As fruit growth is mainly sustained by the supply of current photoassimilate (Ho, 1992), the lower decrease in CER observed in *Verticillium*-inoculated mycorrhizal plants compared to non-mycorrhizal ones could benefit the development of peppers in plants associated with AMF.

Tzeng et al. (1985) found that the greatest impact of *Verticillium*-wilt on lint yield occurred on cotton plants in which foliar symptoms appeared around the time of the beginning of boll set. Thus, in our experiment, the coincidence of the development of the first symptoms with fruit initiation could explain why the inoculation with the pathogen during the vegetative stage had greater impact on pepper yield than the inoculation at flowering. We also found differences when comparing non-mycorrhizal and mycorrhizal controls from the experiment in which the pathogen was inoculated during the vegetative period. The lower fruit set, DM and yield in the mycorrhizal treatment could be due to the partial transport of photoassimilates from leaves to AMF in roots (Bethlenfalvay et al., 1982) in detriment to fruit development (Ho, 1992).

Fruit ripening was affected by both *Glomus* and *Verticillium*. The presence of AMF always delayed pepper maturation, which suggests that hormonal balance could differ between non-mycorrhizal and mycorrhizal plants. In fact, higher levels of cytokinins have been measured in mycorrhizal roots (Danneberg et al., 1992; Goicoechea et al., 1996). In non-climacteric fruits, such as pepper, the formation of red colour is avoided by cytokinin production in roots (Agustí, 2000). On the other hand, the effect of *V. dahliae* on fruit maturation was highly dependent on time of inoculation. While an early inoculation caused a significant delay on pepper ripening in non-mycorrhizal plants, inoculation at flowering accelerated fruit maturation in both non-mycorrhizal and mycorrhizal treatments. Such different behaviours could be due to differences in competition between vegetative and reproductive organs in both cases (Ho, 1992). When *V. dahliae* was inoculated during the vegetative stage, the development of axillary branches close to the fruit set could retard fruit ripening by competition for photoassimilates. When pathogen inoculation was done at flowering, the time span from fruit set to axillary buds activation and the lower development of lateral branches allowed pepper fruits to be a stronger sink for assimilates than vegetative organs.

In summary, results suggest that AMF could reduce the deleterious effect of *V. dahliae* on pepper

growth and yield. However, bioprotection against *Verticillium*-wilt was conditioned by plant phenology at the moment of pathogen attack. When pathogen infection occurred during the vegetative growth of plants, AMF allowed leaf RWC to be maintained for longer and delayed both the appearance of disease symptoms and the decrease of photosynthesis in *Verticillium*-inoculated plants. Although the colonisation of pepper roots by AMF delayed fruit ripening, benefits of symbiotic association on the physiology of *Verticillium*-inoculated plants favoured fruit qualities and yield.

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