

## Review. Arbuscular mycorrhizal fungi (AMF) as bioprotector agents against wilt induced by *Verticillium* spp. in pepper

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### Abstract

*Verticillium dahliae* Kleb. is a vascular pathogen that alters water status and growth of pepper plants and causes drastic reductions in yield. Its control is difficult because it can survive in field soil for several years. The application of arbuscular mycorrhizal fungi (AMF) as bioprotector agents against *V. dahliae* is an alternative to the use of chemicals which, in addition, is more respectful with the environment. The establishment of the mutualistic association of plant roots and AMF involves a continuous cellular and molecular dialogue between both symbionts that includes the preactivation of plant defense responses that may enhance the resistance or tolerance of mycorrhizal plants to soil-borne pathogens. Some AMF can improve the resistance of *Capsicum annuum* L. against *V. dahliae*. This is especially relevant for pepper cultivars (*i.e.* cv. Piquillo) that exhibit high susceptibility to this pathogen. Compared with non-mycorrhizal plants, mycorrhizal pepper can exhibit more balanced antioxidant metabolism in leaves along the first month after pathogen inoculation, which may contribute to delay both the development of disease symptoms and the decrease of photosynthesis in *Verticillium*-inoculated plants with the subsequent benefit for yield. In stems, mycorrhizal pepper show earlier and higher deposition of lignin in xylem vessels than non-mycorrhizal plants, even in absence of the pathogen. Moreover, AMF can induce new isoforms of acidic chitinases and superoxide dismutase in roots. Mycorrhizal-specific induction of these enzymatic activities together with enhanced peroxidase and phenylalanine ammonia-lyase in roots may also be involved in the bioprotection of *Verticillium*-induced wilt in pepper by AMF.

**Additional key words:** biological control, *Capsicum annuum*, mycorrhizal symbiosis, *Verticillium dahliae* Kleb.

### Resumen

**Revisión. Los hongos micorrízico arbusculares (MA) como agentes bioprotectores del pimiento frente a la seca inducida por *Verticillium* spp.**

*Verticillium dahliae* Kleb. altera el estado hídrico y el crecimiento de las plantas de pimiento y reduce la producción del fruto. El empleo de hongos micorrízico arbusculares (MA) como bioprotectores contra *V. dahliae* es una alternativa al uso de productos químicos más respetuosa para el medio ambiente. La asociación entre las raíces de las plantas y los hongos MA conlleva un diálogo celular y molecular entre ambos simbioses, que incluye la preactivación de respuestas de defensa en la planta, lo cual puede incrementar la resistencia o tolerancia de las plantas micorrizadas hacia los patógenos edáficos. Algunos hongos MA incrementan la resistencia del pimiento (*Capsicum annuum* L.) contra *V. dahliae*. Esto es especialmente relevante cuando el cultivar (cv. Piquillo, por ejemplo) es muy susceptible hacia el patógeno. En comparación con las plantas no micorrizadas, las hojas de las plantas de pimiento micorrizadas muestran un metabolismo antioxidante más equilibrado a lo largo del primer mes tras la inoculación del patógeno, lo que puede retrasar el descenso de la fotosíntesis, con el consiguiente beneficio para la producción. La deposición de lignina en el xilema es más temprana en los tallos de las plantas micorrizadas y los hongos MA inducen nuevas isoformas de enzimas quitinasa ácida y superóxido dismutasa en las raíces. La induc-

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ción específica de estas isoenzimas unida a unas actividades peroxidasa y fenilalanina amonio-liasa más elevadas en las raíces de las plantas micorrizadas también pueden favorecer la bioprotección del pimiento frente a la seca inducida por *Verticillium*.

**Palabras clave adicionales:** *Capsicum annum*, control biológico, simbiosis micorrícica, *Verticillium dahliae* Kleb.

## Introduction

Peppers belong to the family of Solanaceae and the genus *Capsicum*. Although this genus includes 25 species, most of the peppers cultivated in temperate and tropical areas belong to the species *C. annum*, thought to originate in Mexico and Central America (Andrews, 1995). The cultivated peppers are herbaceous, frost-sensitive plants that can be grown as an annual or as perennial crop in field or greenhouses. This crop is widely consumed as a fresh vegetable or condiment and used for pharmaceutical and cosmetic purposes (Bosland, 2003).

Pepper plants can suffer disorders, diseases and pests that reduce fruit quality and yield. Diseases can be caused by a wide range of biological agents, including bacteria, fungi, viruses, parasitic higher plants, insects, nematodes, birds and mammals (Bosland, 2003). The most important diseases caused by fungi and Oomycetes are Anthracnose, *Cercospora*, Leaf Spot, Charcoal Rot, Choanephora Blight, Damping-Off and Root Rot, Fusarium Stem Rot, Fusarium Wilt, Gray Leaf Spot, Gray Mold, Phytophthora Blight, Powdery Mildew, Southern Blight, White Mold and Verticillium Wilt. Verticillium Wilt constitutes a relevant economic problem because it produces great yield losses in America (Evans and McKeen, 1975) and Mediterranean countries (Thanassouloupoulos and Kitsos, 1972; García-Mina *et al.*, 1996; Pomar *et al.*, 2001) and it can affect pepper cultivated in both field (García-Mina *et al.*, 1996) and greenhouse (Gayoso *et al.*, 2007). Verticillium Wilt of pepper can be caused by both *Verticillium albo-atrum* Reinke and Berthlod and *V. dahliae* Klebahn, being the latter the most frequently reported as the causal organism (Goldberg, 2003).

### Causal agents of Verticillium Wilt in pepper

Both *Verticillium albo-atrum* and *V. dahliae* are virulent plant pathogenic species with low saprotrophic

abilities. However, while *V. dahliae* form microsclerotia, which are black melanised clumps formed by budding of mycelial cells, *V. albo-atrum* produces only dark and melanised resting mycelium (Barbara and Clewes, 2003). Those resting structures are responsible for the great difficulties to control *Verticillium* wilt diseases. Moreover, the control becomes extremely difficult when the pathogen forms microesclerotia because these resting structures can survive 10 to 15 years in the soil (Heale and Karapapa, 1999). According to Barbara and Clewes (2003), *V. dahliae* should include all isolates which produce only microsclerotia.

The epidemiology of *V. dahliae* has been widely studied. As explained by Zhou *et al.* (2006), the infection process of *V. dahliae* can be divided into two phases. Phase I begins with the germination of microsclerotia in response to plant root exudates (Mol and Scholte, 1995). The fungus enters the root through epidermis or wounds, crossing cortical root to gain access to immature xylem elements. Then phase II takes place, which includes hyphal proliferation and conidia production (Beckman, 1987). The conidia are spread in the vascular elements, where they germinate and colonization occurs. The new set of conidia continues the colonization of the upstream vessels (Beckman, 1987; Gold *et al.*, 1996). In a later stage and coinciding with senescence of leaves, the fungus enters a saprophytic stage. It goes out of the xylem elements and colonizes the surrounding nonvascular tissues in shoots and roots. Afterwards, new microsclerotia that appear in the dying stems and leaves (Mol, 1995) will be incorporated in the soil and will constitute a new source of inoculum. *Verticillium dahliae* causes, therefore, monocyclic disease, which means that only one cycle of disease and inoculum production take place during a growing season (Fradin and Thomma, 2006). In contrast to *V. dahliae*, *V. albo-atrum*, during the saprophytic stage, may produce conidia on infected plant tissues that can become airborne. The dispersion of conidia favors fungal spread so that another cycle of disease can be initiated

Abbreviations used: AMF (arbuscular mycorrhizal fungi), CAT (catalase), CER (CO<sub>2</sub> exchange rate), GPX (guaiacol peroxidase), g<sub>w</sub> (leaf conductance), PAL (phenylalanine ammonia-lyase), RWC (relative water content), SOD (superoxide dismutase), T (transpiration rate), Ψ (water potential), Ψ<sub>p</sub> (pressure potential).

(Jiménez-Díaz and Millar, 1988). This is the reason why the diseases caused by *V. albo-atrum* may sometimes be polycyclic (Fradin and Thomma, 2006). In the dormant phase, *V. albo-atrum* only produces dark and melanised resting mycelium which exhibits shorter survival potential than microsclerotia produced by *V. dahliae*.

### **Growth, physiology, metabolism and fruit yield of pepper plants infected by *Verticillium* spp.**

Symptom severity of Verticillium Wilt in pepper widely varies with soil and air temperatures and nutrient availability (Goldberg, 2003). In addition, the pathogenicity of *V. dahliae* to pepper can be determined by the previous cropping history and the host of origin (Novo *et al.*, 2006). Moreover, when research studies are performed under controlled conditions, the effect of *V. dahliae* on pepper growth is also dependent on plant phenology when the inoculation with the pathogen is carried out (Goicoechea *et al.*, 2000, 2001; Garmendia *et al.*, 2004a). In fact, while a marked reduction in shoot growth occurred when *V. dahliae* infected pepper plants during their vegetative growth, the pathogen did not affect plant height when it was inoculated at flowering. Furthermore, the negative effect of *V. dahliae* on total plant biomass is also dependent on the degree of tolerance exhibited by pepper cultivars. For example, the decrease in fresh weight was 69% in the tolerant cultivars Padrón and Yolo Wonder and achieved 81% in the susceptible cv. Luesia (Pomar *et al.*, 2004). In a recent study, Novo *et al.* (2006), working with Yolo Wonder and Luesia pepper cultivars, used the reduction in plant dry weight to classify different *V. dahliae* isolates according to their aggressiveness. Difference in the degree of aggressiveness among the different isolates suggested high variability among sampling populations independent of their original location. When applied real-time PCR techniques for detecting fungal mycelium in infected pepper, Gayoso *et al.* (2007) found good correlation between the severity of the symptoms displayed by *Verticillium*-infected plants and the relative amount of fungal DNA measured as fungus/plant DNA ratio.

*Verticillium dahliae* can also activate axillary buds in infected pepper (Garmendia *et al.*, 2004a), suggesting 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. When the development of axillary branches is close to the fruit set, the growth of secondary shoots may result in enhanced competition for assimilates to the detriment of both fruit production and ripening.

Goicoechea *et al.* (2000) observed that the leaf water potential ( $\psi$ ) in *Verticillium*-infected pepper declined before any change in the relative water content (RWC) was still observed. In addition, the decline in leaf  $\psi$  occurred before the appearance of visible symptoms of the disease, which indicates that *V. dahliae* released toxic substances that altered the physiology of the plant host even when the fungus was still localized in the roots. In fact, both *V. dahliae* and *V. albo-atrum* are reported to produce phytotoxins and other molecules that induce host cell death (Pegg, 1965). Once the infection had progressed, leaf RWC sharply declined and this reduction was concomitant with drastic increases in free proline and total soluble sugars (TSS), suggesting that the accumulation of proline and TSS could be sensor of the damage caused by the fungal infection (Goicoechea *et al.*, 2000).

*Verticillium dahliae* reinforces the reduction in the rate of CO<sub>2</sub> assimilation and leaf conductance experienced by pepper plants as a consequence of the natural senescence process. However, the decline of photosynthesis in infected pepper can be greater than that measured in the leaf conductance even without any significant change in the total chlorophyll content (Goicoechea *et al.*, 2001), suggesting that other factors apart from stomatal closure and chlorophyll degradation —*i.e.* Rubisco activity— can be also involved in the CO<sub>2</sub> exchange rate reduction. Defoliation is another factor to take into account when talking about gas exchange. When infected with defoliating *V. dahliae* strains, the reduced gas exchange of the entire plant can be mainly due to decreased leaf area rather than to reduced photosynthetic efficiency (Gent *et al.*, 1995).

Pomar *et al.* (2004) found that, in the tolerant pepper cv. Yolo Wonder, changes in stem lignins (monomer composition and crosslinking) and peroxidase activity were related with the maintenance of leaf photosynthetic integrity during Verticillium Wilt. Conversely, in the susceptible cv. Luesia, photochemical processes were strongly damaged as a consequence of the disease. The increased lignin contents in stems of tolerant pepper cultivar did not stop fungal hyphae penetration but could have restricted the growth of the fungal hyphae in the xylem. Recently, Novo *et al.* (2007), also working with Yolo Wonder and Luesia cultivars, mea-

sured increases in elemental sulphur levels similar to the accumulation of phenolic compounds previously reported by Pomar *et al.* (2004) in the mentioned cultivars after *V. dahliae* inoculation, which suggests that, in pepper plants, sulphur—though clearly not conferring full resistance—may form part of a multifaceted response that creates physical and chemical barriers to colonization by *V. dahliae*. Working with a very susceptible cultivar of pepper (cv. Piquillo), Garmendia *et al.* (2006) observed that *V. dahliae* neither stimulated the phenylpropanoid pathway nor elicited hydrolytic activities in infected pepper roots. However, this apparent maintenance of enzymatic activities could be due to the time interval (one week) between plant harvests, which may possibly be too long to detect temporal changes related to the defense response.

*Verticillium dahliae* can accelerate the reproductive ability of pepper plants, possibly through some changes in host hormonal balance. However, many of the flowers may fall prematurely in inoculated plants (Goicoechea *et al.*, 2001), which produces a significant decline in fruit set and, consequently, in yield (Garmendia *et al.*, 2004a). Total yield per plant may also decrease because of the small size achieved by individual fruits in *Verticillium*-infected plants (Garmendia *et al.*, 2004a). Fruit ripening can be altered by the infection with *V. dahliae*. However, in experiments carried out under controlled conditions, it is possible to find contrasting results depending on plant phenology when the pathogen is inoculated. If the pathogen attack takes place during the vegetative growth of pepper plants, fruit maturation may be delayed. In contrast, when *V. dahliae* is inoculated at flowering, fruit ripening may be accelerated (Garmendia *et al.*, 2004a). Such different behaviors could be due to differences in competition between vegetative and reproductive organs in both cases. As explained previously, when the activation of axillary buds in *Verticillium*-infected plants occurs close to the fruit set, the growth of secondary shoots may result in enhanced competition for photoassimilates to the detriment of both fruit production and ripening.

## Control of *Verticillium* Wilt

For intensively managed crops, it has been common practice to fumigate with methyl bromide (MB) prior to planting in order to reduce soil-borne pathogens (Jarvis, 1993; Chellemi, 2000). However, MB causes

soil and water contamination and severely reduces the diversity of the microflora in the treated soil. These reasons, together with the inclusion of MB among the substances with high ozone-depleting potential by the Montreal Protocol, an international treaty sponsored by the United Nations Environment Programme (UNEP), have led to the search for alternatives to the use of MB. Chloropicrin (CP) (trichloronitromethane) has traditionally been used in formulations together with MB to broaden the spectrum of activity and enhance the control of soil-borne fungal pathogens (Wilhelm *et al.*, 1961). In the European Union, however, the use of 1,3-dichloropropene and CP may be forbidden in the future. For this reason, many governments are in urgent need of non-chemical alternatives to the use of MB, 1,3-dichloropropene and CP to control soil pests.

Crop rotation has been used to reduce, at least to some extent, propagules that remain in the soil and may infect susceptible plants. However, the efficacy of this management strategy may be reduced because some propagules of *Verticillium* spp. can persist by saprophytic colonization of plant debris and reproduction on nonhosts or weed species (Goldberg, 2003). In cases where the profit obtained from a crop plant is high, farmers prefer the use of chemicals to crop rotation. In addition, some «monocultures» have little or no possibilities for crop rotations. This is the case of peppers in Spain. In this country, the National Project on Methyl Bromide Alternatives was focused on studying the major problems that the «phased-out» use of MB could create in relation to the exploitation of highly economical and socially important horticultural crops, such as strawberries, peppers and some «cut flower» ornamentals (*i.e.*, the carnation). The Spanish Ministry of Agriculture requested that INIA — the National Institute for Agricultural and Food Research and Technology— developed a Research Program aimed at finding potentially viable approaches for «Alternatives to Methyl Bromide Conventional Uses». The Research Program was launched in June 1997, finished in 2002 and included several projects focused on the search for possible cultural practices, other chemical products or a combination of the two as alternatives to the use of MB. These research projects were developed in collaboration with farmers' cooperatives and producers' associations and field trials were carried out in the same cropping farms and geographical areas of the cropping species under study. After two year trials, results showed that chemical treatments based on 1,3-dichloropropene + chloropicrin (approx. 65:35%) gave

similar results to MB in terms of crop yield for pepper plants. Physical methods for soil pest control, such as «bio-fumigation + solarization» also gave good results under appropriate conditions. However, data on solarization or bio-fumigation alone have to date shown poorer performance of these individual treatments, as compared to MB, under the experimentally applied conditions. On 22<sup>nd</sup> November 2006, the Spanish government published a new Resolution to continue the search for chemical, physical, cultural and biological alternatives to the use of MB.

There are several alternatives to the application of MB and other chemicals to control *Verticillium* Wilts: the use of resistant cultivars, resistance induction, biological methods and the application of appropriate management strategies. These alternatives can be applied alone or may be included in a wider program of integrated control.

Resistance is considered to be the most prudent prevention strategy for the control of diseases due to its effectiveness, ease of use and lack of potential negative effects on the environment (Bosland, 2003). In addition, manipulation and enhancement of plant defense responses using genetic engineering technology has the potential to produce crop plants with improved resistance to pathogens (McFadden, 2000). Polygenic resistance to *Verticillium* spp. has been identified in several plant species, including alfalfa, cotton, potato and strawberry while single dominant resistance genes have been identified in cotton, sunflower, potato and tomato species (see review by Fradin and Thomma, 2006). In tomato, the *Ve* locus that provides resistance against *Verticillium* Wilt has been used by plant breeders for 60 years and is introduced in most cultivated tomatoes. Unfortunately, although there are some tolerant pepper cultivars (*i.e.*, Padrón and Yolo Wonder), resistance genes have not been identified for the *C. annuum*-*V. dahliae* interaction (Pomar *et al.*, 2004). In addition, as all cultivated species of *Capsicum* have  $2n = 24$  chromosomes, the crossability among the species is limited and breeders have only been able to make little differences in disease resistance (Greenleaf, 1986). In fact, no commercial cultivars resistant to the *Verticillium* Wilt are currently available (Goldberg, 2003). However, studies of Jung *et al.* (2003, 2005) constitute an important advance in the knowledge on genes involved in the response of pepper plants when subjected to pathogens, abiotic and environmental stresses. These authors reported the isolation and functional analysis of pepper *CALTP* genes encoding three lipid transfer

proteins, *CALTP*I and *CALTP*II and *CALTP*III. While *CALTP*I and *CALTP*III genes were mainly expressed in various pepper tissues infected by pathogens, *Phytophthora capsici*, a pathogenic fungus that causes wilt disease in pepper (Aguirreolea *et al.*, 1995), did not induce the transcription of the *CALTP*II gene. The identification of antifungal proteins and their expression in transgenic plants provides new possibilities of introducing resistance to pathogens in susceptible cultivars. *Capsicum annuum*, however, is one of the most recalcitrant dicotyledonous species for genetic engineering. In fact, there is not a universal procedure suitable to transform different cultivars within each species via *Agrobacterium tumefaciens* (Smith & Townsend) Conn. However, Pozueta-Romero *et al.* (2001) developed a tissue culture regeneration protocol that opened new prospects for *Agrobacterium*-mediated transformation in bell pepper.

Soil solarization using clear plastic mulch for pre-plant soil treatment is a common practice in modern agricultural systems as an alternative to MB. Indeed, this approach has been reported to be an effective technique to eradicate microsclerotia of *V. dahliae* in the soil and to control *Verticillium* Wilt in several herbaceous and woody hosts. However, the efficacy of soil solarization can be influenced by particular environmental and soil conditions such as physical soil characteristics and irrigation (López-Escudero and Blanco-López, 2000). Biofumigation is a non-chemical approach that combines organic matter or green manure with solarization to control diseases, nematodes and weeds (Kirkegaard *et al.*, 2000). In some cases, however, the joint application of solarization and biofumigation to control some pathogens led to a marked decrease in soil mycoflora (Martínez *et al.*, 2004).

Another management strategy that can be useful for controlling *V. dahliae* is the application of soil amendments. The main soil amendments used to control *Verticillium* Wilts as well as the major problems and limitations associated with their use have been recently discussed by Goicoechea (2009). As exposed in this review, the numerous factors that can influence the efficacy of organic amendments against *Verticillium*-induced wilts have led some researchers to be pessimistic about the chances of success for the application of such materials in Agriculture. However, the organic industry is rapidly becoming a significant player in the global agricultural production scene. According to the New South Wales (NSW) Department of Primary Industries of Australia (2008), the organic industry is fast moving

away from its «niche» industry status and into mainstream agriculture, with an estimated annual growth of up to 30% per year. Management of soil-borne pathogens (including *Verticillium* spp.) with organic amendments would be, as expressed by Lazarovits (2001), «a disease control strategy salvaged from the past». The type and level of organic amendment would clearly vary depending on soil characteristics, climate of the area, kind of crop and even the *Verticillium* isolate to be suppressed. However, all of these parameters must also be taken into account when applying chemical products to control pests and diseases and some of the main limitations previously ascribed to organic amendments (*i.e.* potential risk for human health) may be even more pronounced for some synthetic chemical compounds. Therefore, the use of organic products may represent a very interesting tool for the suppression of *Verticillium*-induced wilt, especially when they are included in a wider program of integrated control.

Biological control agents for plant diseases are currently being examined as alternatives to synthetic pesticides due to their perceived higher level of safety and minimal environmental impact. However, such agents may have potential risks to other organisms present in the ecosystem and these should also be considered (Brimmer and Boland, 2003). As microsclerotia are the most important structures that ensure the survival of *V. dahliae*, several biocontrol strategies have focused on (a) the inhibition of microsclerotia formation on diseased plant tissues after plant death, (b) reduction in survival of microsclerotia, (c) prevention of their germination and/or (d) prevention of root infection by germinating microsclerotia (Tjamos, 2000). In the early 1970s, several researchers identified microbial populations in the rhizosphere as constituting the first barrier to pathogen infection. Nowadays, it is well known that some soils are naturally suppressive to some soil-borne plant pathogens including *Fusarium*, *Gaeumannomyces*, *Rhizoctonia*, *Pythium* and *Phytophthora*. The groups of micro-organisms with antagonistic properties towards plant pathogens are diverse and include both plant-associated prokaryotes and eukaryotes (Barea *et al.*, 2005). The use of antagonistic microorganisms is a widely applied strategy for controlling *V. dahliae*, with *Talaromyces flavus* (Klöcker) Stolk & Samson being the most widely studied biological control agent (Tjamos, 2000). Other genera that have been reported as biocontrol agents against vascular or soil-borne fungal pathogens are *Bacillus*, *Pseudomonas*, *Chryseomonas*, *Penicillium*, *Sphingomonas*, *Stenotro-*

*phomonas*, *Streptomyces* and *Serratia*. For example, Larena *et al.* (2003) proposed that *Penicillium oxalicum* Thom. could be used as biocontrol agent against *Verticillium* Wilt of tomato under both greenhouse and field conditions. More recently, Berg *et al.* (2006) confirmed that *Pseudomonas*, *Serratia*, *Bacillus* and *Streptomyces* were the main groups of *Verticillium* bacterial antagonist in the rhizosphere of strawberry and oilseed rape. Apart from bacteria, certain fungal root endophytes can be used to control plant diseases biologically (Brimmer and Boland, 2003), including wilt induced by *V. dahliae*. A group of rhizospheric fungi that are of increasing interest are the arbuscular mycorrhizal fungi (AMF) and this is because the majority of crop and horticultural plants are associated with them to the mutual benefit of both plant host and fungus.

### The use of AMF as an alternative for controlling *Verticillium* Wilt in susceptible pepper cultivars

In Navarra, Northern Spain, wilt induced by *V. dahliae* is one of the most common diseases that affects pepper, and drastically reduces yield. In addition, the most appreciated pepper cultivar in this area, *C. annuum* cv. Piquillo, is very susceptible to *V. dahliae*. This cultivar, which mainly grows in the Southwest of Navarra, has Guarantee of Origin and it is very appreciated for catering. In the context of Sustainable Agriculture, the lack of genetic resistance in pepper cv. Piquillo together with restrictions on chemical products induce to look for other alternatives. As exposed previously, one possibility is the use of AMF. Since AMF can benefit plant growth and health there is an increasing interest in ascertaining their effectiveness in particular plant production practices. In fact, the mutualistic symbiosis between AMF and plant roots plays a crucial role in nutrient cycling in the ecosystem and can protect plants against soil-borne pathogens (Azcón-Aguilar and Barea, 1997). Several mechanisms can be involved in bioprotection by AMF (Azcón-Aguilar *et al.*, 2002): (a) the improvement of plant nutrient status can help mycorrhizal plants to overcome a pathogen's attack; (b) the increase in root biomass in mycorrhizal plants can compensate tissue damage by the pathogen; (c) mycorrhizal and pathogenic fungi may compete for host photosynthates and root colonization sites; (d) AMF can induce changes in the anatomy and architec-

ture of the root system and in the rhizosphere; (e) AMF can activate plant defense mechanisms. Effective bioprotection is a cumulative result of all these mechanisms working separately and/or together (Harrier and Watson, 2004).

### Influence of AMF on physiology and biochemistry of pepper plants inoculated or not with *V. dahliae*

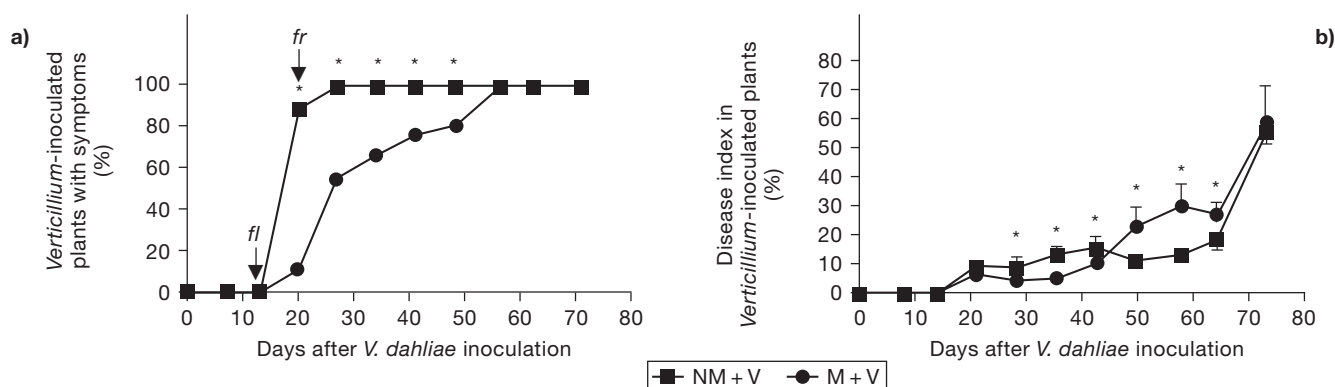
#### Leaves

The development of visible symptoms of the disease in pepper inoculated with *V. dahliae* can differ depending on the presence or absence of AMF associated with plant roots. In fact, Garmendia *et al.* (2004a) observed that, while 100% of non-mycorrhizal plants exhibited wilted, chlorotic and/or necrotic leaves 3 weeks after pathogen inoculation, at that time, foliar disease symptoms had appeared in only 15% of plants associated with *Glomus deserticola* (Trappe, Bloss and Menge) (Fig. 1a). These same authors, working with *C. annuum* cv. Piquillo, also found that the disease index remained unchanged in the mycorrhizal treatment between the third and the sixth weeks after inoculation, coinciding with the fruit set period (Fig. 1b).

The study of water status parameters has revealed that the reduction in leaf  $\Psi$  in non-mycorrhizal diseased plants (Fig. 2a1) occurs before any decrease in RWC is still observed (Fig. 2b1) (Garmendia *et al.*, 2004a). As it has been widely demonstrated, most wilt

pathogens increase the resistance to water movement. The decrease in leaf  $\Psi$  could be a mechanism developed by infected pepper plants in order to achieve the required tension to let water move from soil to shoot (Goicoechea *et al.*, 2000). However, Garmendia *et al.* (2004a) demonstrated that, in mycorrhizal plants inoculated with *V. dahliae*, leaf RWC always remained unchanged (Fig. 2b2) despite the reduction in leaf  $\Psi$  (Fig. 2a2). The relationship between RWC and  $\Psi$  has sometimes been used to quantify the dehydration tolerance of tissues (Sánchez-Díaz and Kramer, 1971). It has been suggested that tissues able to retain a high RWC as  $\Psi$  declines are more tolerant to dehydration. Also, maintenance of RWC at any given  $\Psi$  may reflect a greater rigidity of the cell walls and their ability to withstand mechanical collapse as water is being lost from the tissue (Bennet-Clark, 1959). Garmendia *et al.* (2004a) also observed that, two months after pathogen inoculation, leaf RWC in pepper associated with *G. deserticola* (Fig. 2b2) was higher than that measured in non-mycorrhizal plants (Fig. 2b1) despite their similar leaf  $\Psi$  (Fig. 2a1, 2a2), suggesting an improved hydraulic conductance in mycorrhizal plants (Mushin and Zwiazek, 2002). Moreover, Garmendia *et al.* (2005) measured higher pressure potential ( $\Psi_p$ ) in leaves of mycorrhizal pepper compared to that found in non-mycorrhizal plants, which may be due to different wall elasticity in the leaf cells from the two types of plants.

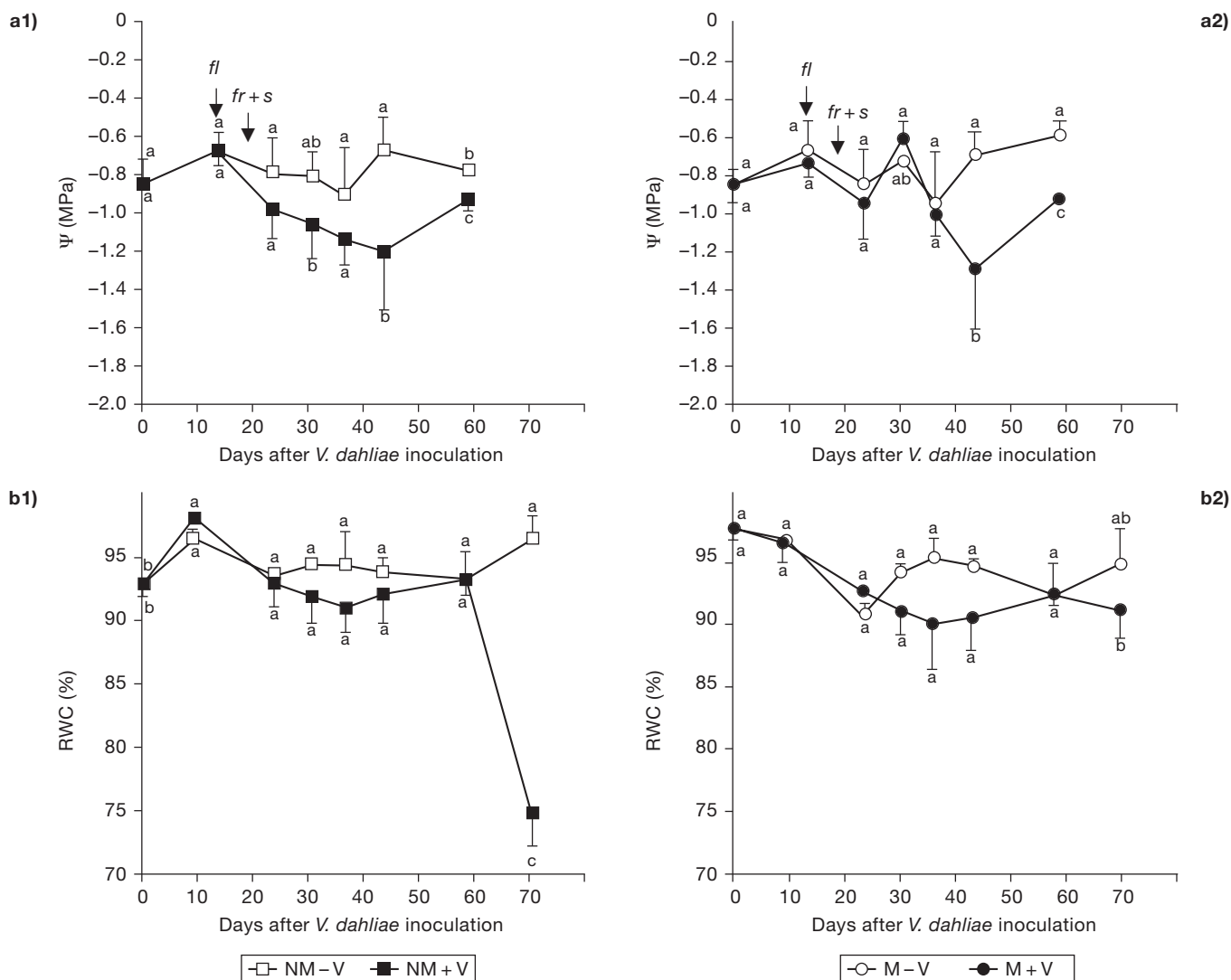
*Verticillium dahliae* can cause significant decreases of CO<sub>2</sub> exchange rate (CER), leaf conductance ( $g_w$ ) and transpiration rate (T) in both non-mycorrhizal and mycorrhizal plants (Fig. 3) according to Garmendia *et*



**Figure 1.** Percentage of plants with visible symptoms of disease (a) and disease index (%) (b) in non-mycorrhizal (NM, ■) and mycorrhizal (M, ●) pepper inoculated with *V. dahliae*. Percentages of plants showing disease symptoms (Fig. 1a) were subjected to arc-sin transformation before applying  $\chi^2$  test. Each point in Figure 1b represents the mean  $\pm$  SD of 8-10 plants. Means were compared with the Student's t-test. Within each graph, asterisks indicate significant differences ( $p \leq 0.05$ ). *fl*: flowering. *fr*: beginning of fruit set. This figure has been extracted from the European Journal of Plant Pathology 110, 227-238 (2004) with kind permission of Springer Science and Business Media.

al. (2004a). However, these authors found some differences between non-mycorrhizal and mycorrhizal diseased plants. First, pepper plants colonized by *G. deserticola* exhibited greater CER (Fig. 3a2),  $g_w$  (Fig. 3b2) and T (Fig. 3c2) than non-mycorrhizal ones (Figs. 3a1, 3b1, 3c1). Second, the declines in photosynthesis and  $g_w$  were firstly detected in non-mycorrhizal plants (Figs. 3a1, 3b1). Third, while the reduction in  $g_w$  in non-mycorrhizal plants was concomitant with the development of the first visible symptoms of the disease (Fig. 3b1), the decrease in  $g_w$  in mycorrhizal pepper

occurred 15 days after symptoms were first observed (Fig. 3b2). All these findings reinforce the idea that AMF may be especially important for plants subjected to adverse conditions (Peña *et al.*, 1988; Sánchez-Díaz *et al.*, 1990; Goicoechea *et al.*, 1997, 2004). 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 *G. deserticola*.

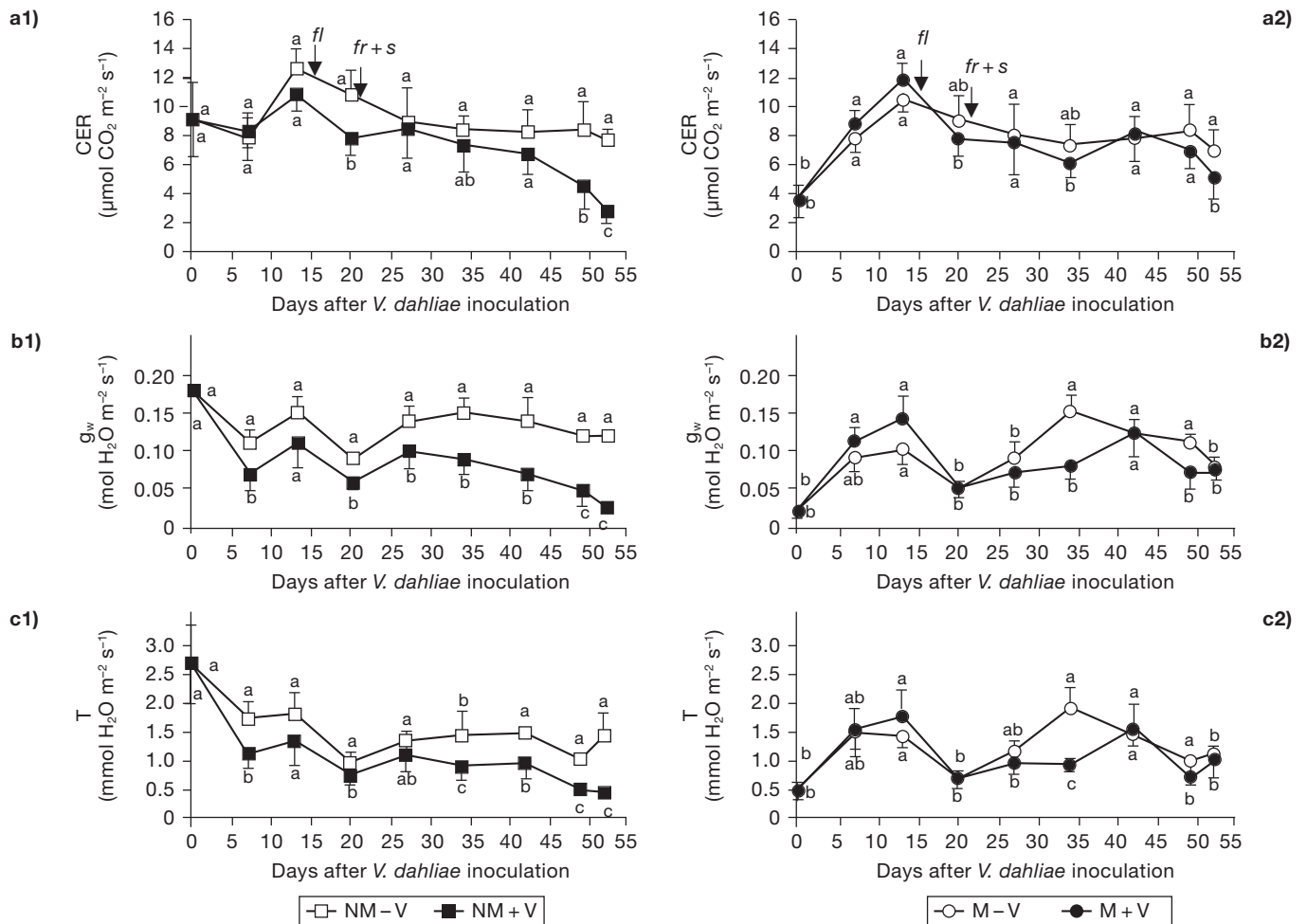


**Figure 2.** Water potential ( $\Psi$ ) (MPa) (a) and relative water content (RWC) (%) (b) in leaves of non-mycorrhizal (NM) plants inoculated (+V, ■) or not (-V, □) with *V. dahliae* (a1, b1) and in mycorrhizal (M) plants inoculated (+V, ●) or not (-V, ○) with *V. dahliae* (a2, b2). On each day, values were analysed with one-way ANOVA. Means  $\pm$  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 do not differ significantly ( $p \leq 0.05$ ). *s*: first foliar disease symptoms. *fl*: flowering. *fr*: beginning of fruit set. This figure has been extracted from the European Journal of Plant Pathology 110, 227-238 (2004) with kind permission of Springer Science and Business Media.



Studies focused on the antioxidant metabolism in pepper infected with *V. dahliae* and associated or not with AMF (Garmendia *et al.*, 2004b) demonstrated that such metabolism was modified in leaves of *Verticillium*-inoculated plants before symptoms of the disease were evident (Fig. 4), which agrees with the idea that *Verticillium* may release toxic substances that alter the metabolism and physiology of the host plant even when the fungus is still localized in the roots (Lorenzini *et al.*, 1997). However, the study performed by Garmendia *et al.* (2004b) showed the existence of some differences between the antioxidant metabolism of non-mycorrhizal and mycorrhizal pepper. When pathogen-inoculated plants were compared with their respective healthy controls on day 13 after the inoculation with *V. dahliae*, there was enhanced superoxide dismutase (SOD) acti-

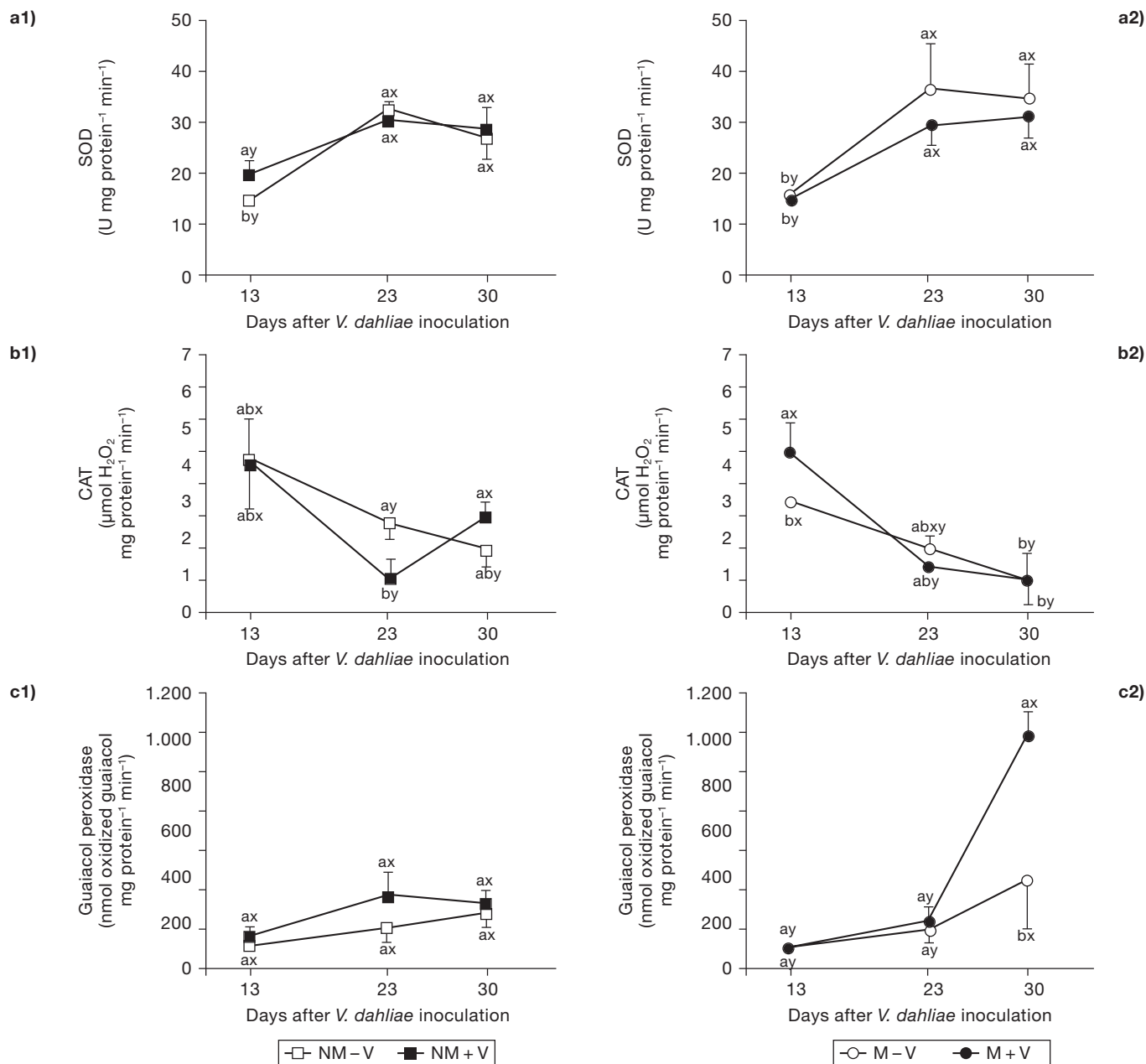
vity in leaves of non-mycorrhizal pepper infected with the pathogen (Fig. 4a1) and increased catalase (CAT) activity in leaves from plants colonized by *G. deserticola* and inoculated with *V. dahliae* (Fig. 4b2). García-Limones *et al.* (2002) observed higher activities of both SOD and CAT in stems of a susceptible cultivar of chickpea infected by *Fusarium oxysporum*, and no changes were found when the interaction between chickpea and *F. oxysporum* was incompatible. The early enhancement of SOD activity in non-mycorrhizal pepper (Fig. 4a1) which was not co-ordinated with increases in CAT (Fig. 4b1) or other peroxidase activities (*i.e.* guaiacol peroxidase, GPX, Fig. 4c1) could have resulted in an accumulation of H<sub>2</sub>O<sub>2</sub> in leaves and subsequent oxidative damage (Arcocha *et al.*, 2001). In addition, decreased CAT activity in *Verticillium*-inoculated non-



**Figure 3.** CO<sub>2</sub> exchange rate (CER) ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ) (a), leaf conductance (g<sub>w</sub>) ( $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ) (b) and transpiration rates (T) ( $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ) (c) in non-mycorrhizal (NM) plant 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). Otherwise, as for Figure 2. This figure has been extracted from the European Journal of Plant Pathology 110, 227-238 (2004) with kind permission of Springer Science and Business Media.

mycorrhizal plants on day 23 (Fig. 4b1) suggests that the amount of  $H_2O_2$  accumulated in leaves could even have increased from day 13. Active oxygen species can reinforce plant cell-walls, act as toxic agents against the pathogens and/or participate as second messengers in routes leading to the activation of plant defense-

related genes (García-Limones *et al.*, 2002). However, in *C. annuum* cv. Piquillo, the hypothetical accumulation of  $H_2O_2$  could be related to the accelerated senescence (Fig. 1a) and the decline in photosynthesis in plants infected with *V. dahliae* (Fig. 3a1), especially when pepper was not associated with *G. deserticola*



**Figure 4.** Superoxide dismutase (SOD) (U mg protein<sup>-1</sup> min<sup>-1</sup>) (a1, a2), catalase (CAT) (μmol H<sub>2</sub>O<sub>2</sub> mg protein<sup>-1</sup> min<sup>-1</sup>) (b1, b2) and guaiacol peroxidase (GPX) (oxidized nmol guaiacol mg protein<sup>-1</sup> min<sup>-1</sup>) (c1, c2) specific activities in non-mycorrhizal (NM) (a1, b1, c1) and mycorrhizal plants (M) (a2, b2, c2) inoculated (+V) or not (-V) with *V. dahliae*. Means ± SD (n=4 plants) were analyzed with one-way analysis of variance (ANOVA) and, when the F-ratio was significant, least significant differences were compared with the Tukey-b test. Within each parameter and day after pathogen inoculation, data with the same letter (a, b) do not differ significantly. Within each parameter and treatment, data with the same letter (x, y) do not differ significantly. Significance levels were always set at 5% level of significance.

**Table 1.** Total phenolic compounds (mg gallic acid g<sup>-1</sup> DM) in stems and roots from non-mycorrhizal (NM) plants and plants associated with *Glomus deserticola* (M), inoculated (+V) or not (-V) with *Verticillium dahliae*. Determinations were performed at the moment of the pathogen inoculation (day 0) or 70 days later. Means  $\pm$  SD ( $n = 4-6$  plants) were compared with the Tukey-*b* test. Values followed by a common letter within each column (day and plant organ) (a, b, c) or line (treatment and plant organ) (x, y, z) are not significantly different ( $p \leq 0.05$ )

Treatment	Days after pathogen inoculation			
	0		70	
	Stem		Root	
NM-V	4.12 $\pm$ 1.65 <sup>ax</sup>	1.94 $\pm$ 0.45 <sup>cy</sup>	2.07 $\pm$ 0.30 <sup>ax</sup>	2.11 $\pm$ 0.30 <sup>bx</sup>
NM+V	4.12 $\pm$ 1.65 <sup>ax</sup>	4.04 $\pm$ 0.55 <sup>ax</sup>	2.44 $\pm$ 0.55 <sup>ax</sup>	2.99 $\pm$ 0.57 <sup>bx</sup>
M-V	4.88 $\pm$ 1.15 <sup>ax</sup>	1.66 $\pm$ 0.47 <sup>cy</sup>	2.07 $\pm$ 0.30 <sup>ax</sup>	2.73 $\pm$ 0.68 <sup>bx</sup>
M+V	4.88 $\pm$ 1.15 <sup>ax</sup>	2.55 $\pm$ 0.79 <sup>by</sup>	2.44 $\pm$ 0.55 <sup>ay</sup>	3.46 $\pm$ 0.43 <sup>ax</sup>

(Garmendia *et al.*, 2004a,b). The increase in CAT activity in leaves of pathogen-inoculated non-mycorrhizal plants between days 23 and 30 (Fig. 4b1) observed by Garmendia *et al.* (2004b) did not seem to be high and/or early enough to avoid the development of disease symptoms. In contrast, GPX activity increased in leaves of pepper colonized by *G. deserticola* one month after pathogen inoculation (Fig. 4c2). This mechanism of H<sub>2</sub>O<sub>2</sub> detoxification could be related to the slower development of the disease observed in these plants when compared with non-mycorrhizal ones (Fig. 1a). The more balanced SOD, CAT and GPX activities in mycorrhizal plants could have also contributed to maintain control photosynthetic rates for longer (Fig. 3a2) and, consequently, would have benefit fruit yield (Table 2) in plants associated with *G. deserticola* (Garmendia *et al.*, 2004a,b).

### Stem

According to Pomar *et al.* (2004), working with *C. annuum* cvs. Padrón, Yolo Wonder and Luesia, the phenolic metabolism of pepper is altered during the Verticillium Wilt. In fact, the levels of total free phenolics clearly increased in stems of diseased plants, although the amount of chlorogenic acid, the main free phenolic in peppers (Díaz *et al.*, 1997), was comparable in healthy and diseased plants. In contrast, the amount of free phenolic compounds in stems of *C. annuum* cv. Piquillo had not increased two months after their inoculation with *V. dahliae* (Table 1) (Garmendia *et al.*, unpublished results). However, on day 70<sup>th</sup>, plants inoculated with the pathogen exhibited higher content of

phenolics than healthy plants, being levels greater in non-mycorrhizal than in mycorrhizal plants.

It is known that AMF can induce the synthesis of lignin and/or other bound phenolics acids (Morandi, 1996) that can play a role in strengthening the cell-walls and preventing invasion by pathogens. Lignins are three-dimensional phenolic heteropolymers covalently associated with polysaccharides in plant cell walls (Lewis *et al.*, 1999) and they are mainly localized in supporting tissues and in the xylem. Lignin deposition provides mechanical resistance to the stems and protects cellulose fibers from chemical and biological degradation in the face of fungal attack (Grabber *et al.*, 1998). Since lignification mainly occurs in the secondary xylem cell wall, which is in potential contact with *V. dahliae*, it may be expected that xylem lignification acts as a physical barrier against *V. dahliae* (Smit and Dubery, 1997). According to microscopic observations of cross-stem sections taken from pepper cv. Piquillo and stained with fast green-safranin (Fig. 5), there were not clear differences between non-mycorrhizal (NM+V) and plants associated with *G. deserticola* (M+V) one month after their inoculation with *V. dahliae* (Garmendia *et al.*, unpublished results). However, when compared healthy non-mycorrhizal (NM-V) and mycorrhizal (M-V) plants, lignin deposition was higher in the xylem of stems belonging to plants colonized by *G. deserticola*. The early enhancement of peroxidase activity in roots of plants associated with *G. deserticola* (Garmendia *et al.*, 2006), could be involved in higher stem lignification of mycorrhizal peppers. It is known that peroxidases catalize the oxidative polymerization of phenylpropanols to produce lignin and the cross-linking of cell wall proteins, con-

**Table 2.** Fruit set (%), fruit DM (g fruit<sup>-1</sup>), length (cm) and diameter (cm) of peppers, and fruit yield (g plant<sup>-1</sup>) in non-mycorrhizal (NM) plants and plants associated with *Glomus deserticola* (M), inoculated (+V) or not (-V) with *Verticillium dahliae*. Means ( $n = 4-6$  plants) were compared with the Tukey-*b* test. Within each column values followed by the same letter are not significantly different ( $p \leq 0.05$ )

Treatment	Fruit set (%)	Fruit DM (g fruit <sup>-1</sup> )	Fruit length (cm)	Fruit diameter (cm)	Pepper yield (g DM plant <sup>-1</sup> )
NM-V	34.65 <sup>a</sup>	2.90 <sup>a</sup>	6.46 <sup>a</sup>	2.72 <sup>a</sup>	4.82 <sup>a</sup>
NM+V	8.46 <sup>c</sup>	1.05 <sup>b</sup>	3.99 <sup>b</sup>	2.16 <sup>a</sup>	0.73 <sup>d</sup>
M-V	22.68 <sup>b</sup>	1.67 <sup>b</sup>	6.56 <sup>a</sup>	2.63 <sup>a</sup>	3.31 <sup>b</sup>
M+V	11.40 <sup>c</sup>	1.95 <sup>ab</sup>	5.99 <sup>a</sup>	2.75 <sup>a</sup>	1.45 <sup>c</sup>

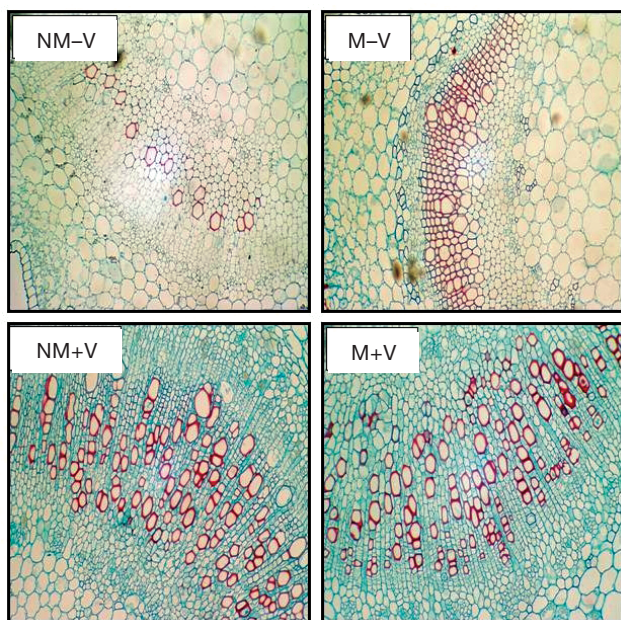
DM: dry matter. This table has been extracted from the European Journal of Plant Pathology 110, 227-238 (2004) with kind permission of Springer Science and Business Media.

tributing to enhance cell wall reinforcement during fungal penetration (Mittler, 2002). It is not clear if the enhanced lignin deposition in mycorrhizal pepper plants cv. Piquillo was a direct consequence of plant response to fungal colonization or it was due to possible changes in plant phenology. In any case, lignin deposition could have improved host plant ability to restrict the fungal growth in the xylem.

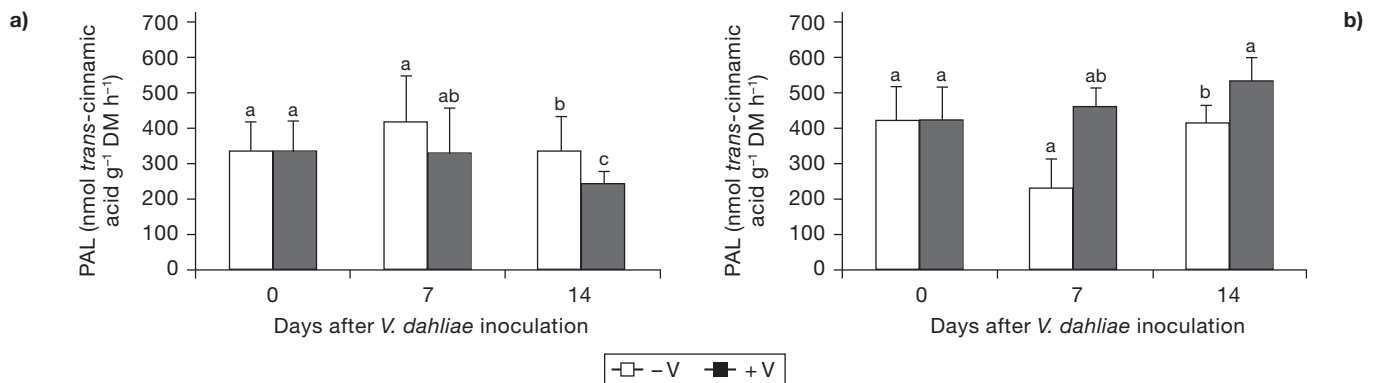
### Root

Garmendia *et al.* (2006) found that phenylalanine ammonia-lyase (PAL) activity remained unchanged or

even slightly decreased in non-mycorrhizal pepper plants cv. Piquillo when they were inoculated with *V. dahliae* (Fig. 6a). Resistant plants are characterized by a rapid increase in PAL activity once they are attacked by *Verticillium* spp. (Fradin and Thomma, 2006) but *C. annuum* cv. Piquillo does not exhibit genetic resistance against *V. dahliae* (Palazón, 1985). However, there can be other reasons to explain the lack of PAL enhancement in roots of *Verticillium*-infected pepper plants observed by Garmendia *et al.* (2006). First, in this study, pepper inoculated with *V. dahliae* had not still shown visible symptoms of the disease two weeks after pathogen inoculation. Guillon *et al.* (2002) observed that the highest PAL transcript only occurred when lesions on the hypocotyls were very evident in bean plants attacked by *Rhizoctonia solani*. Second, the possibility of a transient induction of PAL at early stages of the infection of *C. annuum* cv. Piquillo with *V. dahliae* cannot be discarded. Moreover, the reduction in PAL activity can coincide with high expression of *pal* genes (Kervinen *et al.*, 1998). Although colonization of roots by AMF can stimulate the phenylpropanoid pathway (see Morandi, 1996 for review), the presence of *G. deserticola* never induced PAL activity in roots of healthy pepper (Fig. 6b) in the study carried out by Garmendia *et al.* (2006), which agrees with observations of Guillon *et al.* (2002). The determination of total soluble phenolics in roots of four months old plants corroborated that healthy non-mycorrhizal and pepper plants associated with *G. deserticola* had similar levels of these compounds (Table 1) (Garmendia *et al.*, unpublished results). However, when compared PAL activity in roots of *V. dahliae*-infected pepper, plants colonized by *G. deserticola* exhibited the highest value two weeks after pathogen inoculation (Fig. 6b) (Garmendia *et al.*, 2006). Similarly, Dehne and Schönbeck (1979) observed that the simultaneous



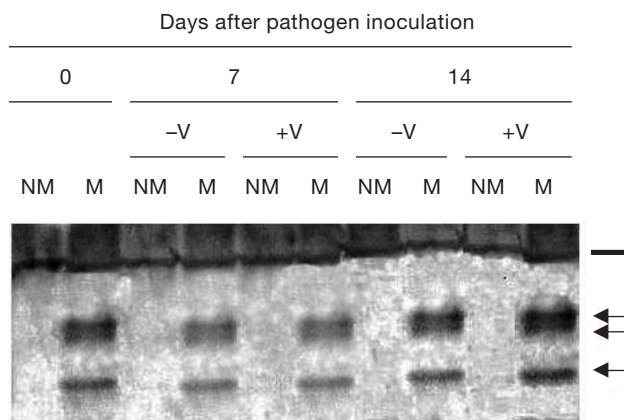
**Figure 5.** Lignin deposition (red colour) on xylem vessels in cross-stem sections of non-mycorrhizal (NM) and mycorrhizal (M) pepper plants inoculated (+V) or not (-V) with *V. dahliae* one month after pathogen inoculation.



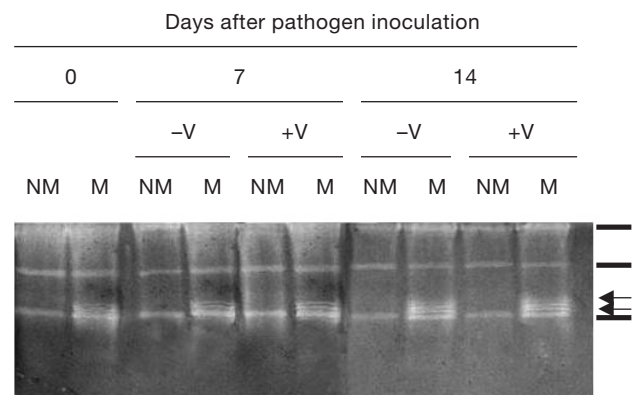
**Figure 6.** Phenylalanine ammonia-lyase (PAL) activities (nmol *trans*-cinnamic acid g<sup>-1</sup> DM h<sup>-1</sup>) in roots from non-mycorrhizal (NM) (a) and mycorrhizal (M) (b) plants inoculated (+V) or not (-V) with *V. dahliae*. Means (n = 3 plants) ± SD just before inoculating *Verticillium* were compared with Student's *t*-test and data on days 7 and 14 after pathogen inoculation were analysed by Tukey-b-test. Histograms followed by a common letter are not significantly different ( $p \leq 0.05$ ). This figure has been extracted from the journal *BioControl* 51, 293-310 (2006) with kind permission of Springer Science and Business Media.

infection of tomato with *G. mosseae* and *F. oxysporum* increased PAL activity of roots more than the mycorrhizal fungus alone or the pathogen alone. The increased PAL activity in roots of plants colonized by *G. deserticola* and infected with *V. dahliae* (Garmendia *et al.*, 2006) could explain the enhanced amount of soluble phenolic compounds in mycorrhizal roots two months after pathogen inoculation (Table 1) (Garmendia *et al.*, unpublished results).

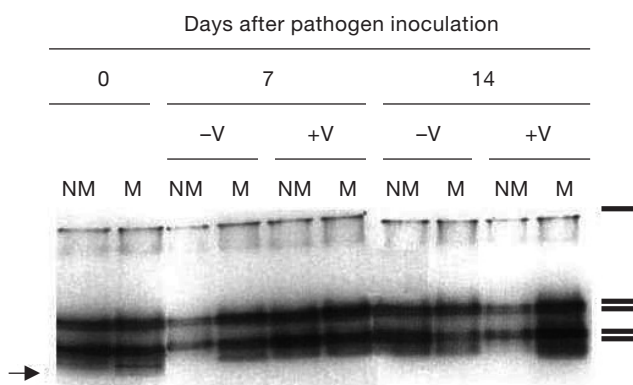
The presence of *G. deserticola* induced three new acidic chitinase (Fig. 7), two new SOD (Fig. 8) and one new peroxidase (Fig. 9) isoforms in roots of *C. annuum* cv. Piquillo at early stages of the symbiosis in healthy plants (Garmendia *et al.*, 2006). Association of roots with AMF can elicit plant chitinase (Pozo *et al.*, 1996), peroxidase (Gianinazzi and Gianinazzi-Pearson, 1992) and SOD (Lambais *et al.*, 2003) activities. The establishment of the mutualistic association between the roots of host plants and AMF involves a continuous



**Figure 7.** Acidic chitinase activity after separation of proteins under native conditions by David system in 15% (w/v) polyacrylamide gel containing glycol chitin as substrate. Roots extracts in McIlvaine buffer (7 µg of protein per sample) from non-mycorrhizal (NM) and mycorrhizal (M) plant inoculated (+V) or not (-V) with *V. dahliae* were analyzed just before inoculating the pathogen as well as 1 and 2 weeks later. Bars indicate constitutive isoforms and arrows mark additional isoforms. This figure has been extracted from the journal *BioControl* 51, 293-310 (2006) with kind permission of Springer Science and Business Media.



**Figure 8.** Superoxide dismutase (SOD) isoenzymes after separation of proteins under native conditions using David system in 15% (w/v) polyacrylamide gels. Root protein extracts in McIlvaine buffer (7 µg of protein per sample) from non-mycorrhizal (NM) and mycorrhizal (M) plants inoculated (+V) or not (-V) with *V. dahliae* were analyzed just before inoculating the pathogen as well as 1 and 2 weeks later. Bars indicate constitutive isoforms and arrows mark additional isoforms. This figure has been extracted from the journal *BioControl* 51, 293-310 (2006) with kind permission of Springer Science and Business Media.

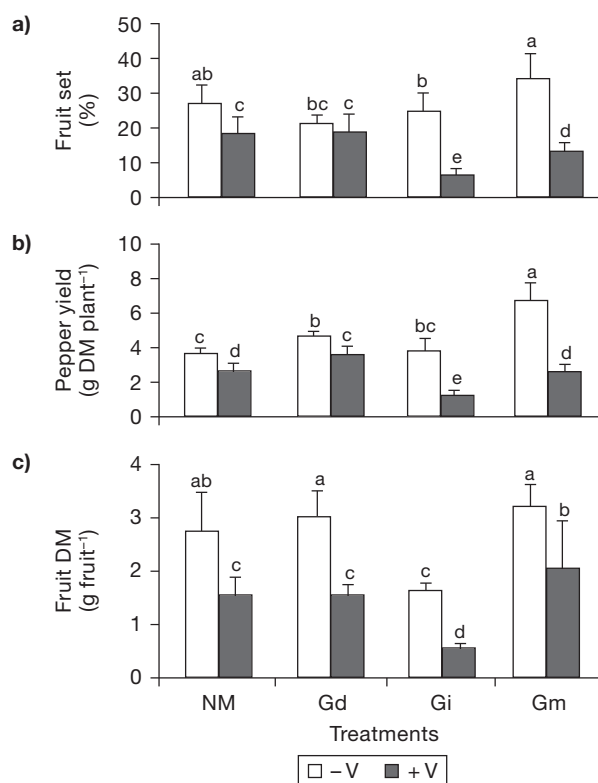


**Figure 9.** Peroxidase activity after separation of proteins under native conditions by David system in 15% (w/v) polyacrylamide gels. Root protein extracts (7  $\mu$ g of protein per sample) from non-mycorrhizal (NM) and mycorrhizal (M) plants inoculated (+V) or not (-V) with *V. dahliae* were analyzed just before inoculating the pathogen as well as 1 and 2 weeks later. After protein separation, gels were incubated in 0.1 M Tris-HCl (pH 7.6) with 4-chloro-1-naphthol and  $H_2O_2$ . Bars indicate constitutive isoforms and arrows mark additional isoforms. This figure has been extracted from the journal *BioControl* 51, 293-310 (2006) with kind permission of Springer Science and Business Media.

cellular and molecular dialogue between both symbionts (Bonfante-Fasolo, 1984) that includes the pre-activation of defense responses that can contribute to enhance the resistance of mycorrhizal plants to soil-borne pathogens (Azcón-Aguilar *et al.*, 2002).

### Influence of AMF on yield in pepper plants inoculated or not with *V. dahliae*

When studying fruit characteristics and yield in healthy plants, Garmendia *et al.* (2004a) observed, in some cases, decreases in fruit set, individual fruit dry matter (DM) and total yield per plant in mycorrhizal pepper compared to non-mycorrhizal plants (Table 2). The lower fruit set, DM and yield in plants associated with *G. deserticola* 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). However, the association of pepper with AMF not always causes reduction in the production of fruits (Fig. 10b) (Garmendia *et al.*, 2004c). In contrast, the inoculation of *C. annuum* cv. Piquillo with *V. dahliae* always had detrimental effects on yield (Garmendia *et al.*, 2004a,c). It has been found that *V. dahliae* reduces both the quantity and quality of pepper fruits in plants cultivated in both greenhouse



**Figure 10.** Fruit set (%) (a), pepper yield (g plant<sup>-1</sup>) (b) and fruit DM (g fruit<sup>-1</sup>) (c) in non-mycorrhizal (NM) plants and plants associated with *Glomus deserticola* (Gd), *G. intraradices* (Gi) or *G. mosseae* (Gm), inoculated (+V) or not (-V) with *Verticillium dahliae*. Values are means  $\pm$  SD ( $n = 4-6$  plants). Means were compared with the Tukey-*b* test. Within each graph, values followed by the same letter are not significantly different ( $p \leq 0.05$ ). DM: dry matter. This figure has been extracted from the journal *Biological Control* 31, 296-305 (2004) with kind permission of Elsevier (License Number 2158840328065).

(Garmendia *et al.*, 2004a) and field (García-Mina *et al.*, 1996) due to the relevant decrease of photosynthesis combined with the premature fall of flowers (Goicoechea *et al.*, 2001). The reduction in the percentage of fruit set and pepper yield as a consequence of pathogen inoculation was more evident in non-mycorrhizal plants and in plants colonized by *G. intraradices* (Schenck and Smith) and *G. mosseae* (Nicol. and Gerd.) Gerd. and Trappe than in pepper associated with *G. deserticola* (Table 2, Figs. 10a, 10b) (Garmendia *et al.*, 2004a,c). In addition, only non-mycorrhizal plants showed a reduction in fruit length and DM compared to their respective healthy controls (Table 2) (Garmendia *et al.*, 2004a). As suggested previously, the lower decrease in CER observed in plants associated with *G. deserticola* (Fig. 3a2) after their inoculation with the pathogen compared to non-mycorrhizal ones

(Fig. 3a1) would have benefited the development of fruits in plants associated with the mentioned AMF (Table 2) (Garmendia *et al.*, 2004a).

In summary, slight but significant improvement of fruit yield per plant has been observed after the application of *G. deserticola* as bioprotector agent of pepper cv. Piquillo against Wilt induced by *V. dahliae*. Taking into account the high number of pepper plants cultivated in greenhouses, this slight improvement of yield per plant would imply relevant profits for farmers.

## Final considerations

Despite the positive results when applying *G. deserticola* to protect *C. annuum* cv. Piquillo against *V. dahliae*, the effectiveness of AMF in controlling soil borne pathogens can be affected by several biotic and abiotic factors (Singh *et al.*, 2000). In fact, different *Glomus* species can exhibit different degree of success as bioprotector agents against a particular pathogen (Habte *et al.*, 1999; Declerck *et al.*, 2002). In a study focused on testing the ability of *G. deserticola*, *G. mosseae* and *G. intraradices* as hypothetical bioprotector agents against *Verticillium*-induced wilt in pepper, Garmendia *et al.* (2004c) concluded that only *G. deserticola* was effective to avoid great yield reductions in diseased pepper plants (Fig. 10b). The enhanced growth showed by plants inoculated with *G. mosseae* was not concomitant with an improvement of fruit production when they were infected with *V. dahliae*. Only in healthy plants, the association of pepper with *G. mosseae* benefited yield (Fig. 10b). The decline in yield observed in diseased non-mycorrhizal plants and diseased plants colonized by *G. intraradices* or *G. mosseae* was due to both reduced fruit set (Fig. 10a) and fruit size (Fig. 10c). In plants associated with *G. deserticola*, the negative effect of the pathogen on total yield per plant was only due to decreased fruit size (Fig. 10c).

The bioprotective effect of AMF on the development of pepper wilt is also determined by the plant phenology at the moment when *V. dahliae* is inoculated (Garmendia *et al.*, 2004a). In fact, the highest effectiveness of *G. deserticola* occurred when the pathogen was inoculated during the vegetative stage of pepper plants.

In addition, the aggressiveness of *V. dahliae* can be affected by other abiotic factors, such as soil moisture. Garmendia *et al.* (2005) observed that the deleterious effect of this soil borne pathogen on fruit set and yield can be amplified when pepper plants undergo a drought

period before their inoculation with *V. dahliae*. These authors also found that the water deficit did not improve the effectiveness of *G. deserticola* as bioprotector agent against *Verticillium* Wilt in pepper.

The interactions between AMF and rhizosphere micro-organisms can also influence the biological control of root pathogens (Azcón-Aguilar and Barea, 1997; Barea *et al.*, 2005). As exposed by Barea *et al.* (2005), a key point is to know to what extent an antifungal biocontrol agent will negatively affect AMF. As several studies (see Barea *et al.*, 2005 for review) have demonstrated that microbial antagonists of fungal pathogens (*i.e.* PGPR, other fungi) do not exert any antimicrobial effect against AMF, the dual inoculation of AMF and PGPR would mean new possibilities for the biocontrol of plants against soil borne pathogens, including *V. dahliae*.

In conclusion, pepper plants cv. Piquillo associated with *G. deserticola* exhibited more balanced antioxidant metabolism in leaves after pathogen inoculation than non-mycorrhizal plants, which could contribute to delay both the development of disease symptoms and the decrease of photosynthesis in *Verticillium*-inoculated plants with the subsequent benefit for yield. In addition, the higher deposition of lignin in the xylem vessels of stems together with the induction of hydrolytic and antioxidant enzymes in roots could also be involved in the bioprotector effect of *G. deserticola* against *Verticillium* Wilt in pepper cv. Piquillo. However, the effectiveness of AMF in protecting pepper against *Verticillium* Wilt can vary among different *Glomus* species. In addition, high effectiveness in improving plant nutrition and growth is not necessary related to great effectiveness as plant bioprotector agents (Garmendia *et al.*, 2004c). Moreover, bioprotection against *Verticillium* Wilt can be conditioned by plant phenology at the moment of pathogen attack (Garmendia *et al.*, 2004a) and other abiotic factors—such as drought—can also influence both the aggressiveness of *V. dahliae* and the effect of AMF as bioprotector agents against *Verticillium*-induced wilt in pepper (Garmendia *et al.*, 2005).

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