INDUCED PROTECTION BY *Rhizophagus intraradices* AGAINST

**Fusarium Wilt of Tomato**

Rosario Alicia Fierro-Coronado, Mercedes Guadalupe Castro-Moreno, Rey David Ruelas-Ayala, Miguel Ángel Apodaca-Sánchez and Ignacio Eduardo Maldonado-Mendoza

**SUMMARY**

Fusarium wilt of tomato, caused by Fusarium oxysporum f. sp. lycopersici (Fol) is a common disease in tomato. The arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* (previously called *Glomus intraradices*) was evaluated on its bioprotective ability against Fol in tomato cv. Missouri plants. Pathogenicity assays with Fol isolates from races 1, 2 and 3, showed that races 2 and 3 are the most aggressive for this cultivar, being the isolate R_C; from race 3 the most virulent one. The results using this isolate showed that root rot index (RRI) caused by Fol is lower in tomato mycorrhizal plants grown under a low phosphate (20µM) fertilization regime (Fol +AMF +Pi 20µM) compared to non-colonized plants (Fol +Pi 20µM). Fol infested plants subjected to high phosphate (200µM) fertilization (Fol +Pi 200µM) had an intermediate RRI value that was not significantly different in the two treatments. These findings suggest that *R. intraradices* confers tolerance to Fol in tomato cv. Missouri plants and that this mechanism may partially be influenced by improved phosphate nutrition.

**Introduction**

Tomato is an important horticultural species worldwide (Giaconi and Escaff, 1995). This crop is cultivated in more than 100 countries with a yearly production close to 2×10^6 metric tons, and Mexico occupies the tenth place (FAO-STAT, 2010). During the autumn-winter 2009-2010 crop season, a total of 53572ha of tomato were cultivated in Mexico, and Sinaloa State contributed with 28.63% of the grown area in the country (SIAP, 2010).

Tomato is susceptible to a wide variety of plagues and diseases, such as the ones caused by pathogenic fungi (Apodaca-Sánchez et al., 2002; Carrillo-Facio et al., 2003). The most important fungal disease in tomatoes is Fusarium wilt caused by *Fusarium oxysporum* Schlechtend. Fr. f. *sp. lycopersici* (Sacc.) W.C. Snyder & H.N. Hans (Fol), which can affect yield up to 60%, as well as fruit quality (Agrios, 2004). Three pathogenic races have been described for this fungus: race 1 (Saccardo, 1886), race 2 (Alexander and Tucker, 1945) and race 3 (Grattidge and O’Brien, 1982). *Fusarium* wilt of tomato is a hypoplastic disease, which causes reduced development, and is similar in most respects to vascular fusarioses of various other plants. However, in *Fusarium* wilt no cortical necrosis of the root system occurs under any set of environmental conditions that have been tested (Walker and Foster, 1946).

Practices to control Fol disease include the employment of chemical fungicides (Song et al., 2004). However, their application is becoming more restricted due to the negative side effects on the environment and human health. Other alternatives are the use of resistant varieties, management practices such as flooding and solarization, and most recently, biological control (Mandal et al., 2009). The most commonly used microorganisms for biological control include some fungal and bacterial species, such as *Trichoderma* and *Bacillus* spp. (González et al., 2004). Some of the microorganisms exhibiting biological control of Fol are able to develop symbiotic interactions with plants, such as the arbuscular mycorrhiza fungi (Steinkellner et al., 2011), a mutualistic interaction that is established between members of the phylum Glomeromycota and vascular plants (reviewed in Harrison, 1997). Arbuscular mycorrhiza fungi (AMF) are an essential component of the rhizosphere and they have been identified as organisms causing beneficial effects in growth and yield of different crops (Cavagnarao et al., 2006). AMF improve the uptake of different nutrients such as phosphorus, nitrogen, micronutrients and others (Clark and Zeto, 2000; Javaid, 2009). AMF play an important role in disease tolerance (Akhtar and Siddiqui, 2008) and have been used to decrease soil pathogen incidence from genera such as *Aphanomyces*, *Cylindrocladium*, *Fusarium*, *Macrophomina*, *Phytophthora*, *Pythium*, *Rhizoctonia* and *Verticillium* (Harrer and Watson, 2004).

There is a wide range of plant diseases that AMF are able to affect by diminishing their effects. Pepper plants pre-colonized with *Rhizophagus* intraradices (previously named *Glomus intraradices*) showed lower severity and higher survival (100%) of the...
La marchitez del tomate causada por Fusarium oxysporum f. sp. lycopersici (Fol) es una enfermedad común del tomate. El hongo micorrízico arbuscular (FMA) Rhizophagus intraradices (previamente denominado Glomus intraradices) fue evaluado en cuanto a su capacidad bioprotectora contra Fol en plantas de tomate cv. Missouri. Los ensayos de patogenicidad con aislados de Fol de las razas 1, 2 y 3 mostraron que las razas 2 y 3 fueron las más agresivas para este cultivar, siendo el aislado R$_3$C$_5$ de la raza 3 el más virulento. Los resultados de protección inducida usando R. intraradices contra el aislado de Fol R$_3$C$_5$ mostraron que el índice de pudrición radial causado por Fol es más bajo en plantas de tomate micorrizadas (Fol +HMA +Pi 20µM) creciendo en un regímen de bajo fosfato (20µM) comparado a plantas no colonizadas con HMA (Fol +Pi 20µM). Las plantas infectadas con Fol y sometidas a fertilización con 200µM de fosfato (Fol +Pi 200µM) tuvieron un valor del índice de pudrición radial intermediario que no fue diferente estadísticamente en los dos tratamientos. Esto sugiere que R. intraradices confiere tolerancia a Fol en plantas de tomate cv. Missouri y que este mecanismo puede ser parcialmente influido por una mejora en la nutrición fosfatada.

**RESUMEN**

La marchitez del tomate causada por Fusarium oxysporum f. sp. lycopersici (Fol) es una enfermedad común del tomate. El hongo micorrízico arbuscular (FMA) Rhizophagus intraradices (previamente denominado Glomus intraradices) fue evaluado en cuanto a su capacidad bioprotectora contra Fol en plantas de tomate cv. Missouri. Los ensayos de patogenicidad con aislados de Fol de las razas 1, 2 y 3 mostraron que las razas 2 y 3 fueron las más agresivas para este cultivar, siendo el aislado R$_3$C$_5$ de la raza 3 el más virulento. Los resultados de protección inducida usando R. intraradices contra el aislado de Fol R$_3$C$_3$ mostraron que el índice de pudrición radial causado por Fol es más bajo en plantas de tomate micorrizadas (Fol +HMA +Pi 20µM) creciendo en un regímen de bajo fosfato (20µM) comparado a plantas no colonizadas con FMA (Fol +Pi 20µM). Las plantas infectadas con Fol y sometidas a fertilización con 200µM de fosfato (Fol +Pi 200µM) tuvieron un valor del índice de pudrición radial intermediario que no fue diferente estadísticamente en los dos tratamientos. Esto sugiere que R. intraradices confiere tolerancia a Fol en plantas de tomate cv. Missouri y que este mecanismo puede ser parcialmente influido por una mejora en la nutrición fosfatada.

**RESUMO**

O apodrecimento causado pelo Fusarium oxysporum f. sp. lycopersici (Fol) é uma doença frequente do tomate. O fungo micorrízico arbuscular (FMA) Rhizophagus intraradices, (antes denominado Glomus intraradices) foi avaliado por sua capacidade bio-protetora contra o Fol nas plantas de tomate cv Missouri. Os testes de patogenicidade com as amostras do Fol das cepas 1, 2 e 3 mostraram que as cepas 2 e 3 foram mais agressivas para este tipo de cultivar, sendo as amostras R$_3$C$_3$ da cepa 3 as que são mais virulentas. Os resultados de proteção induzida usando R. intraradices contra isolado Fol R$_3$C$_3$ revela que o índice de podridão radical causado pelo Fol é mais baixo nos cultivos de tomate micorrizadas (FOL +HMA +Pi 20µM) crescendo num meio baixo em fosfato (20µM) comparado a plantas não enxertadas com FMA (FOL +Pi 20µM). As plantas tocadas com o FOL e submetidas a fertilização com 200µM de fosfato (FOL +Pi 200µM) tiveram um valor de índice de podridão radical intermediário, que não difere estatisticamente nos dois tratamentos. Isto revela que R. intraradices concede tolerância a Fol nos cultivos de tomate cv. Missouri, e que este mecanismo pode ser parcialmente beneficiado pela melhoria na nutrição fosfatada.

**MATERIALS AND METHODS**

*Fusarium* isolates were obtained from infected tomato stem tissue collected in Culiacan Valley, Sinaloa, while the three isolates belonged to race 3 and were widespread throughout Sinaloa. The main goal of this study was to evaluate the protective effect that AMF may exert against *Fusarium*. The present work, reports the bioprotective effect of *R. intraradices* against a highly virulent Folk race 3 isolate in tomato cv. Missouri. In addition, some *Fusarium* isolates obtained from different tomato fields affected by *Fusarium* wilt in Culican Valley in 2004-2005 were characterized molecularly and their race identity confirmed by genotyping as belonging to races 1, 2 and 3.

**Materials and Methods**

*Fusarium* pathogenicity assays on tomato cv. *Missouri*

*Fusarium* isolates were obtained from infected tomato stem tissue collected in Culiacan Valley, Sinaloa, while the three isolates belonged to race 3 and were widespread throughout Sinaloa. The main goal of this study was to evaluate the protective effect that AMF may exert against *Fusarium*. The present work, reports the bioprotective effect of *R. intraradices* against a highly virulent *Fusarium* race 3 isolate in tomato cv. *Missouri*. In addition, some *Fusarium* isolates obtained from different tomato fields affected by *Fusarium* wilt in Culican Valley in 2004-2005 were characterized molecularly and their race identity confirmed by genotyping as belonging to races 1, 2 and 3.

**Materials and Methods**

*Fusarium* pathogenicity assays on tomato cv. *Missouri*
Bioprotection of tomato plants with *R*. *intraradices* against Fol

The material used in this study was obtained from monoxenic cultures consisting of hairy root cultures of *Daucus carota* clone DC2 colonized with the AM fungal species *R*. *intraradices* (Bécard and Piché, 1992). Spores produced under these in vitro conditions were used for inoculation of tomato roots. *R*. *intraradices* was established in culture with transformed carrot roots as described by Bécard and Fortin (1988) and Doner and Bécard (1991).

Fol inoculum was obtained and adjusted to 6×10^6 conidia/ml. Fol inoculation consisted on the application of 2ml of the conidia water suspension directly to 21-day seedling roots. The plants were grown under greenhouse conditions (~13h of natural light), with temperatures between 15 and 25°C. Irrigation and fertilization was performed once every three days using 50ml of Hoagland’s solution (Millner and Kitt, 1992). The substrate pH was typically 6.5. Twenty days post-inoculation the *Fusarium* wilt symptoms were evaluated according to a severity scale designed to evaluate symptoms on the aerial part of the plant (Marlatt *et al.*, 1996). The pathogenicity assay consisted in a complete randomized design with five replicates per treatment, nine treatments in total (three isolates per race) and a control. Treatments in total (three iso-replicates per treatment, nine assays consisted in a complete assay. Symptoms were evaluated according to the severity scale described by Marquardt (1990). The rest of the replicates were harvested and stained with trypan blue, after clarification of root tissue (Phillips and Hayman, 1970), to evaluate colonization efficiency of *R*. *intraradices* (McGonigle *et al.*, 1990).

**Results**

Tomato cv. Missouri was susceptible to R1C2, R3C1, R1C3 and R1C, isolates, which were identified as Fol races 2 and 3 and tolerant to R1C2, R2C3 and R2C5 isolates which are Fol isolate 1 and to the Fol 2 isolate R1C3 (Table I). Forty five days after inoculation with R1C2, R2C3, or R1C3 race 1 isolates, plants only showed slight rot symptoms and some shortening with respect to the control (Figures 1a, b). Severity index analysis (Marlatt *et al.*, 1996) showed that they were statistically similar between both control and all three Fol race 1 (R1C1, R1C2, R1C3) infected plants (Table I). R1C2 and R3C1 show isolates slight unilateral wilt in older leaves (Figure 1c). Fol race 3 isolate R3C3 showed *Fusarium* wilt symptoms in tomato cv. Missouri and was different to control non-inoculated plants (Figure 1d). Fol race 3 isolate R3C3 showed the most severe symptoms with chlorosis, wilting, dwarfism and death of the plants (Figure 1e).

In terms of severity index of disease (Marlatt *et al.*, 1996), the isolate belonging to race 3 R1C3 was statistically similar to the R1C1, R3C1 and R3C3 isolates (Table I). Race 3 isolates were different to the control isolates, R1C3 and race 1 isolates (R1C1, R2C3, and R3C3). Tomato cv. Missouri was tolerant to R1C1, R1C3, R2C5 and R2C3 isolates, showing very mild symptoms that were not statistically different from the control non-inoculated plants and a lower tolerance to race 2 isolates (R1C2 and R2C5). Race 3 Fol isolates were the most
virulent to this cultivar, particularly isolate R\(_{3C5}\).

**Disease protection of tomato plants with* R. intraradices **against Fol

Bioprotection assays were initiated once the fungal colonization with *R. intraradices* had been established for five weeks. Efficiency of colonization at this time averaged 23.33% of the root system. The Fol isolate used for this assay was R\(_{3C5}\), since it presented the highest level of damage to the plants when analyzed in the previous assay.

Typical Fol symptoms obtained at the end of this assay were coincident with those observed in the previous pathogenicity assays. Absolute control plants did not show any symptoms of Fol disease (Figure 2a). Even though all treatments without Fol did not present any characteristic symptoms associated to the pathogen, leaf rolling and dark leaf coloration was observed in those treatments were Pi 20\(\mu\)M was used (Figures 2b, c). These symptoms were attributed to nutritional stress for phosphate (Taiz and Zeiger, 2010) and the limitations of growth in the root tissue due to the length of time that the plants needed to be maintained in the pots. Control plants inoculated with Fol +Pi 20\(\mu\)M suffered plant death (not shown) and, in some cases, moderate chlorosis, wilting, shortening and defoliation compared to the absolute control plants (Figure 2d).

Uninfested AMF colonized plants (AMF +Pi 20\(\mu\)M) did not show any disease symptoms (Figure 2b) and showed a better foliar appearance with respect to the absolute control plants (Pi 20\(\mu\)M; Figure 2c). In Fol infested mycorrhizal plants (Fol +AMF +Pi 20\(\mu\)M) symptoms were found only in one out of five plants, showing chlorosis, wilting and plant shortening. The other replicates only showed a slight wilting of basal leaves, and chlorosis was absent (Figure 2e).

Non-inoculated plants with 200\(\mu\)M phosphate (Pi 200\(\mu\)M) did not show any symptoms and developed more biomass compared to the plants grown on 20\(\mu\)M phosphate either AMF colonized (Figures 2e, f) or non-colonized (Figures 2b, c). Two out of five plants infested with Fol (Fol +Pi 200\(\mu\)M) and fertilized with 200\(\mu\)M phosphate showed severe chlorosis, wilting and plant shortening (not shown) with respect to the Fol uninfested ones with high phosphate control (Pi 200\(\mu\)M; Figure 2a). The other replicates showed wilting in basal leaves and plant shortening (Figure 2d).

Biomass measured as dry weight in control plants and plants infected with Fol, and colonized or not-colonized with AMF, did not differ statistically among all 20\(\mu\)M phosphate treatments (Table II). Variance analysis for biomass in shoots and roots showed that plants grown under a high phosphate regime developed much better and were statistically different when compared to the 20\(\mu\)M plant treatments.

*R. intraradices* decreased significantly the disease severity measured as root rot index (RRI; Gardezi et al., 2001) of AMF colonized plants growing in Fol infested soil. Arbuscular mycorrhiza decreased plant damage under a low phosphate fertilization regime (20\(\mu\)M). The RRI in Fol infested plants grown under high phosphate (Fol +Pi 200\(\mu\)M) was statistically similar to both the Fol +Pi 20\(\mu\)M and the Fol +AMF +Pi 20\(\mu\)M low phosphate treatments, whereas these last two conditions were different among them (Table II).

By the end of the experiments (75 days post inoculation with *R. intraradices*) when differential responses were observed, plants colonized with AMF (Fol +AMF +Pi 20\(\mu\)M; AMF +Pi 20\(\mu\)M) had an efficiency of colonization of 93.3 and 90% respectively (Table II). All types of AMF internal structures and a good external hyphal network
TABLE II
BIOPROTECTION OF TOMATO PLANTS INOCULATED WITH Rhizophagus intraradices AGAINST FOL 45 DPI AFTER INOCULATION WITH FOL ISOLATE R3C5

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Shoot dry weight (g)</th>
<th>Root dry weight (g)</th>
<th>RRI*</th>
<th>Myc (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pi 20μM</td>
<td>0.845 b</td>
<td>0.447 b</td>
<td>1.0</td>
<td>c</td>
</tr>
<tr>
<td>Fol +Pi 20μM</td>
<td>0.593 b</td>
<td>0.290 b</td>
<td>3.6</td>
<td>a</td>
</tr>
<tr>
<td>AMF +Pi 20μM</td>
<td>0.863 b</td>
<td>0.472 b</td>
<td>1.0</td>
<td>c</td>
</tr>
<tr>
<td>Fol +AMF +Pi 20μM</td>
<td>0.755 b</td>
<td>0.437 b</td>
<td>2.3</td>
<td>b</td>
</tr>
<tr>
<td>Pi 200μM</td>
<td>3.223 a</td>
<td>2.907 a</td>
<td>1.0</td>
<td>c</td>
</tr>
<tr>
<td>Fol +Pi 200μM</td>
<td>3.680 a</td>
<td>4.078 a</td>
<td>3.0</td>
<td>ab</td>
</tr>
</tbody>
</table>

*According to the scale reported by Gardezi et al. (2001).

Similar letters indicate that treatments with those isolates do not differ statistically. The data were analyzed by ANOVA test and the means were separated using LSD, α= 0.05. RRI: root rot index, Myc: mycorrhization.

were observed at this time (data not shown).

Discussion
In several regions of the world, tomato plants are attacked by Fol, generating great economical losses (Ascencio-Álvarez et al., 2008). This disease is considered one of the most important affecting this crop, and its control can be difficult. This difficulty has stimulated the search for alternatives of biological control (Fravel et al., 2003).

We tested the susceptibility of tomato cv. Missouri in pathogenicity assays and found that it was tolerant to race 1, but susceptible to races 2 and 3 of Fol. A race 3 isolate (R3C5) was identified as the most pathogenic one to tomato cv. Missouri (Table I) among the tested isolates.

The interaction between plant roots and arbuscular mycorrhiza can increase the tolerance to some biotic and abiotic stress factors. Different mechanisms involved in this tolerance include a higher capability of the arbuscular mycorrhiza to incorporate water, toxic compounds, mineral nutrients and to tolerate diseases (Augé 2001; Elsen et al., 2003; Quoreshi and Khasa, 2008; Hernández-Ortega et al., 2012). The bioprotective effect of R. intraradices against Fol (isolate R3C5) showed typical Fol invasion symptoms in some plants (data not shown). Nevertheless, at the end of the bioassay the symptoms were less severe than in non-mycorrhizal plants infected with Fol (Figure 2). Similar observations are described with other biological control agents, such as Paenibacillus lentimorbus and Trichoderma sp. used against F. solani in tomato (González et al., 2004), as well as with arbuscular mycorrhiza used against F. oxysporum f. sp. gladioli in Gladiolus grandiflorus (Gardezi et al., 2001).

Previous work using G. mosseae (Steinkellner et al., 2011) and R. intraradices (Akköprü and Demir, 2005) showed a bioprotective effect against Fol. Fol infected plants colonized by R. intraradices showed higher phosphate levels either when alone or combined with different rhizobacteria compared to Fol infected plants grown under a low phosphate regime (Akköprü and Demir, 2005). This suggests that the severity of Fol disease might be affected by the phosphate status of the plants. In the present study it was found that arbuscular mycorrhiza caused a protective effect against Fol, but when compared with the plants growing in high phosphate it was observed that phosphate also increased tolerance to Fol (Table II). This suggests that the tolerance mechanism mediated by R. intraradices may be at least partially due to an improved phosphate status of the plant. These results agree with those of other authors who also suggest that the increased tolerance by AMF can be associated with improved nutrients, especially phosphate concentration (Davis and Menge, 1980; Graham and Menge, 1982). However, some researchers presumed that the increased tolerance by AMF might not be completely related to this factor (Caron et al., 1986; Akköprü and Demir, 2005; Kapoor, 2008). Several hypotheses have been proposed to explain the mechanisms of the increased resistance in mycorrhizal plants: improvement of plant nutrition (Davis and Menge, 1980; Graham and Menge, 1982), competition for space (Azcón-Aguilar and Barea, 1996), modified microbial flora in the rhizosphere (Filion et al., 1999; Ren et al., 2010), and induced systemic resistance in the plant (Whipps, 2004).

The profuse root colonization by R. intraradices, as shown in this study, could have helped to protect the plant tissue against the pathogen by competing for the same niche. At the end of the bioprotection assay the root tissue was completely colonized by the AMF (>90%). The AM fungal symbiont probably can compete for space with any other organisms that invade the root system when the percentage of root colonization is high. Nevertheless, in this case, at the time the plants were infected with Fol, the percentage of colonization was only 23%.

Thus, explaining the induction of tolerance to Fol in tomato cv. Missouri only by space competition would be insufficient. It has been shown that the pattern of global gene expression is modified when a plant interacts with R. intraradices (Paszkowski et al., 2006; Liu et al., 2007), indicating that there is a systemic response that allows for the protection of the root system, as well as the induction of tolerance to foliar pathogens such as Xanthomonas campesiris pv. vescicatoria in Medicago truncatula (Liu et al., 2007). This induced protection by AMF colonization is accompanied by an induction on the expression of a great number of phosphate-related and defense-related genes.

In conclusion, the present results suggest that AMF colonization shows a bioprotective effect against Fol in tomato cv. Missouri and that this mechanism may be partially mediated by an improved phosphate nutritional state. These findings suggest that AMF sometimes may act as bioprotective agents especially in soils where phosphate might be low or unavailable by increasing the nutritional status of the plants.

ACKNOWLEDGMENTS
The authors thank Melina López-Meyer for critical reading of the manuscript and valuable comments, and acknowledge funding by IPN (SIP 20080715) and CECyT-Sinaloa (2007 and 2008).

REFERENCES


