ASSOCIATION OF *Fusarium solani* WITH *rolABC* AND WILD TYPE TROYER CITRANGE

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**SUMMARY**

To determine if *rolABC* genes of *Agrobacterium rhizogenes* could modify the susceptibility of Troyer citrange to dry root rot, conidial suspensions of a *Fusarium solani* strain isolated from citrus roots (PVCT1A) were used in cutting, leaf midrib and root inoculation assays of wild type (wt) and *rolABC* Troyer citrange. In all assays, the pathogen was reisolated equally well from wt and transformant. Interveinal chlorosis, wilt and defoliation of inoculated cuttings and leaf midribs, observed both in *rolABC* and wt, were more severe in transgenic lines. Two months after root inoculation with the pathogen, root weight was significantly reduced in *rolABC* lines. No root rot symptoms were observed on *rolABC* or wt lines, but wilting was more severe on wt. Cell-free fungal culture filtrate induced leaf wilt and defoliation within 10 days from immersion in the filtrate, without any significant difference between *rolABC* and wt lines. Scanning electron microscopy observations of infected roots showed that fungal penetration and colonization was similar in both lines. Evidence is presented that *F. solani* colonizes Troyer citrange tissues even in absence of visible symptoms and without differences between wt and *rolABC* lines.

Key words: *Fusarium solani*, Troyer citrange, *rolABC*, transgenic plants, pathogenicity.

**INTRODUCTION**

*Fusarium solani* (Mart.) Appel & Wollenw. emend. Snyder & Hans. (teleomorph *Nectria haematococca*) is a worldwide soil-borne fungus attacking a wide range of host plants including citrus (Sherbakoff, 1953; Nemec, 1975), with a great overall impact on productivity. Studies conducted by Menge and Nemec (1997) showed that the most common citrus rootstocks are susceptible to dry root rot caused by *F. solani*, and their results tally with other field observations (Klotz, 1973).

*F. solani* is regarded as a mild pathogen, its damage being sometimes underestimated (http://www.up.ac.za/academic/fabi/citrus/rootrot.html). The fungus can induce symptomless infection in citrus roots but, under stress conditions, it causes severe dry root rot, so called for distinguishing it from the common *Phytophthora* foot rot. Dry root rot cankers in fact, unlike *Phytophthora*-induced lesions, usually do not ooze gum.

The most conspicuous symptom above ground is a fatal collapse of the tree; leaves wilt suddenly and in a few days dry up, remaining attached on the tree. Normally, however, the course of the disease is chronic and symptoms resemble those of other root rots caused by *Phytophthora*, *Armillaria*, rodent damage, etc. Other symptoms include twig dieback, chlorosis of the main leaf-veins, yellowing and dropping of the leaves (Fawcett, 1936; Klotz et al., 1967; Bender et al., 1982; Menge, 1988). Isolates of the fungus have been categorised as mild, intermediate, and severely pathogenic, the latter causing extensive root rot and scorching associated with the production of phytotoxins (Strauss and Labuschagne, 1995).

Sour orange, the most common citrus rootstock used in Italy is being replaced with Troyer and Carrizo citranges for their resistance to *Citrus tristeza virus*. Recently, a devastating decline has been observed in some citrus groves established with citrange rootstock (Polizzi et al., 1992; Ippolito and De Cicco, 1995). *Fusarium solani* was consistently isolated from discoloured wood of affected trees (Polizzi et al., 1992).

Because of the difficulties of conventional breeding, genetic engineering appears a promising technique for citrus, allowing specific characters to be inserted into desirable genotypes without affecting other traits (Peña and Navarro, 1999). Troyer citrange was recently transformed with *rolABC* genes from *Agrobacterium rhizogenes* to modify the growth habit (Gentile et al., 2004; La Malfa et al., 2004). Based on previous publications morphological and physiological modifications were monitored which included dwarfing effect (up to 50% height reduction), increase of root system density and increased content of phenolic compounds, PAL and peroxidase activity (Gentile et al., 2004; La Malfa et al., 2004).
The rolABC genes affect phytohormone balance of the plants so as to induce dwarfing, increase rooting, alter flowering, cause leaves to wrinkle and/or increase branching (Christey, 2001). These genes can also increase the sensitivity of transgenic tissues to both cytokinins and auxins (Estruch et al., 1991a, 1991b).

Although numerous reports have examined the phenotypical alterations of rol plants (reviewed in Christey, 2001), only a few papers have dealt with the effects of microorganisms on transgenic rol/ABC plants (Cirvilleri et al., 2005) and their potentially modified susceptibility to pathogens (Fladung and Gieffers, 1993; Bettini et al., 2001; Ballestra et al., 2001; Spina et al., 2008).

Transgenic Troyer citrange plants have been previously examined for soil microbial populations (Cirvilleri et al., 2005) and similar studies have been conducted on transgenic cherry plants overexpressing the phytochrome A gene (Spina et al., 2006; Cirvilleri et al., 2008). In the present study we investigated whether the presence of rolABC genes could modify the susceptibility of Troyer citrange to *F. solani*. To this aim, various established inoculation methods were used for pathogenicity test on cuttings, leaf-midribs and roots. Colonization of *F. solani* strain PVCT1A in inoculated tissues and symptom development were monitored. Culture filtrates of the pathogen were used to evaluate phytotoxicity on cuttings of wild type (wt) and rol/ABC. Troyer citrange, and infected roots were also studied by scanning electron microscopy (SEM).

**MATERIALS AND METHODS**

**Plant material and growth conditions.** Seedlings of wt and transgenic rol/ABC Troyer citrange (Gentile et al., 2004) were planted in 2 liter plastic pots in a pasteurized (80°C for 1h) soil-sand-peat (1:1:1) compost, and grown in a conditioned greenhouse at a temperature of 24±2°C during the day (16 h) and 18±2°C during the night (8 h). Natural light was reduced by 25%, covering the greenhouse with a black net.

**Inoculation of plant cuttings and leaf-midribs.** *Fusarium solani* PVCT1A used in this study was isolated from symptomatic Troyer citrange roots in Sicily, single-spored and identified according to Balmas et al. (2000). A spontaneous benomyl-resistant strain of *F. solani* PVCT1A was cultured on potato-dextrose agar (PDA) amended with 10 µg ml⁻¹ of benomyl (Benlate, Du Pont De Nemours, USA) at 25°C for seven days. Conidial suspensions of the pathogen in sterile distilled water (SDW) were adjusted to a concentration of 10⁶ conidia ml⁻¹.

Shoots 15 cm long with five to eight leaves, taken from three-year-old wt and rol/ABC plants, were plunged in sterile tubes containing 10 ml conidial suspension of *F. solani* PVCT1A and kept at an average temperature of 26°C under a 16 h photoperiod (35 µmol m⁻² s⁻¹) for 14 days. The experiments were replicated five times on ten shoots of each line at one time. Shoots plunged in 10 ml of SDW were used as control.

For midrib inoculation assays, shoots were plunged into sterile tubes containing 10 ml SDW and the leaves inoculated with 10 µl of *F. solani* PVCT1A conidial suspension, wounding the midribs near the petiole with a sterile needle to facilitate conidial penetration. All the cuttings were kept at an average temperature of 26°C under a 16 h photoperiod (35 µmol m⁻² s⁻¹) for 14 days. The experiments were replicated five times on thirty leaves of each line at one time. Leaves inoculated with SDW were used as control.

Colonization of *F. solani* was monitored by longitudinal sections of inoculated cuttings and inoculated leaf midribs. Three sections 5 mm long (inoculation point, middle and upper point) were surface-sterilized for 30 sec in 10% sodium hypochlorite, rinsed twice in SDW, plated on PDA enriched with streptomycin sulphate (25 mg ml⁻¹), 25% lactic acid and 10 µg ml⁻¹ benomyl, and incubated at 25°C in the dark. Results were recorded as percentage of *F. solani*-colonized tissue sections. Non-inoculated cuttings and midribs were used as control.

**Inoculation of roots.** Three-month-old wt and rol/ABC Troyer citrange seedlings, grown in steamed soil-sand-peat (1:1:1) medium, were inoculated by dipping the roots in a conidial suspension of *F. solani* PVCT1A (10⁶ conidia ml⁻¹). Seedlings dipped in SDW were used as control. All plants were replaced in their pots and maintained in a greenhouse at 18/26°C night/day temperature regime under a 16 h photoperiod (35 µmol m⁻² s⁻¹). Pots were thoroughly watered after planting and left standing for 30 min to allow water to drain. Plants were watered every second day. The experiments were carried out five times on ten seedlings of each line at one time.

Two months after inoculation, random samples of feeder roots were collected (two replicates for each plant) and dilution series were plated using a Spiral Plater Eddy Jet (IUL Instruments, Spain) on PDA enriched with streptomycin sulphate and 25% lactic acid, with and without 10 µg ml⁻¹ benomyl. Fungal colonies were counted and examined microscopically. The number of total fungi and *F. solani* propagules was determined. Mean values of cfu g⁻¹ of fresh weight were converted to log values, before performing analysis of variance (ANOVA) by COSTAT software. Means were supported by least significant difference at P = 0.05.

**Effects of culture filtrate.** *F. solani* PVCT1A was grown in Czapek-Dox medium (3 g/l NaNO₃, 1 g/l K₂HPO₄, 0.5 g/l MgSO₄·7H₂O, 0.01 g/l FeSO₄·7H₂O, 30 g/l sucrose, 1 g/l yeast extract) to stimulate toxin production (Tatum and Baker, 1983). Cultures were incubated in the dark at 25°C on a reciprocal shaker (67 oscilla-
tions min\(^{-1}\)) and after seven days were strained through four layers of sterile cheesecloth. Filtrates were centrifuged for 30 min at 2800 rpm, passed through a Millipore filter (0.45 micron pore size) and stored at -20°C.

For phytotoxicity bioassay, cell-free culture filtrate was diluted 1:1 with 1 mM KH\(_2\)PO\(_4\), as reported by Hartman et al. (2004). Cuttings of three-month-old wt and rolABC Troyer citrange seedlings were immersed in 5 ml of culture filtrate diluted 1:1, and incubated at an average temperature of 26°C under a 16 h photoperiod (35\(\mu\)mol·m\(^{-2}\)·s\(^{-1}\)). The experiments were carried out five times on ten cuttings of each line at one time.

**Data analysis.** In all replicated experiments, disease severity ratings were recorded 14 days after inoculation, based on a 1-5 arbitrary scale where 1 = no symptoms; 2 = interveinal chlorosis/partial defoliation, 1-20% foliage affected; 3 = interveinal chlorosis/partial defoliation, 21-50% foliage affected; 4 = yellowing of leaves/partial defoliation, 51-80% foliage affected; 5 = wilt/browning/total defoliation. Data were converted to percents of mid-values (Hartman et al., 1997), where severity rating of 1 = 0%, 2 = 10%, 3 = 35%, 4 = 65% and 5 = 90%. Percentage values were converted to arcsin values, before performing ANOVA by COSTAT software. Means were supported by least significant difference at \(P=0.05\).

Two months after root inoculation, plant height, root weight and root length were determined.

**Scanning electron microscopy.** Penetration of the pathogen into the root systems was observed at three and six days after inoculation. Root pieces, randomly selected from five seedlings of each line (inoculated and non-inoculated wt and rolABC seedlings) at three and six days, were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4, followed by rinsing for 20 min in the same buffer. Samples were dehydrated in graded ethanol dilutions, critical-point dried in CO₂, mounted on standard copper stubs with silver paint, sputter-coated with gold and examined with a DSM 940A Zeiss scanning electron microscope (Labuschagne et al., 1987).

**RESULTS**

**Inoculation of cuttings and leaf midribs.** *F. solani* PVCT1A was always re-isolated from infected cuttings and midribs eight days after inoculation without significant differences (\(P=0.05\)) between rolABC and wt lines (Table 1). The percentage of infected cuttings and leaf midribs was always higher at the inoculation point, but no significant differences were recorded among the portions of sampled tissues (Table 1). *F. solani* PVCT1A was never recovered from non-inoculated cuttings and leaves.

Interveinal chlorosis, wilt and defoliation occurred on inoculated cuttings and leaves of rolABC and wt Troyer citrange within 14 days after inoculation (Fig. 1). Overall, disease severity ratings (Table 2) were significantly higher (\(P=0.05\)) in transgenic than in wt cuttings and leaves, and significantly higher percentage values were recorded in the inoculated rolABC cuttings and leaves, compared to wt. Control cuttings and leaves never showed symptoms.

**Inoculation of roots.** *F. solani* PVCT1A was consistently re-isolated from inoculated rolABC and wt roots without significant differences between rolABC (log 2.70) and wt (log 2.61) lines (\(P=0.05\)) (Fig. 2). Significant differences were recorded in total fungal populations isolated from rolABC inoculated (log 3.65) and non-inoculated (log 5.16) roots. Total fungal popula-

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**Table 1.** Infection of cuttings and leaf midribs of rolABC and wt Troyer citrange lines in replicated growth chamber experiments eight days after inoculation with *F. solani* PVCT1A.

<table>
<thead>
<tr>
<th>Inoculated organ</th>
<th>Site</th>
<th>Infected tissues (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rolABC</td>
<td>Wild type</td>
</tr>
<tr>
<td>Cuttings</td>
<td>Inoculation point</td>
<td>88a</td>
</tr>
<tr>
<td></td>
<td>Middle</td>
<td>63a</td>
</tr>
<tr>
<td></td>
<td>Upper</td>
<td>56a</td>
</tr>
<tr>
<td>Leaf midribs</td>
<td>Inoculation point</td>
<td>75a</td>
</tr>
<tr>
<td></td>
<td>Middle</td>
<td>41a</td>
</tr>
<tr>
<td></td>
<td>Upper</td>
<td>30a</td>
</tr>
</tbody>
</table>

*Values on the same line followed by the same letter do not differ significantly from one another according to Student-Newman-Keuls test (\(P=0.05\)). The test was performed on previously transformed data arc sin \(\sqrt{\%}\). Values are means of five experiments.

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**Table 2.** Disease severity ratings of rolABC and wt Troyer citrange cuttings and leaf midribs in replicated growth-chamber experiments 14 days after inoculation with *F. solani* PVCT1A.

<table>
<thead>
<tr>
<th>Inoculated organ</th>
<th>Disease severity ratings (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rolABC</td>
</tr>
<tr>
<td>Cuttings</td>
<td>37b</td>
</tr>
<tr>
<td>Leaf midribs</td>
<td>64b</td>
</tr>
</tbody>
</table>

*Values on the same line followed by different letter differ significantly from one another according to Student-Newman-Keuls test (\(P=0.05\)). The test was performed on previously transformed data arc sin \(\sqrt{\%}\). Values are means of five experiments.
**Fig. 1.** Intereval chlorosis on rolABC (A, C) and wt (B, D) Troyer citrange cuttings (A, B) and leaves (C, D), 14 days after inoculation with *F. solani* PVCT1A.

**Fig. 2.** Total fungal and *F. solani* population on the roots of rolABC and wt Troyer citrange seedlings in replicated growth-chamber experiments two months after root-inoculation with *F. solani* PVCT1A. Values are means of eight replications (two replicates for each plant of each line for five experiments). Student-Newman-Keuls test (P=0.05) was performed on previously transformed data arc sin √%. Comparing rolABC to wt lines.

*F. solani* PVCT1A was not re-isolated from non-inoculated roots.
tions were significantly higher in rolABC roots not inoculated with the pathogen. *F. solani* PVCT1A was never recovered from non-inoculated roots (Fig. 2).

Two months after *F. solani* PVCT1A inoculation, stem height, root length and root weight were not significantly reduced, compared to non-inoculated seedlings, without significant differences between rol/ABC and wt lines (Table 3). Root weights were significantly higher only in non-inoculated rolABC seedlings. Wilting and defoliation (disease severity ratings) were significantly higher on inoculated wt than on rolABC seedlings (Table 3). No symptoms were observed in non-inoculated rolABC and wt seedlings.

**Effects of culture filtrate.** Wilt and defoliation occurred on inoculated cuttings of rolABC and wt Troyer citrange within 10 days from immersion in *F. solani* PVCT1A culture filtrate (Fig. 3A,B), without differences (*P*=0.05) between the two seedling lines (data not shown). Control cuttings did not show any symptom (Fig. 3 C, D).

**Scanning electron microscopy.** SEM observations of *F. solani* PVCT1A root colonization were made three and six days after inoculation and no differences were observed between rolABC (Fig. 4 A, B) and wt (Fig. 4 C, D) citrange lines. Hyphae could be seen growing superficially along the root surface (Fig. 4B, D) and penetration into and exit from the root (Fig. 4 A, C) occurred.

Table 3. Mean values of stem height, root length and root weight and mean severity ratings of rolABC and WT Troyer citrange seedlings in replicated growth-chamber experiments two months after root-inoculation with *F. solani* PVCT1A.

<table>
<thead>
<tr>
<th>Inoculated organ</th>
<th>Disease severity ratings (%)</th>
<th>rolABC</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cuttings</td>
<td></td>
<td>37b</td>
<td>27a</td>
</tr>
<tr>
<td>Leaf midribs</td>
<td></td>
<td>64b</td>
<td>10a</td>
</tr>
</tbody>
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* Values on the same line followed by different letter differ significantly from one another according to Student-Newman-Keuls test (*P*=0.05). The test was performed on previously transformed data arc sin *Y* %. Values are means of five experiments.

**DISCUSSION**

Citrus species are subject to many biotic stresses so that development of disease-resistant plants is a high priority for breeding programs (Peña and Navarro, 1999). This is particularly important for Troyer citrange which is severely damaged by dry root rot caused by *F. solani*. In fact, the fungus is widespread in citrus orchards and represents an economic problem throughout the world. Control measures against Fusarium root rot comprise elimination of conditions causing stress to the trees (Labuschagne et al., 1996), biological control practices, and use of antagonistic microorganisms (Nemec et
al., 1996; Lim et al., 1991; Cirvilleri et al., 2005), but use of resistant rootstocks is also a promising strategy (Labuschagne et al., 1996).

Since rolABC genes induce genetic modification affecting hormone sensitivity and plant morphology, an effect on plant resistance to pathogens may be expected. To date, the few studies considering pathogen resistance modification in transgenic rol plants have produced conflicting results. Fladung and Gieffers (1993) observed increased susceptibility to *Alternaria alternata*, *Botrytis cinerea* and *Erwinia carotovora* subsp. *atroseptica* of rolC transgenic potato leaves positively correlated with their fructose and glucose content, whereas tubers showed enhanced resistance positively correlated with glucose, dry matter and starch content. Bettini et al. (2001) found higher resistance to toxins of *Fusarium oxysporum* f.sp. *lycopersici* in rolABC tomato plants, suggesting that modifications induced by the rol genes improved the plant defence response. On the other hand, Balestra et al. (2001) found increased susceptibility to *Pseudomonas syringae* pv. *syringae* and *P. viridiflava* of rolABC kiwi plants, strictly correlated with raised leaf nitrogen content. Till now, the resistance/susceptibility to *Fusarium solani* of citrus plants transformed with rolABC genes of *A. rhizogenes* has not been examined.

Inability to reproduce *Fusarium solani* symptoms in pathogenicity tests has been reported (Graham et al., 1985; Dandurand and Menge, 1993) and partially ascribed to the inoculation methods. After dipping citrus seedling roots in a suspension of *F. solani* conidia for 30 seconds, root rot and wilting of plants 36-60 h after inoculation were observed (Nemec et al., 1986). In soil treated with a conidial suspension of the fungus, no root rot was recorded but root length and seedling weight were reduced (Dandurand and Menge, 1993). On the other hand, using millet seed inoculum of *F. solani*, citrus plants developed wilting and scorching on their shoots as well as severe root rot within 14 days of inoculation (Strauss and Labuschagne, 1995).

In the present work, interveinal chlorosis, wilt and defoliation were observed 14 days after dipping plant cuttings or after leaf inoculation with *F. solani* PVCT1A conidial suspension. There were significant differences between rolABC and wt lines with both methods of inoculation, with rolABC Troyer citrange cuttings and leaves being more sensitive to the pathogen than the wt lines.
Reduction of root weight following F. solani inoculation has been reported (Nemec et al., 1981; Dandurand and Menge, 1993). In the present study, no differences were observed between inoculated and non-inoculated wt Troyer citrange seedlings, whereas root weight of rolABC plants was significantly reduced, compared with the non-inoculated plants. Root rot was not observed, contrary to foliage symptoms, with wt being more sensitive than rolABC lines.

In our study, F. solani symptom expression was observed 14-60 days after inoculation by all methods. Moreover, with these methods it was also possible to highlight disease severity differences between rolABC and wt lines.

The pathogen could always be re-isolated from inoculated tissues in all experiments, without significant differences between rolABC and wt, suggesting lack of correlation between symptom expression and pathogen colonization. Moreover, these results suggest that colonization of F. solani is not influenced by rolABC gene expression.

F. solani produces a variety of phytotoxins (Baker et al., 1981), and their effects on citrus include vein chlorosis, leaf wilt and vessel plugging (Nemec et al., 1988). Naphthazarin toxins are also known to have a significant effect on root growth (Baker et al., 1981) and on growth of citrus seedlings (Janse van Rensburg et al., 1996). In this study, we evaluated the effect of the culture filtrate of F. solani PVCT1A on transgenic and wt Troyer citrange cuttings, and wilt symptoms similar to those obtained after inoculation of F. solani conidia were observed, whereas no difference was recorded in symptom expression between rolABC and wt lines.

SEM observations showed that Troyer citrange roots are readily infected and colonized by F. solani, even in the absence of visible symptoms, and no differences were observed between rolABC and wt. Our observations are in agreement with those by others on naturally infected (Nemec, 1978) and artificially infected citrus trees (Labuschagne et al., 1987). In particular, Labuschagne et al. (1987) showed that F. solani establishes symptomless infection in roots of rough lemon plants, colonizing the epidermal, cortical and vascular tissue without any root rot development.

Over all, our study has shown that all inoculation methods are good for monitoring pathogen colonization and symptom development. Moreover, we showed that integration into Troyer citrange plants of rolABC genes does not significantly affect the susceptibility of young seedlings to F. solani.

Due to the lack of specific symptoms to evaluate infection as well as the need to compare rootstock behaviour to F. solani infection, we suggest that the symptoms we obtained may represent an early indication of dry root rot development, and the tests described can be used to evaluate rootstock sensitivity to the pathogen.

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