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Gene Expression Studies in Sour Orange and C-22 Rootstocks Challenged with the Fungus, *Phytophthora nicotianae* and the Nematode, *Tylenchulus semipenetrans*

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ABSTRACT

Citrus rootstocks differ in response to soil-borne pests and pathogens exhibiting morphological and molecular changes. Such changes were assessed in two citrus rootstocks, sour orange and C-22, inoculated with the fungus, *Phytophthora nicotianae* and the citrus nematode, *Tylenchulus semipenetrans.* These pathogens induce responses and as a result, different genes are expressed. The cDNA-amplified fragment length polymorphism (cDNA-AFLP) was used to identify the differentially expressed genes. Eight differential fragments corresponding to the changes in host gene expression were cloned and sequenced. A homology search at the National Center for Biotechnology Information (NCBI) Genbank database showed one of the clones derived from C-22 rootstock responded to citrus nematode has similarity to the *Citrus tristeza virus* (CTV) resistance gene locus in *Poncirus trifoliata* with 89% identity and an E value of -75. This suggests that a gene supporting nematode resistance in citrus could be related to CTV. The AFLP fingerprint profiles were characterized on agarose and poly(NAT)® Elchrom gels for resolution and clarity. Better results were obtained with poly(NAT)®, and the result was assessed for different profiles generated by different primer combinations. Sour orange and C-22 rootstocks were also evaluated for changes in shoot length, shoot weight and root weight in response to *P. nicotianae* and *T. semipenetrans*. Sour orange seedlings showed tolerance to *P. nicotianae*, compared to C-22, based on percentage reduction in shoot length, shoot weight and root weight; however, differences were not significant.

Keywords: cDNA-AFLP, Elchrom gel, *Citrus tristeza virus*, *Poncirus trifoliata*, resistance gene

Abbreviations: AFLP, amplified fragment length polymorphism; **BAC**, bacterial artificial chromosome; **cDNA**, complementary DNA; ***Ctm***, *Citrus tristeza virus* short-distance movement resistance gene; **CTV**, *Citrus tristeza virus*; ***Ctv***, *Citrus tristeza virus* resistance gene; **DIW**, deionized water; **LRGV**, Lower Rio Grande Valley; **NCBI**, National Center for Biotechnology Information; **PCR**, polymerase chain reaction; **Poly(NAT)**, Poly-*N*-acryloyl-tris(hydroxymethyl) aminomethane; **PR**, pathogenesis-related

INTRODUCTION

The most commonly implicated soil-borne pathogens of citrus are the fungal pathogen, *Phytophthora nicotianae* Breda de Haan (Tsao 1959) and the citrus nematode, *Tylenchulus semipenetrans* Cobb (Baines *et al.* 1978). *Phytophthora* is responsible for 10 to 30% of losses occurring world-wide in citrus production (Graham and Menge 2000). In the Lower Rio Grande Valley (LRGV) of Texas, foot rot is caused by *P. nicotianae* (Gausman *et al.* 1970) and can result in huge economic losses to the citrus industry (Fletcher *et al.* 2001). Citrus nematode, *T. semipenetrans* Cobb, is one of the important plant-parasitic nematodes in the soil rhizosphere, capable of damaging citrus trees and is considered to be the most serious nematode pest of citrus (Van Gundy and Meagher 1977). It infests more than 50% of the citrus-producing areas, and accounts for the worldwide loss of about 40 million tons of fruit (Hamid *et al.* 1985; Duncan and Cohn 1990). In Texas, citrus nematode is wide-spread and causes a slow decline of citrus (O'Bannon and Esser 1985) with initial symptoms of reduced growth, leaf chlorosis and abscission, dieback and small fruits.

Although various management practices such as biological control (Nemec *et al*. 1996) and systemic fungicides (Davis 1981) were used to reduce the losses, the only efficient and economic method available for preventing foot rot is the use of resistant rootstocks (USDA State Statistical Report 2000). Resistant rootstock offers an excellent economical means of reducing the losses occurring worldwide (Whiteside 1973). Evaluation of citrus rootstocks for resistance to *Phytophthora* plays an important role in the development of effective management strategies (Carpenter and Furr 1962; Klotz *et al.* 1968; Tuzcu *et al.* 1984; Graham 1990). Trifoliate orange and sour orange rootstocks are resistant to gummosis caused by *P. nicotianae.* But their hybrids showed less resistance when compared to their parents (Medina *et al.* 2003). More tolerance to root rot was exhibited in trifoliate orange and Swingle citrumelo compared to Carrizo citrange, sour orange, 'Ridge Pineapple' sweet orange, and Cleopatra mandarin (Graham 1995). Sour orange is the predominant rootstock used in Texas. The other trifoliate hybrids that are used as rootstocks in Texas, mainly in non-commercial plantings, are 'Swingle citrumelo', 'Carrizo' and 'Troyer' citranges and trifoliate orange (Bird and Thomson 1980). C-22, a hybrid rootstock of sunki mandarin and Swingle trifoliate orange is resistant to *Phytophthora* disease and is well suited to LRGV soil conditions (Louzada *et al.* 2008). Sour orange is rated as a susceptible rootstock, whereas Swingle citrumelo is rated as a highly tolerant rootstock to citrus nematode (Anciso 2002).

Studies of different rootstocks using new molecular tools can help us develop strategies for engineering synthetic resistance to plant parasitic nematodes (Bird 1996; Bird and Koltai 2000) and was shown to be an environmentally safe approach (Bradley and Duffy 1982). Plants develop defense mechanisms to abiotic and biotic stress factors by accumulation of pathogenesis-related (PR) proteins. High levels of *PR* gene expression during systemic plant defense

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act as markers for defense response (Tornero *et al*. 1997). An in-depth study of more defense-related genes is possible with more reliable techniques such as complementary DNA-amplified fragment length polymorphism (cDNA-AFLP) (Lang *et al*. 2005). cDNA-AFLP analysis is more sensitive and non-biased technique based on PCR amplification (Gellatly *et al*. 2001). This technique is highly specific and has higher reproducibility when compared to hybridization-based technique (De Paepe *et al*. 2004).

The two main objectives of this study are: (1) to test and obtain quantitative results regarding the processes of *P. nicotianae* and nematode infections in sour orange and C-22 rootstocks, and (2) to study the differential gene expression due to *Phytophthora* and nematode inoculation in sour orange and C-22 rootstocks using cDNA-AFLP technique.

MATERIALS AND METHODS

Inoculation of rootstocks with *P. nicotianae* and *T. semipenetrans*

P. nicotianae was isolated from infected citrus roots and soil samples collected from Block 8, TAMUK Citrus Center, Weslaco, and maintained on *P. nicotianae* selective corn meal agar medium supplemented with antibiotics (PARPH), 10 mg/L Pimaricin (Sigma-Aldrich, St Louis, MO), 250 mg/L Ampicillin (Sigma-Aldrich), 100 mg/L Rifampicin (Sigma-Aldrich), 100 mg/L Pentachloronitrobenzene (PCNB) (Sigma-Aldrich), and 50 mg/L Hymexazol (Sigma-Aldrich). Small portions of cultures were removed from the margins of the media plates and were transferred onto V-8 agar medium (Ferguson and Jeffers 1999) to prepare inoculum. After storing at 24°C in the dark for 3 d, the V-8 agar cultures were placed under direct continuous light for 3 d to induce sporangia. V-8 agar culture discs (6 mm diameter) were cut, suspended in sterile deionized water (DIW), chilled for 5 min at 4°C, and the cultures were placed at 24°C to release zoospores. Five seedlings of each rootstock were inoculated with approximately 2×10^5 zoospores per plant by dipping the roots into the zoospore suspension. Root samples were collected after 2 and 10 h of *P. nicotianae* inoculation. Moreover, root samples were also collected from uninoculated control plants by dipping the roots in water. The root samples collected were flash frozen in liquid nitrogen, and stored at –80°C.

Second stage juveniles (J2) of citrus nematode, *T. semipenetrans*, were isolated from soil using the Baermann funnel extraction (Nigh 1981). After 48 h, the J2 nematodes were collected and concentrated by vacuum suction and collected on 0.45 µm cellulose nitrate membrane filter (Whatman). The extraction (500 mL) was stained with 16 mL of acid fuchsin dye to count the nematodes. Six-week-old seedlings of sour orange and C-22 were inoculated with approximately 7,000 nematodes per seedling. Nematode suspension of 100 mL was poured into the holes of 5 cm depth made near the base of the plant. A total of 40 seedlings of each rootstock were inoculated and 10 seedlings were used as control. Sterile DIW was added to non-inoculated plants were considered as control. One to two root samples were taken randomly from the second day after inoculation and observed under microscope. At 10 d and 12 d post inoculation, roots were cut into pieces, flash-frozen in liquid nitrogen and stored at –80°C until used. The flash frozen roots from un-inoculated control plants as well as plants inoculated with *P. nicotianae* and *T. semipenetrans* were used in total RNA extraction and further cDNA-AFLP gene expression studies.

Evaluation of rootstocks inoculated with *P. nicotianae* and *T. semipenetrans*

Six-week-old seedlings of sour orange and C-22 of similar size with five to six leaves and an approximate shoot length of 4 cm were selected for evaluation studies. This experiment was carried out in two sets, one with *P. nicotianae* and another with citrus nematode.

For evaluation studies against *P. nicotianae*, Metro Mix 700 was sterilized by autoclaving and then mixed with zoospore suspension and V-8 cultural discs of 6 mm diameter with abundant sporangia. In the control treatment, sterilized Metro Mix 700 was mixed with sterile DIW and V-8 discs without fungal mycelia. For evaluation studies against citrus nematode, seedlings were inoculated with 150 J2 per seedling by pouring the nematode suspension in 5-cm deep holes made in the soil mix. Seedlings were treated with sterile DIW without nematodes to serve as control treatment.

After 60 d, 30 seedlings per treatment were carefully uprooted and data was taken on percentage reduction in shoot length, fresh weight of root system, and fresh weight of shoot system. The experiment was repeated for each treatment and results were combined for data analysis. Data were analyzed using PROC GLM procedure (SAS). Treatment means were compared using least significant difference (LSD) test. F-test was significant at $P < 0.05$.

Synthesis of double-strand cDNA and amplified fragment length polymorphism (AFLP) analysis

First-strand cDNA was synthesized from total RNA according to the manufacturer's instructions using Superscript™ first strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA). Two micro-gram of total RNA was reverse transcribed using 200 U Superscript™ II reverse transcriptase in a 20 µl reaction mixture consisting of 50 µM oligo dT primer, 0.2 mM dNTPs, 2 µl of 10X buffer [200 mM Tris-HCl (pH 8.4)], 15 mM $MgCl_2$, 500 mM KCl], 2 µl of 0.1 M DTT, and 1 µl RNaseOUT (40U/µl). The reaction mix was incubated in a thermalcycler for 60 min at 42°C and 15 min at 70°C. The reaction products were stored at –20°C until further use. Double-stranded cDNA was synthesized from first-strand cDNA using the Superscript™ Double-stranded cDNA Synthesis Kit (Invitrogen). To the 20 µl of first-strand reaction, 130 µl of reaction mixture consisting 91 µl of nuclease-free DEPC treated water, 30 µl of 5X second-strand reaction buffer [250 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 375 mM KCl], 3 µl of 10 mM dNTP mix, 1 µl of *E. coli* DNA ligase (10 U/µl), 4 µl of *E. coli* DNA polymerase I (10 U/µl) and 1 µl of *E. coli* RNase H (2 U/µl) was added. The reaction was mixed gently by tapping and incubated at 16°C for 2 h. Two micro-litres of T4 DNA polymerase (5 U/µl) was added to polish the termini (removing protruding 3′ ends) of the completed double-stranded cDNA followed by incubation at 16°C for 5 min and then cooled on ice. To the above mixture, 10 µl of 0.5 M EDTA, 160 µl of phenol, chloroform and isoamylalcohol (25: 24: 1) were added, vortexed, centrifuged for 5 min at 14,000 × g and then cooled on ice for 5 min. The supernatant of 140 µl was carefully collected and transferred into a 1.5 ml microcentrifuge tube. Further precipitation steps were carried out using 70 µl of 7.5 M NH_4OAc and 0.5 ml ice-cold absolute alcohol and the DNA was dissolved in 10 µl of nuclease-free DEPC treated water.

AFLP analysis was carried out using the AFLP® core reagent kit and the AFLP® starter primer kit (Invitrogen). Pre-selective PCR amplification was carried out in a total volume of 51 µL by adding 5 µL of diluted (1: 1) template DNA to 40 µL of pre-amp primer mix, 5 µL of 10X PCR buffer and 1 µL of HotStarTaq® DNA polymerase (5 U/µL) (Qiagen Inc., Valencia, CA). The contents were mixed gently, centrifuged briefly for 10 sec and were incubated at 95°C for 15 min to activate DNA polymerase enzyme followed by 20 cycles of 94°C for 30 sec, 56°C for 60 sec and 72°C for 60 sec. From the reaction product of 51 µL, 5 µL of template DNA was diluted with same amount of TE buffer. Both the diluted and undiluted (46 µL) samples were stored at –20°C. The selective PCR amplification of restricted fragments was performed with different primer pair combinations: *Mse*I+3/*Eco*RI+3 and *Mse*I+3/*Eco*RI+2 (**Table 1**). The *Eco*RI selective primer had a sequence of 5′-GTATCACGAGGCCCTT-3′ and the *Mse*1 selective primer had a sequence of 5′-AGGCGTCCTACTGCGTAA-3′. The primer pair combinations that worked best with the above two methods were selected and once again subjected to selective PCR amplification.

DNA from 25 differentially expressed amplicons was recovered using QIAquick gel extraction kit (Qiagen Inc, Valencia, CA). Furthermore, only eight of these were successfully re-amplified in the repeated PCR re-amplification reactions using the same set of the primers and reaction conditions that were initially used to amplify these DNA fragments.

Table 1 List of primer pair combination tested and selected for cDNA-AFLP selective amplification.

	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC	√	√		√		√		√
E-AAG			√ *	√	√	√ *	√	√
E-ACA	√ *	√	√					
E-ACC	√	√ *	√ *					√ *
E-ACG	√	√ *	√ *		√ *			
E-ACT	√		√				√	
E-AGC		√						
E-AGG		√	√		√	√	√	
E-AG	√ *	√	√	√				
E-AT		√	√					
E-TC		√			√			√ *
E-TG		√			√ *			

"√" Primer pair combination tested in selective AFLP amplification.
"*" Primer pair combination that worked best.

Cloning and sequencing

Eight fragments that showed differential expression were cloned and sequenced. The differential fragments were cloned into pCR®II-TOPO® plasmid vector (Invitrogen) and transformed into *E. coli* (DH5α-T1R) and sequenced at Iowa State University DNA sequencing facility. The sequences were compared to Genbank data base using the blast N program of NCBI.

Comparison of gels

Agarose gels were compared with 6% poly(NAT)® Elchrom gel (Elchrom Scientific, Switzerland) for resolution and clarity. Ready-to-use poly(NAT)® gels were used in Wide Mini-Sub Cell GT (Bio-Rad Laboratories, Hercules, CA, USA) unit. Electrophoresis was conducted at 105 V (7 V/cm) for 115 min using cold TAE buffer at 10°C. The whole unit was kept in a thermal insulated ice box and all the sides of the unit were covered with ice to maintain the temperature of the TAE running buffer at 10°C during electrophoresis. The gel was stained with ethidium bromide for 30 min and washed with nuclease free water for 30 min. Plastic backing was removed with the nylon thread and the gel was viewed on UV transilluminator.

RESULTS AND DISCUSSION

Inoculation of zoospores

High populations of *P. nicotianae* were recovered within 24 h of baiting and sporangia were seen on the baits after only 3-6 d. These results indicate leaf discs collected within 24 h of baiting showed minimum contamination and high recovery of *P. nicotianae*. The type of fungi that contaminate depends on the soil collected and also incubation temperature (Eden et al. 2000). Zoospores of *P. nicotianae* penetrated both sour orange and C-22 rootstocks at 2 h after inoculation. However, both rootstocks showed a higher degree of cell colonization at 10 h after inoculation (**Figs. 1, 2**).

Inoculation of nematodes

In both rootstocks at 10 d after inoculation, many nematodes were attached to the root and few nematodes had penetrated into the roots. Nematodes were densely attached to the roots of sour orange as compared to the roots of C-22. At 12 d after inoculation, the number of nematodes penetrated into the roots of both rootstocks was doubled when compared to 10 d after inoculation.

Evaluation of rootstocks inoculated with *P. nicotianae*

No significant differences were observed at 60 days after inoculation between inoculated and non-inoculated sour orange seedling growth parameters, shoot length, shoot weight and root weight (**Table 2**). In C-22, shoot lengths of

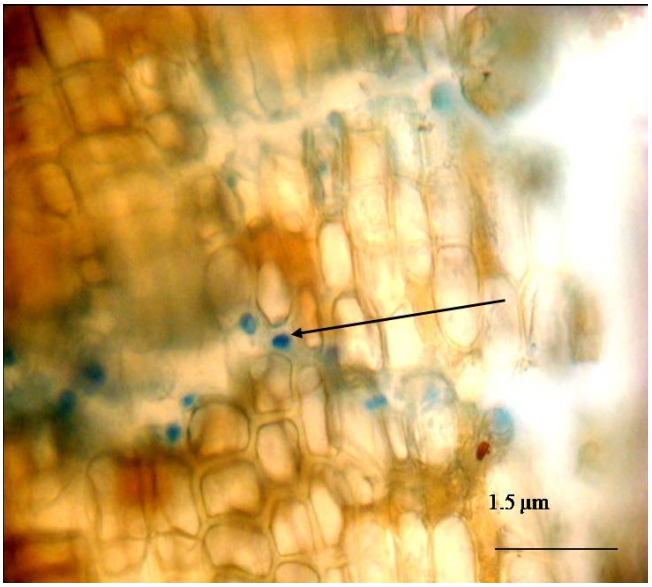

Fig. 1 Penetration of *Phytophthora nicotianae* zoospores through the roots of C-22 10 h after inoculation.

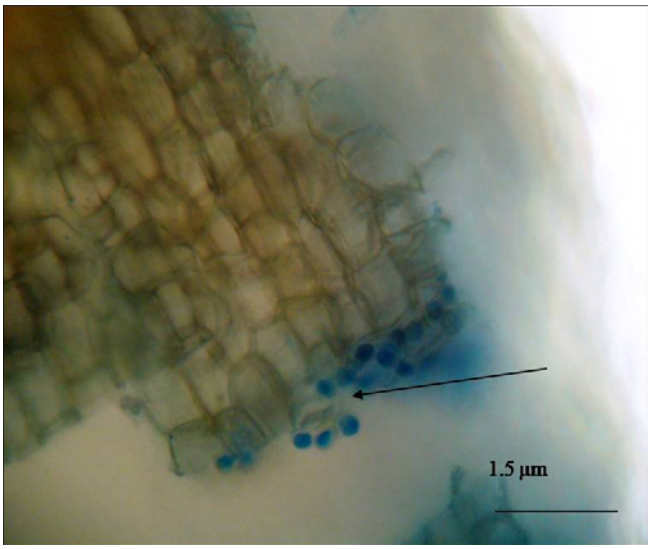

Fig. 2 Penetration of *Phytophthora nicotianae* zoospores through the roots of sour orange 10 h after inoculation.

Phytophthora-inoculated seedlings were not significantly different from non-inoculated control, whereas, shoot weight of inoculated C-22 was significantly lower than the control. Root weights were not significantly different in inoculated or non-inoculated treatments. Only the rootstock type had a significant effect on weight parameters, whereas,

Table 2 Mean shoot length (cm), shoot weight (g) and root weight (g) of inoculated and non-inoculated sour orange and C-22 rootstocks with the fungus *Phytophthora*.

Root stock	Shoot length (cm)			Shoot weight (g)			Root weight (g)		
	Control	Inoculated	*P*	Control	Inoculated	*P*	Control	Inoculated	*P*
Sour orange	7.520 A	7.393 A	0.677	0.687 A	0.625 A	0.075	0.545 A	0.505 A	0.236
C-22	7.586 A	7.170 A	0.174	0.599 A	0.400 B	0.001	0.389 A	0.324 A	0.199

Means with the same letter within a column are not significantly different ($P < 0.01$) according to the student's *t*-test.

Table 3 Significance (*P*-value) of treatment effects on shoot length (cm), shoot weight (g) and root weight (g) when inoculated with the fungus *Phytophthora*.

Treatment factor	*P*-Value		
	Shoot length	Shoot weight	Root weight
Treatment	0.4805	0.3817	0.8930
Rootstock	0.9758	0.0001	0.0004
Treatment * Rootstock	0.7582	0.0174	0.3514

Table 5 Significance (*P*-value) of treatment effects on shoot length (cm), shoot weight (g) and root weight (g) when inoculated with citrus nematode, *Tylenchulus semipenetrans*.

Treatment Factor	*P*-Value		
	Shoot length	Shoot weight	Root weight
Treatment	0.4611	0.8732	0.4194
Rootstock	0.1853	**0.0094**	0.3396
Treatment * Rootstock	0.2848	0.2727	0.8086

inoculation had no significant effect on any of the parameters (**Table 3**). There was no significant interaction between inoculation and rootstock. Inoculations alone had no effect on the growth parameters. Similar results were observed when seedlings were inoculated with nematodes. In the above study, sour orange seedlings showed more tolerance to *Phytophthora* when compared to C-22. However this effect was due to the rootstock factor. High percent reduction in shoot and root weights in C-22 seedlings inoculated with *Phytophthora* may be due to severe root rot. Inoculations alone had no effect on the growth parameters. A similar result was observed when inoculated with nematodes.

Evaluation of rootstocks inoculated citrus nematode

When mean shoot length, fresh shoot weight and fresh root weight of inoculated and non-inoculated seedlings were compared 60 d after inoculation, no significant differences were observed in either sour orange or C-22 (**Table 4**). The result reveals that inoculating sour orange and C-22 rootstocks with nematode did not show differences in growth compared to non-inoculated rootstocks. The rootstock factor had a significant effect on shoot fresh weight (**Table 5**). Treatment alone and also its interaction with rootstock had no significant effect on any of the growth parameters.

Percentage reductions

Shoot length, shoot weight and root weight of infected plants were compared to non-infected controls and expressed as percentage reductions (**Table 6**). Differences among the two rootstocks regarding percentage reductions in shoot weight and root weight due to *P. nicotianae* infection were statistically significant with percent reduction higher in C-22 than sour orange. Percent reduction of all three growth parameters in nematode-inoculated rootstocks was not significantly different from each other.

Number of juvenile nematodes

Initially, all seedlings received equal number of juvenile citrus nematodes as inoculum. The final population densities (number of nematodes per 100 g of soil) of the second stage juvenile nematodes in the soil were less on sour orange than C-22, as C-22 supported significantly higher ($P < 0.0001$) populations of second stage juveniles in the soil (**Table 7**).

At 60 d after inoculation, the population densities of juvenile nematodes were highest in soil samples collected from C-22 seedlings. According to Noling (2002), nematode populations are usually at a lower level in the winter as fibrous root flush is low. Results of this study are in agreement with Noling (2002), as there was no significant difference between inoculated and non-inoculated plants. The period of study was short term and was performed in winter. This could be one possible reason for the presence of negligible number of females in the roots. Nematode inoculations had no effect on the growth parameters in either of the rootstocks.

cDNA-AFLP

Eight differential amplicons (**Figs. 3, 4**) obtained from cDNA-AFLP were selected, cloned and sequenced. In general, clear and sharp bands were observed when the PCR products were run on poly(NAT)® Elchrom gels compared to agarose gels (**Fig. 4**). One of the clones (GenBank accession GU358609) generated from nematode-inoculated C-22 fragment and primer pair EAG-MCAA, showed homology to *Poncirus trifoliata*'s *Citrus tristeza virus* (CTV) resistance gene (*Ctv*) locus with 89% identity and an E value of -75 (accession No. AF506028.1). Analysis of other sequences revealed low homologies without any significant similarity to the known proteins.

The identification of *Poncirus trifoliata*'s CTV resistance gene locus homolog in C-22 suggests that a gene supporting nematode resistance in citrus could be related to related to a plant resistance gene. Conversely, no significant genes were detected from the profiles of sour orange, inoculated either with citrus nematode or with *Phytophthora.*

This study identified a candidate gene involved in the interaction of citrus nematodes and C-22. This gene was up-regulated in the roots of C-22 on the 10th day after inoculation. The sequence analyses in our study indicate that there is a disease resistance (*R*) gene cluster in the *Ctv* region. Such a gene has also been observed by Deng *et al*. (1997) who described that *Ctv* might be linked to the major quantitative trait locus for citrus nematode (*T. semipenetrans*) resistance. The clustering of disease *R* genes is a common occurrence in plant genomes (Michelmore and Meyers

Table 4 Mean shoot length (cm), shoot weight (g) and root weight (g) of inoculated and non-inoculated sour orange and C-22 rootstocks with citrus nematode, *Tylenchulus semipenetrans*.

Root stock	Shoot length (cm)			Shoot weight (g)			Root weight (g)		
	Control	Inoculated	*P*	Control	Inoculated	*P*	Control	Inoculated	*P*
Sour orange	7.633 A	7.260 A	0.202	0.694 A	0.669 A	0.457	0.505 A	0.488 A	0.690
C-22	7.206 A	7.137 A	0.810	0.627 A	0.592 A	0.422	0.453 A	0.324 A	0.450

Means with the same letter within a column are not significantly different ($P < 0.01$) according to the student's *t*-test.

Table 6 Percentage reductions of shoot length, fresh shoot weight, fresh root weight of plants after inoculation with *Phytophthora* and *Tylenchulus semipenetrans* in sour orange and C-22 rootstocks.

Inoculation	Reduction in shoot length (%)		Reduction in shoot weight (%)		Reduction in root weight (%)	
	Sour orange	C-22	Sour orange	C-22	Sour orange	C-22
Phytophthora	18.75 A	21.2 A	28.31 B	55.60 A	33.08 B	62.41 A
Nematode	21.08 A	20.8 A	27.64 B	33.95 B	34.27 B	39.82 B

Means with the same letter within a column are not significantly different ($P < 0.01$) according to the student's *t*-test.

Table 7 Mean number of juvenile nematodes of *Tylenchulus semipenetrans*/100 g of soil.

Root stock	Number of juvenile nematodes/100 g of soil
Sour orange	23.933 A
C-22	73.379 B
P-Value	<0.0001

Means with the same letter are not significantly different ($P < 0.01$) according to the student's *t*-test.

1998), and genes within a single cluster can determine resistance to very different pathogens and activate different defense mechanisms in plants (Hammond-Kosack and Jones 1997). Yang *et al*. (1995) and Deng *et al*. (2001) independently constructed two bacterial artificial chromosome (BAC) libraries as part of a map-based cloning strategy with the idea of identifying BAC clones spanning the genetic region identified as containing gene for resistance to CTV. Deng *et al*. (2001) also found that some DNA fragment markers were found to be associated with *Ctv* and a major gene responsible for the citrus nematode resistance (*Tyr1*).

It was reported that CTV resistance in trifoliate orange was controlled by a single dominant gene *Ctv* (Yoshida 1985; Gmitter *et al.* 1996). In addition to *Ctv*, a second gene called CTV short-distance movement resistance gene (*Ctm*) is involved in the resistance of *P. trifoliata* to CTV (Mestre *et al*. 1997). C-22 is a hybrid rootstock, with a selection of *P. trifoliata* being one of the parents. CTV in progenies derived from *P. trifoliata* behaves as a monogenic trait where the resistance allele is dominant (Yoshida 1985; Mestre *et al.* 1994; Gmitter *et al.* 1996; Fang and Roose 1996). Bulked segregant analysis (Michelmore *et al.* 1991) was used to locate molecular markers linked to disease resistance genes. Its monogenic control along with the fact that hybrids between *P. trifoliata* and *Citrus* spp. can be obtained makes the resistance to CTV a suitable trait.

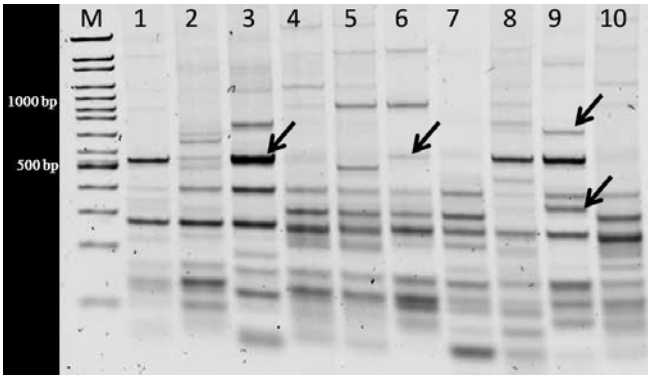

Fig. 4 cDNA-AFLP profiles generated by E-ACA and M-CAG primer pair combination and separated on a poly(NAT)® Elchrom gel. Lanes 1, 2 and 3 are *Phytophthora* control, 2 and 10 h treatments in sour orange. Lanes 4, 5, and 6 are *Phytophthora* control, 2 h and 10 h treatments in C-22. Lanes 7 and 8 are nematode control and inoculation treatments in sour orange. Lanes 9 and 10 are nematode control and inoculation treatments in C-22. M = 100 bp DNA marker.

CONCLUSION

The results obtained in inoculation and evaluation studies demonstrated that sour orange was relatively resistant to the fungus, *Phytophthora* and the nematode, *Tylenchulus.*

In the past, several pathogenesis-related *R* genes have been cloned and identified in many plants. Several classes of *R* genes confer resistance and activate different signaling pathways, thus, coordinating the defense mechanism. This study identified partial sequence of a candidate gene involved in interaction of *Tylenchulus* with C-22, a potential rootstock of the LRGV. The possibility exists for the gene which encodes a protein related to CTV resistance, also responds to the citrus nematode. Breeding and screening prog-

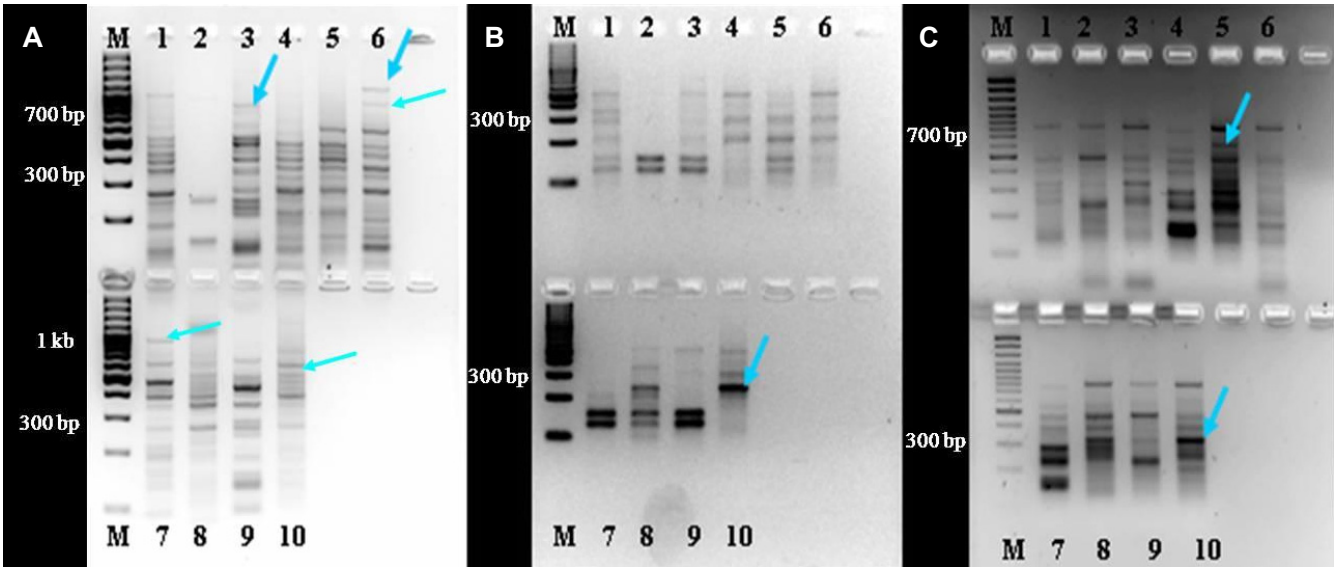

Fig. 3 cDNA-AFLP products separated on agarose gels (panel A, B and C show amplicons derived by using primer pair combinations, EACG-MCAC, EAAG-MCAG and EAG-MCAT, respectively). Lanes 1, 2 and 3 are *Phytophthora* control, 2 h and 10 h treatments in sour orange. Lanes 4, 5, and 6 are *Phytophthora* control, 2 and 10 h treatments in C-22. Lanes 7 and 8 are nematode control and inoculation treatments in sour orange. Lanes 9 and 10 are nematode control and inoculation treatments in C-22. M = 100 bp DNA marker. Arrow indicates differential fragments.

rams for pest and disease resistance in citrus is a challenge and a tedious task. Though it is an initial step towards understanding of mechanisms involved in interaction between citrus nematode and rootstocks, further investigations would provide insight into deep understanding of these mechanisms.

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