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Journal of Citrus Pathology

Brief Report

Citrus dwarfing viroid reduces canopy volume by inhibiting shoot apical growth of navel orange trees grown on trifoliate orange rootstock

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Abstract

Citrus dwarfing viroid (CDVd) infection of navel orange trees (*Citrus sinensis* (L.) Osb.) on 'Rich 16-6' trifoliate orange (*Poncirus trifoliata* (L.) Raf.) rootstock has been previously reported to reduce canopy volume by approximately 50%. We demonstrated that the reduction in tree size of CDVd-infected citrus resulted from a >20% reduction in the apical growth of individual shoots within the tree canopy. We also demonstrated that the reduced canopy volume of the CDVd-infected trees is a long lasting phenotype comparable to that of 'Flying Dragon' rootstock, which is known to reduce citrus tree size.

Keywords: citrus tree size, phytohormones, high-density plantings
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Introduction

At present, seven citrus viroids have been identified, and an eighth species awaits official recognition by the International Committee on Taxonomy of Viruses (Chambers et al. 2018; Di Serio et al. 2014). Citrus viroids are capable of inducing a range of symptoms in specific citrus hosts. In the bioindicator 'Etrog' citron (*Citrus medica* L.) Arizona 861-S-1, citrus dwarfing viroid (CDVd) induces moderate stunting, leaf epinasty, petiole and midvein necrosis (Timmer et al. 2000). In commercial citrus, CDVd typically induces mild symptoms such as tree stunting with reduced canopy volume as reported for Valencia and navel orange (*C. sinensis* (L.) Osb.) and clementine (*C. reticulata* Hort. ex. Tan.) trees grown on 'Rich 16-6' trifoliate orange (*Poncirus trifoliata* (L.) Raf.) rootstock, which in some cases expresses gummy pitting and finger imprint symptoms (Semancik et al. 1997; Vernière et al. 2004; Vidalakis et al. 2004; Vidalakis et al. 2011).

The CDVd infection of navel orange trees grafted on 'Rich 16-6' rootstock, planted in 1998, was reported to reduce tree canopy volume by approximately 50% after 13 years in the field (Vidalakis et al. 2011). To evaluate the long-term CDVd effect on canopy volume, we measured the same 1998-planted CDVd infected navel orange trees after 20 years in the field. The canopy volume of CDVd-infected trees was compared with the canopy volume of two types of controls planted on the same experimental

block: (i) non-infected navel orange trees on 'Rich 16-6' trifoliate rootstock planted in 1998 and (ii) navel orange trees grown on trifoliate rootstock 'Flying Dragon', which is known to reduce tree size, planted in 1984 (Bitters et al. 1979; Davies and Albrigo 1994; Roose 1986). We discovered that CDVd reduced the tree canopy volume by more than 60% in comparison to the non-infected controls and by approximately 6% in comparison to the 'Flying Dragon' controls.

To understand how CDVd reduces canopy volume, we surveyed the navel orange trees vegetative shoots for length and apical growth. We initially measured the length of five nodes, moving back from the tip of selected shoots, and found a statistically significant difference between CDVd-infected trees and non-infected controls. Next, we surveyed the apical growth of selected shoots for two spring flushes. We found that the apical growth of the shoots of the CDVd-infected trees was significantly lower by more than 20% ($p < 0.01$) than that of the non-infected controls. There was no statistical difference in the length or the apical growth of the measured shoots between CDVd-infected trees and 'Flying Dragon' controls. The findings of this study indicate that the effect of CDVd on length and apical growth of vegetative shoots is likely to be the main factor determining the observed reduction in overall citrus tree canopy volume.

Materials and Methods

Plant material and CDVd infection

This study was performed on various commercial varieties of navel oranges used by the California citrus industry at the time of tree planting over 20 years ago.

In the 1984 'Flying Dragon' trifoliate rootstock trial (used in this study as 'Flying Dragon' controls), eight trees in total, two for each of 'Parent Washington', 'Frost', 'Atwood', and 'Carter' navel oranges, were propagated and planted in the experimental block #41 at the University of California, Agricultural and Natural Resources (UC-ANR), Lindcove Research and Extension Center (LREC), in Central California's San Joaquin Valley (SJV).

In the 1998 CDVd trial, 37 'Parent Washington' navel orange trees were propagated on the commercially used trifoliate orange cv. 'Rich 16-6' rootstock. At the time of propagation, the rootstock of 29 of the 37 'Parent Washington' navel orange trees was graft-inoculated with CDVd (GenBank AF18147) (used in this study as CDVd-infected trees) and the remaining eight trees were not inoculated with CDVd (used in this study as non-infected controls) (Semancik et al. 1997; Vidalakis et al. 2011). CDVd-infected and non-infected controls were planted in the same LREC experimental block (#41) as the 1984 'Flying Dragon' rootstock trial trees. The trees in this study were never pruned and all cutting tools for sample collection were disinfected with 1% sodium hypochlorite solution to avoid the accidental spread of CDVd (Roistacher et al. 1969).

In 2018, all 45 trees in this study were tested for CDVd by reverse transcription quantitative polymerase chain reaction (RT-qPCR) and other graft-transmissible citrus pathogens reported from California as previously described (Li et al. 2006; Osman et al. 2017; Osman et al. 2015; Vidalakis and Wang 2013; Vives et al. 2013; Wang et al. 2013; Yokomi et al. 2008).

Tree measurements and statistical analyses

Canopy volume

Tree height (H) and canopy spread (Sp) in two directions were measured in the winter 2017-2018 and 2018-2019. Canopy volume (CV) was calculated as half of prolate spheroid using the formula CV= $2/3\pi ab^2$ with a= H and b= Sp/2 (Morse and Robertson 1987; Serfontein and Catling 1968; Vidalakis et al. 2011).

Length of shoots

The length of five (5) apical nodes from the tip of four (4) citrus shoots, on the north (N) and south (S) side of each of the CDVd-infected and non-infected controls, was measured in winter 2015-2016. In the winter of 2017-2018 and 2018-2019, eight (8) citrus shoots (4 N and 4 S) of the CDVd-infected, non-infected controls and 'Flying Dragon' controls were measured.

Apical growth of shoots

New growth (i.e. light green succulent tissues) of four (4) vegetative shoots (2 N and 2 S) of each tree, were measured in spring 2016. To increase the sample size per tree eight (8) vegetative shoots, (4 N and 4 S) of each tree, were measured in spring 2018.

Statistical Analysis

Canopy volume, shoot length and apical growth data, were analyzed by the Kruskal-Wallis one way analysis of variance on ranks, followed by the all pairwise multiple comparison procedures (Dunn's method; at *P*= 0.05). Analysis was performed using SigmaPlot 14.0 statistical software package (SPSS Science, Chicago, IL).

Results

CDVd infection reduces canopy volume

Visual inspection of the 1998 CDVd trial, navel orange trees on 'Rich 16-6' rootstock, revealed that CDVd-infected trees maintained their overall smaller size compared to the non-infected controls 20 years after planting (Vidalakis et al. 2011) (Figure 1).

Fig. 1. Effect of citrus dwarfing viroid (CDVd) on overall tree size. CDVd-infected trees are noticeably small in size (left image) than non-infected trees (right image). Graduate student was included in the image to allow an effective visual comparison. Images were taken in January 2016.

Only CDVd was detected in the dwarfed trees by qPCR and no other California reported graft-transmissible pathogen of citrus was detected in any of the 45 trees tested (Table 1).

Tree height (H), canopy spread (Sp) and canopy volume (CV) of navel orange trees on 'Rich 16-6' rootstock infected with CDVd were significantly reduced (*p*< 0.01), in comparison with the non-infected controls (H by 23.3%; Sp by 29.6% and CV by 61.2%, respectively) (Table 2 and Figure 1). The reduction of H, Sp and CV of CDVd-infected trees in comparison to the 'Flying Dragon' controls was not statistically significant (*p*> 0.05) (H by 6.7%; Sp by 1.5%; and CV by 6.2%, respectively) (Table 2).

The estimated CV increase per year of the CDVd-infected trees was approximately three times less than that of the non-infected controls and similar to the 'Flying Dragon' controls (Table 2).

Table 1
Quantitative polymerase chain reaction (qPCR) testing of Navel orange on *Poncirus trifoliata* rootstock 'Rich 16-6' for citrus dwarfing viroid (CDVd) and other California reported raft-transmissible pathogens of citrus.

Treatment			qPCR (Cq)		
Citrus trees	n	CDVd	Viroids	Viruses	Bacteria
CDVd-infected	58	27.11 ± 0.80	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Non-infected control	16	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
'Flying Dragon' control	16	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
qPCR controls					
Citrus gene	2	21.11 ± 0.72	19.63 ± 0.71	17.97 ± 0.75	19.61 ± 0.66
Non-inoculated	4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
NTC	4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Each value is the mean and standard deviation of quantification cycle (Cq) of two technical qPCR replicates of 29, 8 and 8 biological/tree replicates, respectively. qPCR positive controls (n= 4): Citrus dwarfing viroid (23.86 ± 0.85); Viroids: Citrus exocortis viroid (22.49 ± 0.55), hop stunt viroid (20.59 ± 0.79), citrus bark cracking viroid (22.85 ± 0.64), citrus bent leaf viroid (24.11 ± 0.75) and citrus viroid V (28.18 ± 1.63); Viruses: Citrus tristeza virus (23.98 ± 0.25), citrus psorosis virus (25.64 ± 0.10), citrus leaf blotch virus (23.02 ± 0.02). Citrus vein enation virus testing was performed with conventional PCR and amplicon had the expected size (425nt). Bacteria: *Spiroplasma citri* (31.60 ± 0.14) and *Candidatus* Liberibacter asiaticus (24.37 ± 0.21). Citrus gene: NADH. NTC: No-template control.

Table 2
Effect of citrus dwarfing viroid (CDVd) on tree size of navel orange on *Poncirus trifoliata* rootstock.

Treatment	Planting Year	n	Height		Spread		Canopy Volume		Estimated Canopy Volume Increase per Year After Field Planting
			m	%	m	%	m³	%	m³
CDVd-infected	1998	58	2.31 ± 0.03 a	76.7	2.43 ± 0.02 a	70.4	7.19 ± 0.16 a	38.8	0.30
Non-infected control	1998	16	3.01 ± 0.06 b	100	3.45 ± 0.05 b	100	18.55 ± 0.80 b	100	0.88
'Flying Dragon' control	1984	16	2.51 ± 0.05 a	83.4	2.48 ± 0.10 a	71.9	8.34 ± 0.75 ac	45.0	0.30

Each value is the mean and standard error of two measurements. Mean values of height, spread and canopy volume were tested by one-way analysis of variance on ranks followed by all pairwise multiple comparison: Canopy volume: CDVd-infected vs. non-infected controls and non-infected controls vs. 'Flying Dragon' controls, $p< 0.01$; CDVd-infected versus 'Flying Dragon' controls, $p= 0.951$. Spread: CDVd-infected vs. non-infected controls and non-infected controls vs. 'Flying Dragon' controls, $p< 0.01$; CDVd-infected versus 'Flying Dragon' controls, $p= 1.000$. Height: CDVd-infected vs. non-infected controls and non-infected controls vs. 'Flying Dragon' controls, $p< 0.01$; CDVd-infected versus 'Flying Dragon' controls, $p= 0.053$. Median values calculated for the statistical test were as follows: Canopy volume, CDVd-infected: 7.10, non-infected controls: 18.10 and 'Flying Dragon' controls: 7.86. Spread, CDVd-infected: 2.46, non-infected controls: 3.46, and 'Flying Dragon' controls: 2.34. Height, CDVd-infected: 2.29, non-infected controls: 2.98 and 'Flying Dragon' controls: 2.44. Different letters are denoting statistically significant differences.

CDVd infection reduces apical shoot length and growth

Since vegetative growth accounts for most of a tree's canopy volume, we examined the length and apical growth of vegetative shoots in CDVd-infected trees and compared them with the non-infected and 'Flying Dragon' controls.

First, we measured the length of winter shoots, as this is when trees are not flushing in California. We repeated the measurements for a total of three winters. We observed a statistically significant reduction of 6% ($p= 0.002$, ANOVA on ranks) in shoot length of the CDVd-infected trees (mean 4.27 ± 0.04cm, n= 580) in comparison to the non-infected controls (mean 4.55 ± 0.08cm, n= 160) (Figure 2). No statistical difference was present between CDVd-infected and 'Flying Dragon' controls (mean 4.09 ± 0.09cm, n= 128) ($p= 0.297$, ANOVA on ranks). The shoot length of navel orange trees grown on 'Flying Dragon' trifoliate rootstock was significantly reduced by 10.1% ($p< 0.001$, ANOVA on ranks) in comparison to the non-infected controls on 'Rich 16-6' trifoliate orange rootstock (Figure 2).
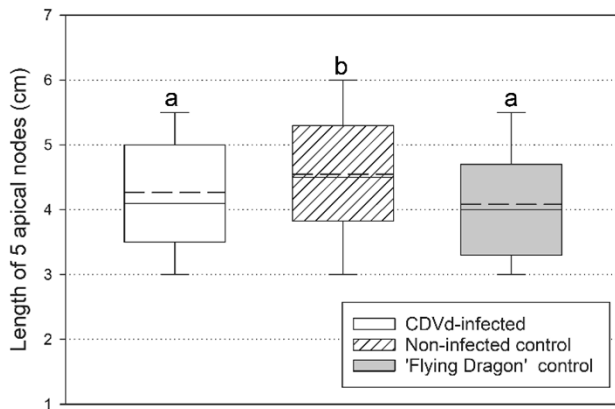
Fig. 2. Effect of citrus dwarfing viroid (CDVd) on length of shoots. Box plots represent multiple measurements of the length of five apical nodes: CDVd-infected trees, n= 580 (29 trees, 20 shoots measured in 3 winters); non-infected controls, n= 160 (8 trees, 20 shoots measured in 3 winters); and 'Flying Dragon' controls, n= 128 (8 trees, 16 shoots measured in 2 winters). Statistical analysis was performed by one-way analysis of variance on ranks followed by all pairwise multiple comparison. CDVd-infected vs. Non-infected control, p= 0.002; Flying Dragon controls vs. CDVd-infected, p= 0.297; and non-infected controls vs. Flying Dragon controls, p< 0.001. Median values presented in box plots were calculated for the statistical analysis were as follows: CDVd-infected: 4.10, Non-infected control: 4.50 and Flying Dragon controls: 4.00. Median and mean values are presented as a solid and dashed line, respectively. Different letters are denoting statistically significant differences.

Next, we monitored new apical growth of shoots over an active growth period (spring) and compared CDVd-infected trees with non-infected and 'Flying Dragon' controls. We observed a statistically significant reduction of 19.9% ($p= 0.010$, ANOVA on ranks) in apical growth of shoots of the CDVd-infected trees (mean 4.28 ± 0.17cm, n= 348) in comparison to the non-infected controls (mean 5.34 ± 0.34cm, n= 96) (Figure 3). The apical growth of shoots of the CDVd-infected trees was significantly greater ($p= 0.018$, ANOVA on ranks) than that of the 'Flying Dragon' control (mean 3.06 ± 0.25cm, n= 96). The apical

growth of shoots of navel orange trees grown on 'Flying Dragon' trifoliate rootstock was significantly reduced by 42.7% ($p< 0.001$, ANOVA on ranks) in comparison to the non-infected controls on 'Rich 16-6' trifoliate orange rootstock.
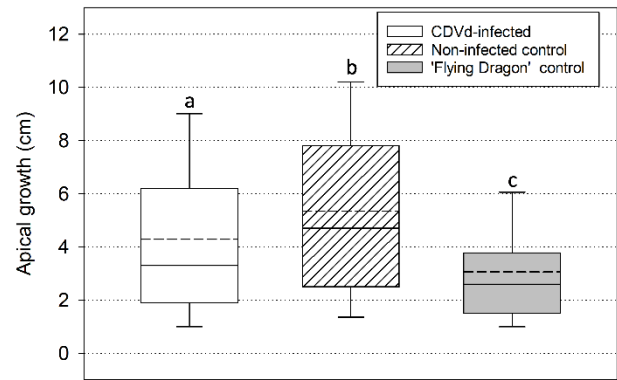
Fig. 3. Effect of citrus dwarfing viroid (CDVd) on apical growth of shoots.
Box plots represent multiple measurements of new apical growth of shoots: CDVd-infected trees, n= 348 (29 trees, 12 shoots measured in 2 springs); non-infected controls, n= 96 (8 trees, 12 shoots measured in 2 springs); and 'Flying Dragon' controls, n= 96 (8 trees, 12 shoots measured in 2 springs). Mean values were tested by one-way analysis of variance on ranks followed by all pairwise multiple comparison: CDVd-infected vs. Non-infected control, p= 0.010; 'Flying Dragon' controls vs. CDVd-infected, p= 0.018; and non-infected controls vs. 'Flying Dragon' controls, p< 0.001. Median values calculated for the statistical test were as follows: CDVd-infected: 3.30, Non-infected control: 4.70 and Flying Dragon controls: 2.60. Median and mean values are presented as a solid and dashed line, respectively. Different letters are denoting statistically significant differences.

Discussion

As described in several previous reports, CDVd (syn. citrus viroid III) infection can reduce the tree size of citrus grown on trifoliate rootstock (Semancik et al. 1997; Vernière et al. 2004; Vidalakis et al. 2011). However, no previous study has looked in detail at the tree canopy growth patterns that collectively result in the dwarfing phenotype observed with CDVd infection. In this study, we demonstrated that the observed reduction in the size of CDVd infected citrus trees resulted from reduced growth of individual shoots within the citrus canopy. We also demonstrated that the reduced canopy volume of the CDVd-infected trees is a long lasting phenotype that is comparable to that of 'Flying Dragon' rootstock, which is well known to reduce citrus tree size (Bitters et al. 1979; Davies and Albrigo 1994; Roose 1986).

The identification of the shoot growth as a key element of the reduced tree size highlights the need for future research of the plant cellular and physiological mechanisms modulated by infection with CDVd. Phytohormones are master regulators of plant growth and development, and most likely play a role in this phenotype, and as such could be the focus of future studies (Buchanan et al. 2015; Taiz et al. 2015).

The importance of elucidating the CDVd dwarfing mechanism lies in the potential to develop commercial citrus dwarfing applications that do not require an infectious agent. Dwarfed trees are fundamental for successful high-density plantings that in the future will be critical for meeting challenges posed by water shortages, disease spread, farmland reduction, and mechanization of citrus horticulture to address increasing labor costs (Hutton et al. 2000; Platt 1973; Stover et al. 2008). Even though 'Flying Dragon' rootstock is proven to produce smaller citrus trees it is well known that nursery production, propagation and growth of such trees are truly challenging (Roose 1986). In addition, recent reports on the development of new rootstocks with some dwarfing properties (Bowman et al. 2016) will have to be evaluated in long-term field trials for tree size, yield, fruit quality and bud union health, under different growing conditions and with different rootstock-scion combinations. Such questions have been addressed in the case of CDVd (Semancik et al. 1997; Vernière et al. 2004; Vidalakis et al. 2011). Therefore, understanding viroid-induced citrus tree size reduction can currently provide a platform for innovation in citriculture and for the re-engineering of the citrus orchard.

Acknowledgments

References

Bitters WP, Cole DA, McCarthy CD. 1979. Facts about dwarf citrus trees. Citrograph. 64:54-56.

Bowman KD, Faulkner L, Kesinger M. 2016. New citrus rootstocks released by usda 2001–2010: Field performance and nursery characteristics. HortScience. 51(10):1208-1214.

Buchanan BB, Gruissem W, Jones RL. 2015. Biochemistry and molecular biology of plants. John Wiley & Sons.

Chambers GA, Donovan NJ, Bodaghi S, Jelinek SM, Vidalakis G. 2018. A novel citrus viroid found in australia, tentatively named citrus viroid vii. Arch Virol. 163(1):215-218.

Davies FS, Albrigo LG. 1994. Dwarfing rootstocks. Citrus. Wallingford, UK: CAB International. p. 100-1001.

Di Serio F, Flores R, Verhoeven JTJ, Li S-F, Pallás V, Randles J, Sano T, Vidalakis G, Owens R. 2014. Current status of viroid taxonomy. Arch Virol. 159(12):3467-3478.

Hutton RJ, Broadbent P, Bevington KB. 2000. Viroid dwarfing for high density citrus plantings. Hortic Rev. 24:277-317.

Li W, Hartung JS, Levy L. 2006. Quantitative real-time PCR for detection and identification of *Candidatus Liberibacter* species associated with citrus huanglongbing. J Microbiol Meth. 66(1):104-115.

Morse J, Robertson C. 1987. Calculating canopy area of citrus trees and surface area of fruits. The Florida Entomologist. 70(1):168-171.

Osman F, Dang T, Bodaghi S, Vidalakis G. 2017. One-step multiplex rt-qpcr detects three citrus viroids from different genera in a wide range of hosts. J Virol Methods. 245:40-52.

Osman F, Hodzic E, Kwon S-J, Wang J, Vidalakis G. 2015. Development and validation of a multiplex reverse transcription quantitative pcr (rt-qpcr) assay for the rapid detection of citrus tristeza virus, citrus psorosis virus, and citrus leaf blotch virus. J Virol Methods. 220:64-75.

Platt R. 1973. Planning and planting the orchard. . In: Reuther W, editor. Citrus industry. Riverside, CA: University of California, Division of Agricultural Sciences. p. 48–81.

Roistacher C, Calavan E, Blue R. 1969. Citrus exocortis virus-chemical inactivation on tools, tolerance to heat and separation of isolates. Plant Dis Rep. 53:333-336.

Roose ML. 1986. Dwarfing rootstocks for citrus. Proc Intl Soc Citrus Nurserymen.18.

Semancik J, Rakowski A, Bash J, Gumpf D. 1997. Application of selected viroids for dwarfing and enhancement of production of 'valencia'orange. J Hortic Sci. 72(4):563-570.

Serfontein C, Catling H. 1968. Determining the canopy area of citrus trees. South African Citrus Journal. 413:14-15.

Stover E, Castle WS, Spyke P. 2008. The citrus grove of the future and its implications for huanglongbing management. Proc Eta State Hort Soc. 121:155-159.

Taiz L, Zeiger E, Møller IM, Murphy A. 2015. Plant physiology and development. Sunderland, MA: Sinauer Associates, Oxford University Press.

Timmer LW, Garnsey SM, Graham JH. 2000. Graft-transmissible, systemic diseases. Compendium of citrus diseases. Second ed. St. Paul, Minnesota: APS press. p. 51-69.

Vernière C, Perrier X, Dubois C, Dubois A, Botella L, Chabrier C, Bové JM, Vila ND. 2004. Citrus viroids: Symptom expression and effect on vegetative growth and yield of clementine trees grafted on trifoliate orange. Plant Disease. 88(11):1189-1197.

Vidalakis G, Gumpf D, Bash J, Semancik J. 2004. "Finger imprint" of poncirus trifoliata: A specific interaction of a viroid, a host, and irrigation. Plant Disease. 88:709-713.

Vidalakis G, Pagliaccia D, Bash J, Afunian M, Semancik J. 2011. Citrus dwarfing viroid: Effects on tree size and scion performance specific to poncirus trifoliata rootstock for high-density planting. Ann Appl Biol. 158(2):204-217.

Vidalakis G, Wang J. 2013. Molecular method for universal detection of citrus viroids. U.S.A. Patent publication number 20130115591.

Vives MC, Velázquez K, Pina JA, MOreno P, Guerri J, Luis N. 2013. Identification of a New Enamovirus Associated with Citrus Vein Enation Disease by Deep Sequencing of Small RNAs. Phytopathology. 103 (10):1077-1086.

Wang J, Boubourakas I, Voloudakis A, Agorastou T, Magripis G, Rucker T, Kyriakopoulou P, Vidalakis G. 2013. Identification and characterization of known and novel viroid variants in the greek national citrus germplasm collection: Threats to the industry. Eur J Plant Pathol. 137(1):17-27.

Yokomi RK, Mello AF, Saponari M, Fletcher J. 2008. Polymerase chain reaction-based detection of spiroplasma citri associated with citrus stubborn disease. Plant Disease. 92(2):253-260.