

Research Article

Vector transmission of lime witches' broom Phytoplasma to Mexican lime seedlings under greenhouse condition

Chamran Hemmati^{1, 2*}, Majeed Askari Seyahooei³, Mehrnoosh Nikooei¹, Seyed Saeid Modarres Najafabadi³, Azadeh Goodarzi³, Mohsen Amiri Mazraie³ and Mohammad Mehdi Faghihi³

1. Department of Agriculture, Faculty of Agriculture and Natural Resources, University of Hormozgan, Bandar Abbas, Iran.

2. Plant Protection Research Group, University of Hormozgan, Bandar Abbas, Iran.

3. Plant Protection Research Department, Hormozgan Agricultural and Natural Resources Research and Education Center, Agricultural Research Education and Extension Organization (AREEO), Bandar Abbas, Iran.

Abstract: Despite successful lime witches'-broom (LWB) phytoplasma transmission by *Hishimonus phycitis* to the lime trees and Bakraee seedlings, there is no published document regarding LWB transmission by *H. phycitis* to lime seedlings. To study the possibility of vector-based transmission to lime seedlings, the feral leafhoppers were collected in LWB-infected lime orchards and caged on one-year old Mexican lime seedlings. Six months after inoculation, 50% of inoculated seedlings showed typical symptoms of LWB and were strongly positive in PCR assays. To our knowledge, this is the first report of transmission of *Ca. P. aurantifolia* to Mexican lime seedlings by *H. phycitis* under greenhouse condition.

Keywords: *Hishimonus phycitis*, Mexican lime seedlings, 16SrII phytoplasma, LWB disease

Introduction

Lime witches'-broom (LWD) disease associated with "*Candidatus Phytoplasma aurantifolia*", has been considered as a lethal disease of Mexican lime in southern Iran. The first report of LWD dates back to 1975 from Oman, causing substantial damage to the Mexican lime orchards (Bové *et al.*, 1988). Subsequently, the disease was observed in the United Arab Emirate in 1989 (Garnier *et al.*, 1991) and later in Iran in 1997 (Bové *et al.*, 2000). Within 19 years, LWB was spread throughout the four main Lime-producing provinces of southern Iran *i.e.*, Sistan-Baluchestan, Hormozgan, Kerman and Fars. The outbreak resulted in devastating 30% of the

Mexican lime trees in southern Iran (Mardi *et al.*, 2011). The disease primarily affects lime (*Citrus aurantifolia*), but in Iran, it is also found on Bakraee (Salehi *et al.*, 2007), Grapefruit (Bagheri *et al.*, 2010) and limequat (Faghihi *et al.*, 2017). The disease has been experimentally transmitted to lime trees by the leafhopper *Hishimonus phycitis* Distant (Hemiptera: Cicadellidae) and to lime seedlings by grafting (Bagheri *et al.*, 2009). The transmission of witches' broom phytoplasma to 15-20-year-old trees grown under semi-natural environment and covered with insect-proof net was successful (Bagheri *et al.*, 2009). However, the transmission of phytoplasma to lime seedlings by the vector had failed in greenhouse condition (Siampour *et al.*, 2006). In the natural environment, no specific symptoms have been recognized on lime or other citrus seedlings, suggesting that in the natural environment, transmission of the disease by vectors hardly

Handling Editor: Mohammad Mehrabadi

*Corresponding author, e-mail: chamran.hemmati@hormozgan.ac.ir
Received: 24 February 2019, Accepted: 17 December 2019
Published online: 18 January 2020

occurs. Based on the previous studies, although the disease had been transmitted to Bakraee seedlings under greenhouse condition, all efforts to transmit the disease by a vector to lime seedlings grown in greenhouse had failed. Taking the above facts into account and given the fact that lime is the main preferred host of this phytoplasma cultivated in Southern Iran, this study was carried out to re-examine the transmission of witches' broom phytoplasma to lime seedlings by *H. phycitis*.

Materials and Methods

To do so, ten one-year old Mexican lime seedlings were provided from "Minab Agricultural and Natural Resources Research and Education Station" and maintained in a net-greenhouse in Bandar Abbas, Hormozgan province, Iran. Before implementing the experiment, all seedlings were checked for phytoplasma infection through extracting the total DNA and performing PCR assays by universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) followed by R16F2n/R16R2 (Table 1) (Gundersen and Lee, 1996) as nested PCR. None of the lime seedlings showed phytoplasma infection.

Table 1 The prime names and sequences used in nested PCR for phytoplasma detection in insects, nursery and experimentally infected Mexican lime.

Primer name	Sequence (5'→3')
P1	AAGAGTTTGATCCTGGCTCAGGATT
P7	CGTCCTTCATCGGCTCTT
R16f2n	GAAACGACTGCTAAGACTGG
R16r2	TGACGGGCGGTGTGTACAAACCCCG

The leafhopper, *H. phycitis* were collected from LWB (+) and LWB (-) lime orchards of Roudan (LWB (+): N27°44'21"; E57°15'85", LWB (-): N27°45'28"; E57°16'78") using D-Vac aspirator in May 2017. Some insects were checked for phytoplasma infection before running the experiment. To do so, total DNA was extracted from the individual leafhoppers using a cetyltrimethyl-ammonium-bromide (CTAB) method in accordance with an adapted

protocol from Reineke *et al.* (1998). Insects collected from WBL (-) were tested for phytoplasma by nested PCR as described above. Transmission assay was conducted by releasing thirty individuals of 4th and 5th nymphal instars and adults per plants which were caged (containing 3-5 individuals in each cage) on different leaves of each seedling (Fig. 1). In addition, leafhoppers collected from LWB (-) orchard were caged and released on plants as negative control. Insects were allowed to complete one generation on the lime seedlings for 30 days. Seedlings were kept in insect-proof chamber and six months after implementing the assay, symptomatic and asymptomatic plants were tested for '*Ca. P. aurantifolia*' infection using PCR assays.



Figure 1 Cages placed on Mexican lime seedlings containing 3-5 individuals per cages.

Total DNA was extracted from both symptomless and symptomatic samples by using an adopted cetyltrimethylammonium bromide (CTAB) extraction procedure described by Sahu *et al.* (2012). A nested PCR was employed for the detection of phytoplasma using the universal primers P1/P7 followed by R16F2n/R16R2. PCR assays were performed as described by Hemmati *et al.*, (2018). A DNA template free and '*Ca. P. trifolii*' were used as negative and positive controls in all PCR tests,

respectively. Afterwards, the PCR products were sequenced bidirectionally using P1/P7 and R16F2n/R16R2 primers by Macrogen Sequencing Service (Republic of Korea). The representative nucleotide sequence of the LWB (+) seedlings and *H. phycitis* were deposited in the GenBank database (accession no: MG822750-2).

The sequences generated from the present study and reference phytoplasma strains' sequences retrieved from GeneBank, were used to construct phylogenetic tree by neighbor joining

method with 1000 replications for each bootstrap value using Mega 6.0 software version (Tamura et al., 2013). The *Acholeplasma laidlawii* was used as out group to root the tree.

Results and Discussion

Six months after releasing leafhoppers under leaf cages, 5 out of 10 inoculated seedlings showed typical symptoms of LWB, including witches' broom, general chlorosis, and little leaf (Fig. 2A, B, C).



Figure 2 Symptoms of witches' broom, little leaf and yellowing (A, B, C) in comparison with LWB (-) Mexican lime seedlings (D).

Symptomatic plants and positive control were strongly positive in PCR assays but no product was obtained from negative control (DNA template free) (Fig. 3).

Amplified P1/P7 and R16F2n/R16R2 primers PCR products from experimentally vector challenged Mexican lime seedlings and *H. phycitis* were sequenced bidirectional. BLAST analysis of the 16S rDNA sequences revealed that the phytoplasma associated with lime seedlings and *H. phycitis* shared 100% identity with 'Ca. P. aurantifolia'-related strain (Acc. No. KY412987). Three isolates of phytoplasma from lime seedlings and *H. phycitis* were identical. The phylogenetic tree was in accordance with BLAST analysis and the sequence from the present study was clustered in group 16SrII (Fig. 4).

Transmission of phytoplasma to Bakraee seedlings and Mexican lime trees were reported previously by Salehi *et al.* (2007)

and Bagheri *et al.* (2009). To our knowledge, this is the first report of greenhouse experimental transmission of *Ca. P. aurantifolia* to Mexican lime seedlings by *H. phycitis* which can be a serious alarm to the lime seedlings producers and new re-cultivated lime orchards of southern Iran. Recently Hassanzadeh *et al.* (2019) confirmed that there were some resistant cultivars to LWB in south Iran which could be the reason why LWB transmission assay on lime seedlings conducted by Siampour *et al.* (2006) failed. Since there are many lime genotypes with different susceptibility to the phytoplasma disease in southern Iran and re-planting of Mexican lime in southern Iran has already started, use of resistant or tolerant lime genotypes for re-cultivation programs is strongly suggested, and otherwise a new phytoplasma epidemic outbreak is not unexpected.

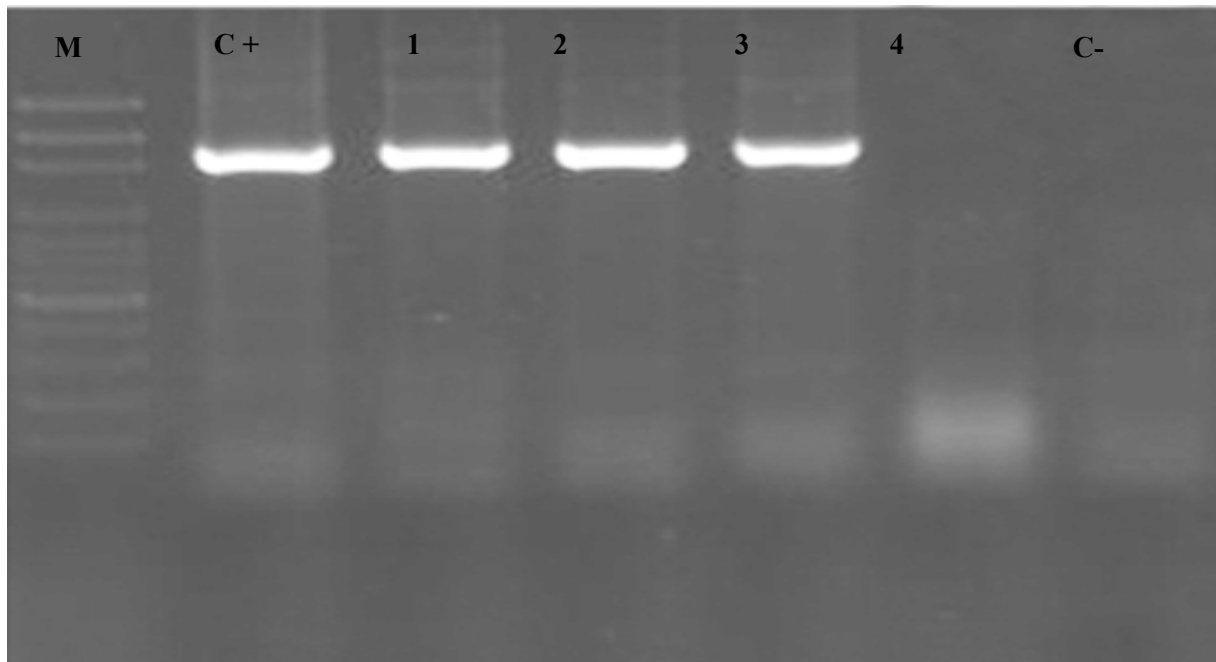


Figure 3 Nested-PCR results of experiment M: marker; C+: positive control; 1: infected insects collected from LWB (+) orchard; 2, 3: experimentally infected plants; 4: Nursery plant; C-: negative control (DNA template free)

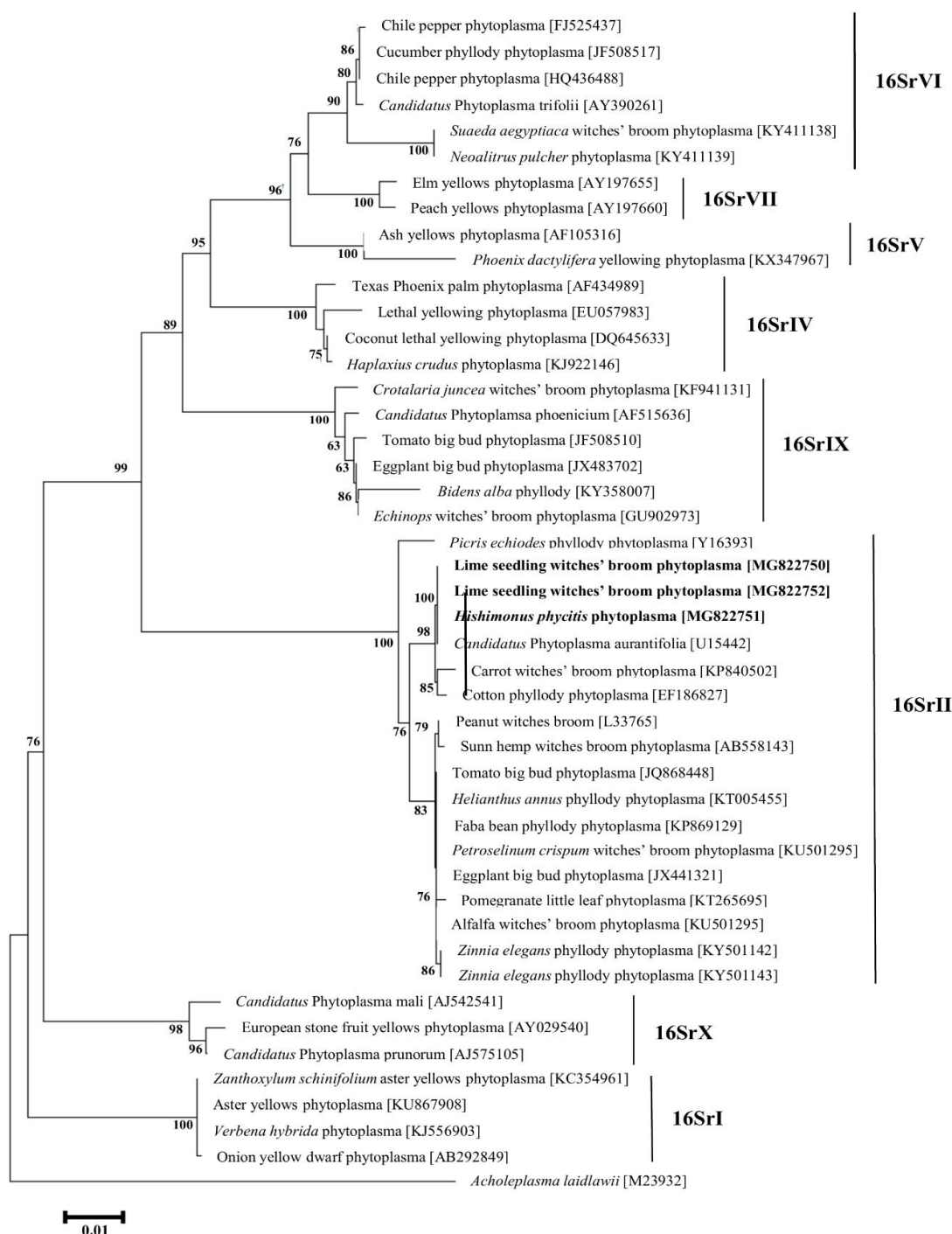


Figure 4 Phylogenetic tree of partial 16S rDNA gene sequence from Mexican lime seedlings witches' broom phytoplasma isolates (marked in bold) and selected phytoplasma reference sequences. GenBank accession numbers are shown in brackets, and 16Sr groups are annotated to the right. *Acholeplasma laidlawii* was used as outgroup to root the tree. The tree was constructed by the neighbor-joining method using MEGA 6 software. The bar indicates the number of nucleotides substitution per site. Bootstrap values are shown at nodes with greater than 50% support.

References

- Bagheri, A., Faghihi, M. M., Salehi, M., Samavi, S., Khanchezar, A. 2010. First report of natural infection of grapefruit trees to lime witches' broom phytoplasma. Proceedings of the 19th Iranian Plant Protection Congress, Shiraz, Iran, 409.
- Bagheri, A., Salehi, M., Faghihi, M. M., Samavi, S. and Sadeghi, A. 2009. Transmission of *Candidatus* Phytoplasma aurantifolia to Mexican lime by the leafhopper *Hishimonus phycitis* in Iran. Journal of Plant Pathology, 91 (4, Supplement): 105.
- Bové, J. M., Garnier, M., Mjeni, A. M., and Khayrallah, A. 1988. Witches' broom disease of small-fruited acid lime trees in Oman: First MLO disease of citrus. In: International Organization of Citrus Virologists Conference Proceedings, 10: 307-309.
- Bové, J. M., Danet, J. L., Bananej, K., Hsaanzadeh, N., Taghizadeh, M., Salehi, M., Garnier, M. 2000. Witches' Broom Disease of Lime (WBDL) in Iran. Proceedings of the 14th International Organization of Citrus Virologists Conference, IOCV, Riverside, CA, 207-212.
- Deng, S. and Hiruki, C. 1991. Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. Journal of Microbial Methods, 14 (1): 53-61.
- Faghihi, M. M., Bagheri, A., Askari Seyahoei, M., Pezhman, A. and Faraji, G. 2017. First report of a '*Candidatus* Phytoplasma aurantifolia'-related strain associated with witches'-broom disease of limequat in Iran. New Disease Reports, 35: 24-24.
- Garnier, M., Zreik, L. and Bové, J. M. 1991. Witches' broom, a lethal mycoplasmal disease of lime trees in the sultanate of Oman and the United Arab Emirates. Plant Disease 75 (6): 546-551.
- Gundersen, D. E. and Lee, I. M. 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathologia Mediterranea, 35 (3): 144-151.
- Hassanzadeh, H., Bahrami, H. R., Faghihi, M. M. and Bagheri, A. 2019. Reaction of some commercial citrus species and Iranian lime biotypes to witches' broom disease of lime. Crop Protection, 122: 23-29.
- Hemmati, C., Nikooei, M. and Pasalari, H. 2018. *Cota tinctoria* and *Orosius albicinctus*: A new plant host and potential insect vector of '*Candidatus* Phytoplasma trifolii'. Australasian Plant Disease Notes, 13 (1):1-5.
- Mardi, M., Khayam Nekouei, S., Farsad, L. K., Ehya, F., Shabani, M., Shafiee, M., Tabatabaei, M., Safarnejad, M. R., Salehi Jouzani, G. and Hosseini Salekdeh, G. 2011. Witches' broom disease of Mexican lime trees: disaster to be addressed before it will be too late. Bulletin of Insectology, 64 (2011): S205-S206.
- Reineke, A., Karlovsky, P. and Zebitz, C. P. W. 1998. Preparation and purification of DNA from insects for AFLP analysis. Insect Molecular Biology, 7 (1): 95-99.
- Sahu, S. K., Thangaraj, M. and Kathiresan, K. 2012. DNA extraction protocol for plants with high levels of secondary metabolites and polysaccharides without using liquid nitrogen and phenol. ISRN Molecular Biology, 2012. <http://dx.doi.org/10.5402/2012/205049>.
- Salehi, M., Izadpanah, K., Siampour, M., Bagheri, A. and Faghihi, S. M. 2007. Transmission of '*Candidatus* Phytoplasma aurantifolia' to Bakraee (*Citrus reticulata* hybrid) by feral *Hishimonus phycitis* leafhoppers in Iran. Plant Disease, 91 (4): 466-466.
- Schneider, B. 1995. Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasma. Mol. Diag. proc. Mycoplasmaology, 1: 369-380.
- Siampour, M., Izadpanah, K., Afsharifar, A. R., Salehi, M. and Taghizadeh, M. 2006. Detection of phytoplasma in insects collected in witches' broom affected lime groves. Iranian Journal of Plant Pathology, 42 (1): 139-158.
- Tamura, K., Stecher, G., Peterson, D., Filipiński, A. and Kumar, S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution, 30 (12): 2725-2729.

انتقال فیتوپلاسمای همراه بیماری جاروک لیموترش به دان نهال های لیمو توسط زنجرک *Hishimonus phycitis* در شرایط گلخانه

چمران همتی^{۱*}، مجید عسکری سیاهوئی^۲، مهرنوش نیکوئی^۱، سیدسعید مدرس نجف آبادی^۳، آزاده گودرزی^۳، محسن امیری مزرعی^۳ و محمد مهدی فقیهی^۳

۱- گروه کشاورزی، مجتمع آموزش عالی میناب، دانشگاه هرمزگان، بندرعباس، ایران.

۲- هسته پژوهشی گیاه پزشکی، دانشگاه هرمزگان، بندرعباس، ایران.

۳- بخش تحقیقات گیاه پزشکی، مرکز تحقیقات و آموزش کشاورزی و منابع طبیعی هرمزگان، بندرعباس، ایران.

پست الکترونیکی نویسنده مسئول مکاتبه: chamran.hemmati@hormozgan.ac.ir

دریافت: ۵ اسفند ۱۳۹۷؛ پذیرش: ۲۶ آذر ۱۳۹۸

چکیده: بیماری جاروک لیموترش که توسط فیتوپلاسمای '*Candidatus Phytoplasma aurantifolia*' ایجاد می شود به درختان لیموترش بالغ و دان نهال بکرانی توسط زنجرک *Hishimonus phycitis* و به دان نهال لیموترش توسط پیوند منتقل شده است، اما انتقال بیماری توسط ناقل حشره ای به دان نهال های لیموترش با شکست روبرو شده است. این پژوهش جهت آزمایش دوباره انتقال فیتوپلاسمای توسط زنجرک *H. phycitis* به دان نهال های لیموترش صورت گرفت. تعداد ده دان نهال یک ساله از شهرستان میناب تهیه و قبل از انجام آزمایش با استفاده از تکنیک nested-PCR و با استفاده از پرایمرهای اختصاصی از لحاظ آلودگی به فیتوپلاسمای تست شدند. پوره و افراد بالغ زنجرک از باغ های آلوده به جاروک لیموترش توسط دستگاه D-vac از منطقه رودان (استان هرمزگان) جمع آوری شد و آلودگی برخی از آن ها به فیتوپلاسمای با PCR تست شد. از بین نمونه های تست شده، حدود ۹۰ درصد از زنجرک ها به فیتوپلاسمای آلوده بودند. دیگر زنجرک های جمع آوری شده روی دان نهال های سالم در داخل قفس پلاستیکی (هر درخت ۵ قفس و هر قفس ۵ حشره) قرار گرفتند و گیاهان در گلخانه مرکز تحقیقات کشاورزی و منابع طبیعی هرمزگان قرار داده شدند. به علاوه، ۱۰۰ زنجرک از باغ های سالم شهرستان رودان جمع آوری و روی دان نهال های سالم به همان روش قبل قرار گرفتند و به عنوان تیمار شاهد در نظر گرفته شدند. شش ماه بعد از رهاسازی زنجرک ها، پنج دان نهال علائم بیماری جاروک شامل کوتاه شدن فاصله برگ ها، ریزبریگی و زردی را نشان داد و علائمی روی دان نهال ها در تیمار شاهد مشاهده نشد. نتایج PCR نیز بیماری گیاهان را تأیید کرد اما هیچ باندی در تیمار شاهد مشاهده نشد. انتقال فیتوپلاسمای همراه بیماری جاروک به دان نهال های لیموترش توسط حشره برای اولین بار گزارش می شود که می تواند یک زنگ خطر جدی برای تولیدکنندگان گیاهان لیموترش و باغ های تازه کشت شده در جنوب ایران باشد.

واژگان کلیدی: زنجرک ناقل، دان نهال لیموترش، فیتوپلاسمای گروه دو، بیماری جاروک لیموترش