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Research Article

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

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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 limequate (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

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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 (+): N27º44'21"; E57º15'85", LWB (-): N27º45'28"; E57º16'78") 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,

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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).

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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.

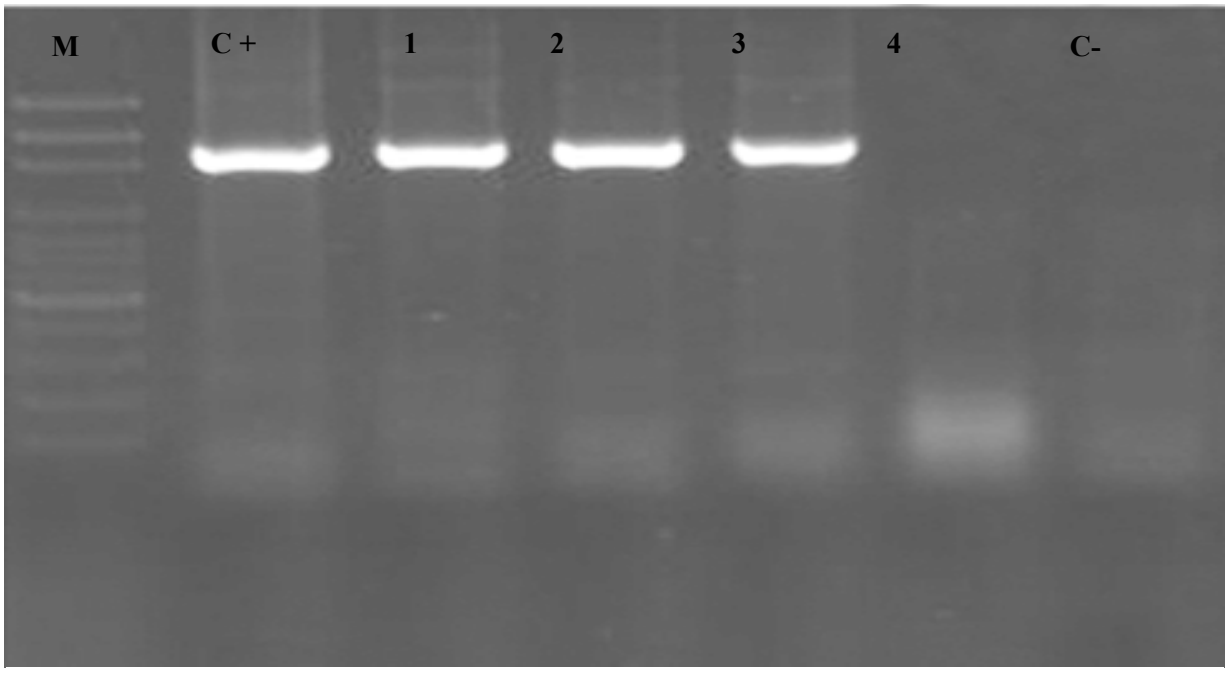

Figure 3 Nested-PCR results of experiment M: marker; C+: positive control; 1: infected insects collected from LWB (+) orchard; 2, 3: experimentally infected plants; 4: Nursery plant; C-: negative control (DNA template free)

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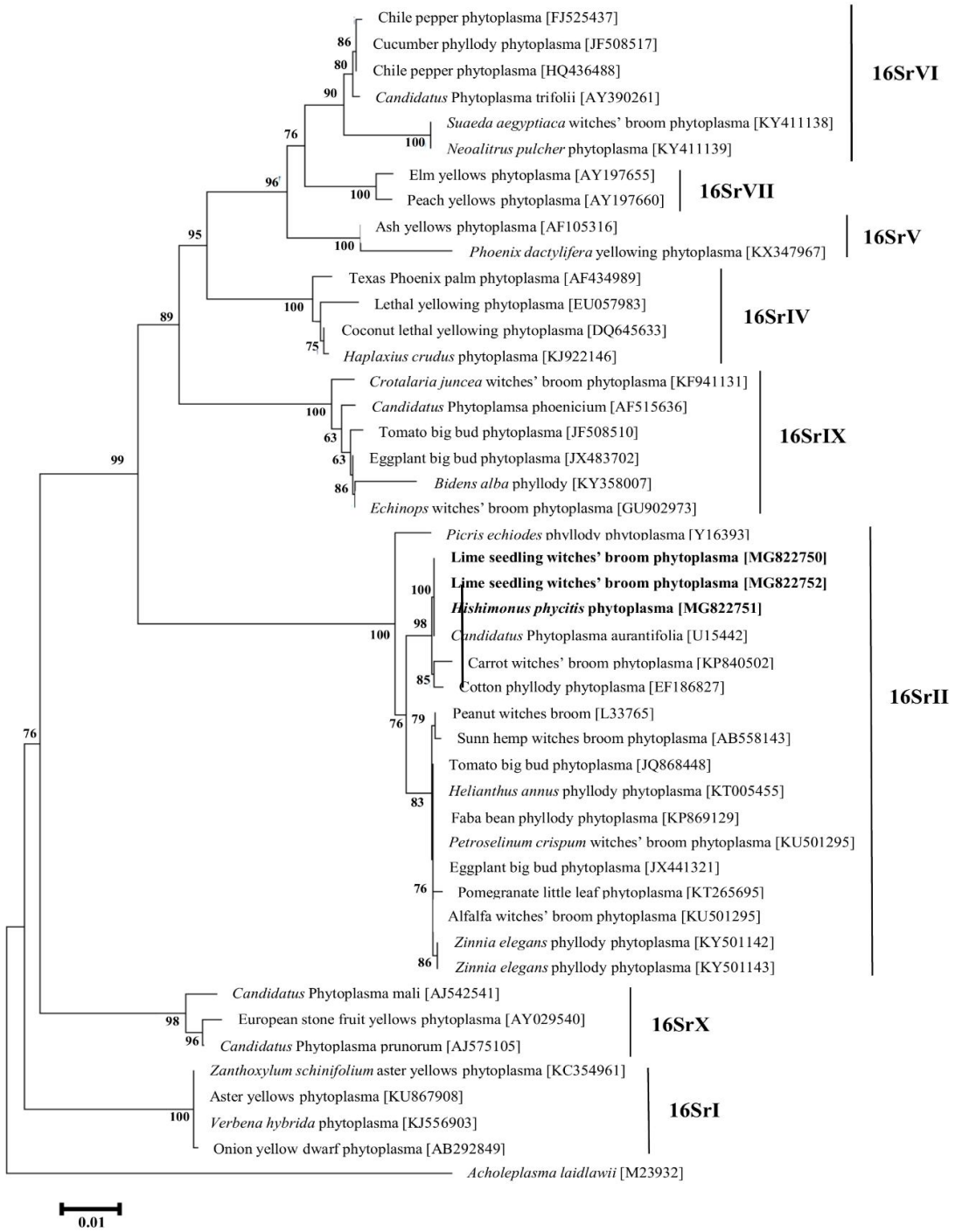

Figure 4 Phylogenetic tree of partial 16S rDNA gene sequence from Mexican lime seedlings witches' broom phytoplasma isolates (marked in bold) and selected phytoplasma reference sequences. GenBank accession numbers are shown in brackets, and 16Sr groups are annotated to the right. *Acholeplasma laidlawii* 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.

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

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چکیده: بیماری جاروک لیموترش که توسط فیتوپلاسمای '*Candidatus* Phytoplasma aurantifolia' ایجاد می‌شود به درختان لیموترش بالغ و دان‌نهال بکرائی توسط زنجرک *Hishimonus phycitis* و به دان‌نهال لیموترش توسط پیوند منتقل شده است، اما انتقال بیماری توسط ناقل حشره‌ای به دان‌نهال‌های لیموترش با شکست روبرو شده است. این پژوهش جهت آزمایش دوباره انتقال فیتوپلاسما توسط زنجرک *H. phycitis* به دان‌نهال‌های لیموترش صورت گرفت. تعداد ده دان‌نهال یک‌ساله 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 فیتوپلاسما تست شدند. پوره و افراد بالغ زنجرک از باغ‌های آلوده به جاروک لیموترش توسط دستگاه D-vac از منطقه رودان (استان هرمزگان) جمع‌آوری شد و آلودگی برخی از آن‌ها به فیتوپلاسما با PCR تست شد. از بین نمونه‌های تست شده، حدود ۹۰ درصد از زنجرک‌ها به فیتوپلاسما آلوده بودند. دیگر زنجرک‌های جمع‌آوری شده روی دان‌نهال‌های سالم در داخل قفس پلاستیکی (هر درخت ۵ قفس و هر قفس ۵ حشره) قرار گرفتند و گیاهان درگلخانه مرکز تحقیقات کشاورزی و منابع طبیعی هرمزگان قرار داده شدند. به‌علاوه، ۱۰۰ زنجرک از باغ‌های سالم شهرستان رودان جمع‌آوری و روی دان‌نهال‌های سالم به‌همان روش قبل قرار گرفتند و به‌عنوان تیمار شاهد درنظر گرفته شدند. شش ماه بعد از رهاسازی زنجرک‌ها، پنج دان‌نهال علائم بیماری جاروک شامل کوتاه شدن فاصله برگ‌ها، ریزبرگی و زردی را نشان داد و علائمی روی دان‌نهال‌ها 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 دان‌نهال‌های لیموترش توسط حشره برای اولین بار گزارش می‌شود که می‌تواند یک زنگ خطر جدی برای تولیدکنندگان گیاهان لیموترش و باغ‌های تازه کشت شده در جنوب ایران باشد.

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

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