Shahid Bahonar University of Kerman
Journal of Advances in Plant Protection
JAPP 2023
Association of Iranian Plant Protection Societies

# Reaction of some commercial citrus genotypes to *Candidatus* Liberibacter asiaticus

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| Article Info. | Abstract |
|---|---|

**Article type:**
Original article

**Article history:**
Received 29 Apr. 2026
Received in revised form 15 Jun. 2026
Accepted 23 Jun. 2026
Available Online 30 Jun. 2026

**Keywords**:
Citrus cultivars,
Disease reaction,
Disease severity,
Huanglongbing.

Citrus huanglongbing (HLB), caused by *Candidatus* Liberibacter spp., is the most destructive bacterial disease affecting citrus production worldwide. In this study, the response of three-year-old seedlings of 10 commercial citrus cultivars and rootstocks to the Asian form of the HLB bacterium (*Ca*. L. asiaticus) (CLas) was investigated through graft inoculation. Seedlings' reactions to the HLB bacterium were evaluated based on PCR detection of CLas after grafting, disease incubation, and disease severity. Based on the timing of positive PCR detection of CLas and the appearance of disease symptoms, Persian lime and Volkamer lemon were ranked at the highest level of tolerance (lowest susceptibility), and Rough lemon was at the next level (intermediate susceptibility). Washington navel orange, Blood orange, Cleopatra mandarin, Clementine mandarin, Bakraei, Alemow, and Rangpur lime were classified as susceptible to the pathogen. In conclusion, all citrus genotypes studied were infected by HLB. However, symptoms varied by cultivar, and symptoms were less severe in Persian limes and Volkamer lemons.

**Cite this article:** Safarpour Kapourchali, S. Faghihi, M. M., Ebadi, N. S., Alizadeh Aliabadi, A., & Hasanzadeh Khankahdani, H. (2026). Reaction of some commercial citrus genotypes to *Candidatus* Liberibacter asiaticus. *Journal of Advances in Plant Protection*, *3*(1), 91–102.


**Publisher**: Shahid Bahonar University of Kerman

## Introduction

Huanglongbing (HLB), previously known as citrus greening, is the most devastating disease of citrus worldwide which is associated with three phloem limited Gram-negative α-proteobacteria, '*Candidatus* Liberibacter asiaticus' (CLas), '*Ca*. L. africanus' (CLaf), and '*Ca*. L. americanus' (CLam) (Bove, 2006). Both CLas and CLam are naturally transmitted through the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Sternorrhyncha: Liviidae), while CLaf is transmitted by the African citrus psyllid *Trioza erytreae* Del Guercio (Sternorrhyncha: Triozidae). HLB-infected trees show different symptoms including blotchy mottle and corky vein in leaves, defoliation, yellowing and die back as well as small misshapen fruits, with color inversion (Bove, 2006).
The Asian form of HLB has been reported across major citrus-producing regions in southern Iran, including the provinces of Sistan-Baluchistan, Kerman, Hormozgan, and Fars. The presence of CLas in symptomatic trees has been consistently confirmed using DNA-based diagnostic methods (Alizadeh et al., 2010; Faghihi et al., 2009; Mohkami et al., 2011; Salehi et al., 2012; Salehi

& Rasoulpour, 2016). Furthermore, HLB has been identified as a primary contributor to citrus decline in specific regions, most notably affecting mandarin orchards in the Siyahoo district of Hormozgan and sweet orange orchards in Kerman province (Alizadeh et al., 2017; Faghihi, 2018; Faghihi et al., 2026; Najafinia & Azadvar, 2016; Passera et al., 2018).

Different strategies have been suggested in many citrus-producing countries with various results that have been indicated to reduce the development of HLB and help maintain tree productivity under field conditions. These measures include the use of insecticides and biological control to control psyllid vectors, improved foliar nutritional programs, the application of inorganic phosphorus (P) solution to the leaves, and the use of antibiotics like ampicillin, oxytetracycline, and streptomycin. Additionally, soil conditioners, plant defense inducers or activators, different graft/rootstock combinations, and thermotherapy may decrease the effects of HLB (Bassanezi et al., 2020).

The development of citrus cultivars resistant to HLB is the most effective long-term control strategy for this disease. Several studies have investigated the influence of rootstocks on the performance of citrus trees under HLB pressure. Previous Research indicated that while HLB was successfully transmitted to 'Mosambi' scions grafted onto Lisbon lemon, Bengal citron, Carrizo citrange, Troyer citrange, and trifoliate orange, these specific combinations exhibited only mild disease symptoms. Therefore, application of tolerant rootstocks was suggested as a part of an integrated control program (Bassanezi et al., 2020). Hajivand et al. (2011) stated that no HLB symptoms were observed in the mandarin scions when grafted on *Citrus grandis* rootstock with *Citrus hystrix* as an interstock. The high vigor-inducing rootstocks, particularly Volker lemon, may enable younger infected trees to survive longer than others (Albrecht et al., 2014). Citrus cultivars and hybrids have variable reactions to HLB bacterium. In general, orange (*Citrus sinensis*), tangerine (*C. reticulata*) and their hybrids are very sensitive, grapefruit (*C. paradisi*) and sour orange (*C. aurantium*) are semi-sensitive, and *Poncirus trifoliata*, Persian lime (*Citrus latifolia*), Eureka lemon (*Citrus limon*) and Mexican lime are tolerant to semi-tolerant to HLB disease (Chika et al., 2013; da Graça 1991; 2008; Deng et al. 2007; Folimonova et al., 2009). Some citrus species, such as *Citrus indica* and *Citrus macroptera,* did not develop disease symptoms under high inbreeding pressure and may be resistant to the disease (Bhagabati, 1993).

Safarpour et al. (2022) reported that Mexican lime and Lisbon lemon were the most tolerant hosts to HLB, while Valencia orange and Orlando tangelo mandarin were the most susceptible.

In Taiwan, the pomelo (*Citrus grandis*) cultivar was previously resistant to HLB, but finally became infected and showed HLB symptoms about 30 years after HLB first appeared (Huang & Chang, 1980). Moreover, kumquat (*Furtunella margarita*) eventually became infected with HLB disease, exhibiting mottling and yellowing symptoms (Tsai et al., 2006).

The identification and development of HLB-resistant or HLB-tolerant citrus cultivars are considered among the most effective long-term approaches for managing huanglongbing. The aim of this study was to investigate the reaction of three-year-old seedlings of some commercial citrus cultivars and rootstocks in Iran to the causal agent of the Asian form of HLB bacterium (*Ca*. L. asiaticus) through grafting infection.

## Materials and Methods

### Plant material and graft inoculation

Experiments were conducted from September 2020 to September 2022 in a greenhouse where temperatures did not exceed 32°C. A number of commercial varieties of citrus including Washington navel, sweet orange (*C. sinensis*), Blood sweet orange (*C. sinensis*), Clementine mandarin (*C. reticulata*) (on Mexican lime rootstock), Cleopatra mandarin (*C. reshni*), Bakraei (*Citrus* sp.), Rangpur lime (*C. limonia*), Rough lemon (*C. jambhiri*), Macrophylla (Alemow) (*C. macrophylla*), Volkamer lemon (*C. volkameriana*) and Persian lime (*C. latifolia*) were prepared and six seedlings from each were kept under controlled conditions. Prior to graft-inoculation, all studied plants analyzed by polymerase chain reaction (PCR) with the primers A2/J5 (Hocquellet et al., 1999) for '*Ca*. Liberibacter asiaticus/africanus'. The sources of inoculum were symptomatic branches of three-year-old Valencia sweet orange seedlings which had previously been infected by CLas through graft-inoculation. Initial source of the infected grafts were mandarin trees severely affected by the Asian form of the HLB disease in an orchard in Siyahoo region (Hormozgan province, Iran).

To evaluate citrus reactions to CLas infection, bark plus wood (budstick) in similar sizes (about 4 cm × 1 cm) were taken from symptomatic branches and used as sources of inoculum. Two budsticks were grafted onto the stem of each plant in two places on the middle part of the seedlings with 10 cm distance. The grafting tissues were firmly fixed by wrapping them with transparent plastic strips for a month. Furthermore, to maintain moisture and promote graft growth, the graft-inoculated part of the plants was kept in a plastic bag for about two weeks. Three seedlings for each genotype were grafted with healthy (HLB-free) scions as negative control. All plants were kept in an insect-free greenhouse at temperatures ranging from 32°C and 14°C (day and night, respectively) and around 12 to 14 hours of natural light (Lopes et al., 2008).

## PCR detection of CLas and disease incubation period

The inoculated plants were inspected regularly for symptom expression of the disease and transmission success of CLas examined at two months intervals up to two years by conventional PCR and nested-PCR analysis of total DNA extracted from leaf samples. Based on the symptom appearance and the incubation period of the disease as well as PCR results, the response of each genotype was evaluated to HLB disease.

The incubation periods were evaluated based on the mean number of days between grafting and the initial PCR detection of CLas in the inoculated seedlings. A completely random design (CRD) with three replications (for each genotype) was used and data analysis was done by analysis of variance (ANOVA).

## Disease severity measurement

Disease severity was evaluated based on symptoms of HLB on the graft-inoculated seedlings according to the rating scale proposed by Hajivand et al. (2010) with brief modifications. Based on the leaf symptoms, the scale included: 0 = No symptom (Resistance), 1 = Mild (mottling and interveinal chlorosis symptoms), 2 = Moderate (Yellowing and/or Fe and Zn deficiencies like symptoms), 3= Severe (defoliation and twigs dieback symptoms)

Disease severity = $\Sigma (a \times b) / N \times Z$

where $\Sigma (a \times b)$ = sum of the symptomatic plant and their corresponding rating, N = total number of sampled plants, and Z = highest rating.

## DNA extraction and PCR assay

Genomic DNA extraction was performed based on the procedure established by Hung et al. (1999), with minor modifications. In order to detection of CLas in the plant samples, the conventional PCR using the specific primers OI1/OI2c (Jagoueixet al., 1996) and a Nested-PCR using OI1/OI2c primer pair (ca. 1160 bp band) in the first round and CGO5R/CGO3F (Zhou et al., 2007) primer pair (ca. 800 bp band) in the second round were performed (Table 1). For both primer pairs, PCR was conducted in 35 cycles of 30 s at 94 °C (4 min at 95 °C for the initial denaturation), 40 s at 55 °C (60 °C for A2/J5 primer pair) (Hocquellet et al., 1999), and 80 s (1 min for A2/J5 primer pair) at 72 °C, and a final extension cycle of 10 min at 72 °C. The PCR reaction mixtures contained 10 µl of PCR Master Mix (Amplicon, Denmark), 1 µl of each primer (10 Mm), and 2 µl of total nucleic acid preparations (approximately 100 ng). One microliter of diluted (1:10) PCR products from the first amplification was used as the template in the second round of PCR. DNA from healthy plants and sterile distilled water were used as the negative controls. Electrophoresis of both conventional PCR and nested PCR amplified fragments was performed using 8 µl of amplified fragments loaded on 1% agarose gel containing 2% safe stain (Sinnaclon, Iran). Afterward, the banding patterns of amplified fragments were observed using UV transilluminator. Then, based on the presence or absence of the expected band, positive and negative reactions were recorded in each experiment.

**Table 1.** Characteristics of primers used in conventional and nested PCR tests to detect *Candidatus* Liberibacter asiaticus.

| Primer name | Sequence | Annealing (°C) | Amplicon size (bp) | Target | Reference |
|---|---|---|---|---|---|
| OI1 | 5′-GCGCGTATGCAATACGAGCGGCA-3′ | 56 | 1160 | 16S rRNA | Jagoueix et al., 1996 |
| OI2c | 5′-GCCTCGCGACTTCGCAACCCAT-3′ | | | | |
| CGO3F | 5′- RGGGAAAGATTTTATTGGAG-3′ | 56 | 800 (nested-PCR) | 16S rRNA | Zhou et al., 2007 |
| CGO5R | 5′-GAAAATAYCATCTCTGATATCGT-3′ | | | | |
| A2 | 5′- TATAAAGGTTGACCTTTCGAGTTT-3′ | 60 | 669 (CLaf) 703 (CLas) | β-operon | Hocquellet et al., 1999 |
| J5 | 5′- ACAAAAGCAGAAATAGCACGAACAA-3′ | | | | |

## Results

## Symptomatology and incubation period

The inoculated plants were monitored in the greenhouse for two years, and different spectrums of disease symptoms were observed at various times post inoculation. These symptoms included mottling in the leaves, yellowing between the veins, symptoms similar to iron and zinc deficiency, yellow vein, and general yellowing of the plant (Fig. 1).

No symptoms were observed in any of the commercial species and rootstocks of citrus until six months post inoculation (6 mpi). About 12 mpi, the symptoms of the disease including mild mottling and yellowing between the veins in the upper leaves of the plant were observed in different cultivars of oranges and mandarins, Bakraei, Rangpur lime, and Alemow. However, no symptoms were observed in the seedlings of Rough lemon, Volkamer lemon, and Persian lime 12 mpi. The symptoms of mild mottling and yellowing between the veins was observed during 16 to 19 mpi in Rough lemon, Volkamer lemon, and Persian lime seedlings (Table 2).

The incubation period of HLB disease was varied among the citrus genotypes. Comparative analysis of the average incubation periods among genotypes revealed the longest incubation periods in Volkamer lemon, Rough lemon, and Persian lime, which differed significantly from the other genotypes evaluated ($P < 0.05$). In contrast, no significant differences ($P < 0.05$) were observed in the incubation periods among Washington navel orange, Blood orange, Clementine mandarin, Cleopatra mandarin, Bakraei, Rangpur lime, and Alemow (Tables 3 and 4).


**Fig. 1.** Symptoms of yellowing and Fe deficiency in Bakraei (A), and Persian lime (B) 12 and 18 months after graft inoculation with *Candidatus* Liberibacter asiaticus, respectively, in greenhouse conditions.

**Table 2.** Symptoms of HLB disease in the graft inoculated hosts at intervals of three months up to two years.

| **Citrus genotypes** | **Symptoms * (months after inoculation)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 3 | 6 | 9 | 12 | 15 | 18 | 21 | 24 |
| Washington navel sweet orange (*Citrus sinensis*) | NS | NS | NS | IC, MM | M, Fe/Zn Df | IC, M, Fe/Zn Df | M, Y, Fe/Zn Df | M, Y, Fe/Zn Df |
| Blood sweet orange (*Citrus sinensis*) | NS | NS | NS | IC, MM | M, Fe/Zn Df | IC, M, Fe/Zn Df | M, Y, Fe/Zn Df | M, Y, Fe/Zn Df |
| Clementine mandarin (*Citrus reticulata*) | NS | NS | NS | MM (upper leaves) | IC, M | IC, M, Fe/Zn Df | IC, M, Y, Fe/Zn Df | IC, M, Y, Fe/Zn Df |
| Cleopatra mandarin (*Citrus reshni*) | NS | NS | NS | MM (upper leaves) | IC, M | IC, M, Fe/Zn Df | IC, M, Y, Fe/Zn Df | IC, M, Y, Fe/Zn Df |
| Bakraei (*Citrus* spp.) | NS | NS | NS | IC, MM (usually upper leaves) | IC, M (usually upper leaves), Fe/Zn Df | IC, M, Fe/Zn Df | IC, M, Fe/Zn Df | IC, M, Fe/Zn Df |
| Rangpur lime (*Citrus limonia*) | NS | NS | NS | MM (upper leaves) | IC, M | IC, M, PY | IC, M, Y, Fe/Zn Df | IC, M, Y, Fe/Zn Df |
| Persian lime (*Citrus latifolia*) | NS | NS | NS | NS | NS | IC, MM | IC, M, Fe/Zn Df | IC, M, PY, Fe/Zn Df |
| Macrophylla (Alemow) (*Citrus macrophylla*) | NS | NS | NS | MM (upper leaves) | IC, M | IC, M, PY | IC, M, Y, Fe/Zn Df | IC, M, Y, Fe/Zn Df |
| Rough lemon (*Citrus jambhiri*) | NS | NS | NS | NS | NS | IC, Fe/Zn Df | IC, M, Fe/Zn Df | IC, M, PY, Fe/Zn Df |
| Volkamer lemon (*Citrus volkameriana*) | NS | NS | NS | NS | NS | IC, Fe/Zn Df | IC, M, Fe/Zn Df | IC, M, PY, Fe/Zn Df |

∗ These symptoms were observed in at least one of the three replicates inoculated for each genotype.

No symptom (NS); Mild mottling (MM); Mottling (M); Interveinal chlorosis (IC); Fe and Zn deficiencies like symptoms (Fe/Zn Df); Yellowing in a part of the plants (PY); General yellowing (Y); Defoliation and die back (DD).

**Table 3.** Analysis of variance related to the effect of citrus genotypes on incubation period of HLB disease.

| Source of variation | df | Sums of squares | Mean of Square | F statistic | C.V% |
|---|---|---|---|---|---|
| Genotype | 9 | 195.4 | 21.7 | 24.1** | 7.2 |
| Error | 20 | 18.0 | 0.9 | - | |
| Total | 29 | 213.4 | - | - | |

**Significant at 1% ($P < 0.01$)

**Table 4.** Mean comparison of the incubation period of HLB disease in citrus genotypes.

| Citrus genotypes | Incubation period (month) |
|---|---|
| Washington navel sweet orange | 11.3b ± 0.67 |
| Blood sweet orange | 11.3b ± 0.33 |
| Clementine mandarin | 12.0b ± 0.58 |
| Cleopatra mandarin | 11.7b ± 0.67 |
| Bakraei | 11.3b ± 0.33 |
| Rangpur lime | 11.7b ± 0.33 |
| Rough lemon | 16.7a ± 0.67 |
| Alemow | 11.7b ± 0.67 |
| Volkamer lemon | 17.3a ± 0.67 |
| Persian lime | 17.3a ± 0.33 |
| LSD | 1.616 |

Means with at least one similar letter had no significant difference based on LSD at 5% of probability level.

## PCR detection of *Ca*. L. asiaticus in the genotypes

Statistical analysis of the interval between graft-inoculation and the first positive CLas detection revealed significant differences among several citrus genotypes (Table 5). The results of direct and nested-PCR detection of *Ca*. L. asiaticus in the graft-inoculated plants at 60-day intervals. As expected, nested-PCR demonstrated higher sensitivity for detecting CLas in seedlings compared to direct-PCR, enabling earlier detection (Table 6). Based on the combined mean of direct and nested-PCR results, CLas was detected earliest in Bakraei 210 days post inoculation (dpi) and Washington navel sweet orange (220 dpi). Conversely, the latest detections occurred in Persian lime (330 dpi) and Volkamer lemon (300 dpi); however, no significant difference was observed between these two genotypes regarding the time to positive PCR detection (Table 6).

Based on the mean interval between graft-inoculation and positive PCR detection of CLas, Persian lime and Volkamer lemon exhibited the greatest resistance to bacterial multiplication, as evidenced by their delayed detection times. These genotypes also demonstrated the longest incubation periods and the least severe symptomatic expression of HLB. Consequently, these cultivars are categorized as the least susceptible among the studied citrus varieties. Rough lemon' displayed an intermediate level of sensitivity, classified as semi-sensitive. In contrast, Washington navel orange, Bakraei, Cleopatra mandarin, Clementine mandarin, Blood orange, Rangpur lime, and Alemow were identified as the most susceptible to CLas infection.

**Table 5.** Analysis of variance of the individual and interaction effects of PCR method and citrus genotype on detection of *Candidatus* Liberibacter asiaticus.

| Coefficient of variation | F statistic | Mean of Square | Sums of squares | df | C.V% |
|---|---|---|---|---|---|
| PCR method | 1 | 50460.0 | 50460.0 | 42.0** | 13.7 |
| Genotype | 9 | 84060.0 | 9340.0 | 7.78** | |
| PCR method × Genotype | 9 | 5340.0 | 593.3 | 0.49ns | |
| Experimental Error | 40 | 48000.0 | 1200.0 | | |
| Total | 59 | 187860.0 | - | | |

** and ns: Significant and non-significant at 1%, respectively.

**Table 6.** Mean comparison of the number of days between grafting and HLB diagnosis times in different citrus cultivars.

| Genotype | First positive PCR detection of CLas (day post inoculation) | | |
|---|---|---|---|
| | Direct-PCR | Nested-PCR | Mean (Host) |
| Washington navel sweet orange | $^{\Psi}240^{cd} \pm 35$ | $200^{de} \pm 20$ | $^{\dagger}220^{B} \pm 20$ |
| Blood sweet orange | $280^{bc} \pm 20$ | $200^{de} \pm 20$ | $240^{B} \pm 22$ |
| Clementine mandarin | $260^{c} \pm 20$ | $200^{de} \pm 20$ | $230^{B} \pm 18$ |
| Cleopatra mandarin | $280^{bc} \pm 20$ | $200^{de} \pm 20$ | $240^{B} \pm 22$ |
| Bakraei | $240^{cd} \pm 0$ | $180^{e} \pm 0$ | $210^{B} \pm 13$ |
| Rangpur lime | $280^{bc} \pm 20$ | $200^{de} \pm 20$ | $240^{B} \pm 22$ |
| Rough lemon | $320^{ab} \pm 20$ | $260^{c} \pm 20$ | $290^{A} \pm 18$ |
| Alemow | $260^{c} \pm 20$ | $200^{de} \pm 20$ | $230^{B} \pm 18$ |
| Volkamer lemon | $320^{ab} \pm 20$ | $280^{bc} \pm 20$ | $300^{A} \pm 16$ |
| Persian lime | $340^{a} \pm 20$ | $320^{ab} \pm 20$ | $330^{A} \pm 13$ |
| **PCR method mean** | $^{\dagger}\mathbf{282^{A} \pm 8}$ | $\mathbf{224^{B} \pm 10}$ | |

†Means having capital in the right column and below row with same letters are not significantly difference according to LSD test ($P < 0.05$).
Ψ Means having small letter in middle columns with same letters are not significantly difference according to LSD test ($P < 0.05$).

## Disease severity

Disease severity was evaluated among different citrus hosts at 12, 18, and 24 months post-inoculation with CLas (Table 7). At 12 months, Rough lemon, Volkamer lemon, and Persian lime remained asymptomatic, whereas mild symptoms were detected in all other hosts (Table 8). The disease severity in the hosts increased over time and the most disease severity for each genotype was observed at 24 months post inoculation. After 24 months of inoculation, the lowest disease severity (0.44; moderate symptoms) was recorded in Volkamer lemon, and Persian lime.

**Table 7.** Analysis of variance in relation to Disease severity progress of HLB in different citrus cultivars.

| Source of variation | df | Sums of squares | Mean of Square | F statistic | Prob. |
|---|---|---|---|---|---|
| Time | 2 | 3.251 | 1.626 | 219.5** | <0.0001 |
| Cultivar | 9 | 0.316 | 0.035 | 4.74** | <0.0001 |
| Time × Cultivar | 18 | 0.180 | 0.010 | 1.35ns | 0.1904 |
| Error | 60 | 0.444 | 0.007 | - | - |
| Total | 89 | 4.191 | - | - | - |

$^{ns, *, **}$ Not Significant, Significant at $P < 0.05$ and $P < 0.01$, respectively.

**Table 8.** Disease severity progress of HLB in different citrus cultivars.

| Genotype | Disease severity | | | Mean of Genotype |
|---|---|---|---|---|
| | 12 months | 18 months | 24 months | |
| Washington navel sweet orange | $^{\Psi}0.2222^{\mathbf{de}} \pm 0.0641$ | $0.4444^{\mathbf{bc}} \pm 0.0641$ | $0.5555^{\mathbf{ab}} \pm 0.0641$ | $^{\dagger}0.4074^{\mathbf{AB}} \pm 0.0586$ |
| Blood sweet orange | $0.2222^{\mathbf{de}} \pm 0.0641$ | $0.4444^{\mathbf{bc}} \pm 0.0641$ | $0.6666^{\mathbf{a}} \pm 0.0000$ | $0.4444^{\mathbf{A}} \pm 0.0693$ |
| Clementine mandarin | $0.2222^{\mathbf{de}} \pm 0.0641$ | $0.4444^{\mathbf{bc}} \pm 0.0641$ | $0.6666^{\mathbf{a}} \pm 0.0641$ | $0.4444^{\mathbf{A}} \pm 0.0717$ |
| Cleopatra mandarin | $0.1111^{\mathbf{ef}} \pm 0.0000$ | $0.4444^{\mathbf{bc}} \pm 0.0641$ | $0.6666^{\mathbf{a}} \pm 0.0000$ | $0.4074^{\mathbf{AB}} \pm 0.0828$ |
| Bakraei | $0.2222^{\mathbf{de}} \pm 0.0641$ | $0.4444^{\mathbf{bc}} \pm 0.0641$ | $0.5555^{\mathbf{ab}} \pm 0.0000$ | $0.4074^{\mathbf{AB}} \pm 0.0556$ |
| Rangpur lime | $0.1111^{\mathbf{ef}} \pm 0.0000$ | $0.4444^{\mathbf{bc}} \pm 0.0641$ | $0.5555^{\mathbf{ab}} \pm 0.0641$ | $0.3703^{\mathbf{ABC}} \pm 0.0717$ |
| Rough lemon | $0.0000^{\mathbf{f}} \pm 0.0000$ | $0.4444^{\mathbf{bc}} \pm 0.0641$ | $0.5555^{\mathbf{ab}} \pm 0.0641$ | $0.3333^{\mathbf{BCD}} \pm 0.0888$ |
| Alemow | $0.1111^{\mathbf{ef}} \pm 0.0000$ | $0.4444^{\mathbf{bc}} \pm 0.0641$ | $0.6666^{\mathbf{a}} \pm 0.0000$ | $0.4074^{\mathbf{AB}} \pm 0.0828$ |
| Volkamer lemon | $0.0000^{\mathbf{f}} \pm 0.0000$ | $0.4444^{\mathbf{bc}} \pm 0.0641$ | $0.4444^{\mathbf{bc}} \pm 0.0000$ | $0.2963^{\mathbf{CD}} \pm 0.0764$ |
| Persian lime | $0.0000^{\mathbf{f}} \pm 0.0000$ | $0.3333^{\mathbf{cd}} \pm 0.0000$ | $0.4444^{\mathbf{bc}} \pm 0.0641$ | $0.2592^{\mathbf{D}} \pm 0.0693$ |
| **Mean of Time** | $^{\dagger}0.1222^{\mathbf{C}} \pm 0.0202$ | $0.4333^{\mathbf{B}} \pm 0.0171$ | $0.5777^{\mathbf{A}} \pm 0.0195$ | |

†Means having capital in the right column and below row with same letters are not significantly difference according to LSD test ($P < 0.05$).
**Ψ** Means having small letter in middle columns with same letters are not significantly difference according to LSD test ($P < 0.05$).

## Discussion

The Asian citrus HLB caused by *Candidatus* Liberibacter asiaticus was first found in Iran in 2008 (Faghihi et al., 2009). The disease is transmitted by the Asian citrus psyllid, *Diaphorina citri,* which is widespread in citrus growing areas in southern Iran (Alizadeh et al., 2022; Salehi et al., 2012).

This study investigated the susceptibility of 10 commercial citrus cultivars and rootstocks (three-year-old seedlings) to *Ca.* L. asiaticus via graft-inoculation. Host response was evaluated based on the PCR detection of CLas after grafting, the disease incubation period, and disease severity.

During graft inoculation, the pathogen is directly transmitted from infected scion tissues to the recipient host plant through the graft union. In this method, unlike the transmission with psyllid and *Cuscuta* spp., the exact time of inoculation, the amount of initial inoculum (the number and size of the scions), and the inoculation location can be controlled to a large extent (Batool et al., 2007). However, there are limitations in this transmission method, such as the survival of the scion, and the degree of compatibility of the scion with the rootstock tissue.

Symptom progression in the inoculated seedlings was recorded regularly and compared with non-inoculated seedlings (negative controls). Furthermore, the presence of CLas infection in the symptomatic plants was confirmed via PCR. The symptoms of HLB disease are not very specific and the type and severity of the symptoms occurred in the greenhouse may be different from the symptoms in nature (due to the age of the trees, climatic conditions, and the presence of the vector). In nature, typical symptoms of the disease include yellowing shoots, blotchy mottle of leaves, corky veins, and localized symptoms of HLB spreads over the canopy, finally leading to defoliation and dieback. Moreover, the infected plants usually produce small, asymmetrical, bitter-tasting fruits with color inversion (Bove, 2006). However, in our greenhouse experiment, only some of the symptoms were observed, and in many cases, the predominant symptoms in the infected seedlings were mottling, general yellowing, interveinal yellowing, and symptoms resembling iron and zinc deficiencies. The chlorotic pattern's symptoms similar to those of zinc and iron deficiencies can be seen in other diseases such as citrus tristeza, citrus stubborn, and phytoplasma infection. In the studied citrus genotypes, the symptoms of the disease appeared between 10 and 18 months on different seedlings, and the incubation period varied between seedlings up to eight months. On the other hands, the CLas could be detected by PCR in the inoculated plants several months before the appearance of the disease symptoms (Table 6). For example, the first disease symptoms were noticed on Washington navel sweet orange at 10 to 12 mpi, while the first positive detection of CLas was found in these seedlings by the nested-PCR method at 6 to 8 mpi and by the direct-PCR assay at 6 to 10 mpi. Therefore, on average, 4 to 6 months before the appearance of HLB symptom, the CLas was detected in inoculated sweet orange seedlings by direct- and nested-PCR tests, respectively. Disease symptoms were appeared in Blood sweet orange, Clementina mandarin, Cleopatra mandarin, Bakraei, Rangpur lime, and Alemow, between 10 and 13 mpi. In these genotypes, the first positive PCR detection of CLas occurred between 6 and 8 mpi, or approximately 4 to 5 months prior to the onset of symptoms. The longest incubation period of the HLB disease was recorded between 16 and 19 mpi in the seedlings of Rough lemon, Volkamer lemon, and Persian lime, while PCR confirmed that these genotypes were infected with CLas between 10 and 12 mpi. Hence, even in more tolerant plants, the CLas could be detected by PCR up to 12 mpi and several months before the appearance of the disease symptoms. It is difficult to detect CLas prior to the symptom expression, especially under natural conditions, due to its non-uniform distribution and low concentration in infected citrus trees. The highly sensitive nested-PCR is an appropriate approach for detecting CLas during the disease's incubation period and can reveal the presence of HLB infection several months before direct-PCR.

Molecular methods such as PCR have been used successfully to identify and detect the different species of *Ca*. Liberibacter (Jagoueixet al., 1996; Zhou et al., 2007; Hocquellet et al., 1999). In some cases, direct-PCR may fail to detect the CLas in infected trees due to the low concentration of the bacterium or present of disturbing metabolites in the tissue extract. In this study, in addition to the direct-PCR, a sensitive nested-PCR was performed to detect the CLas. Overall, there was a significant difference between the nested-PCR and direct-PCR methods for detecting CLas in the inoculated plants. As expected, the nested-PCR had the higher sensitivity, and CLas was detected in the inoculated plants at least two months before direct-PCR.

However, the false positive results may occur, especially in nested-PCR assay, hence DNA from a healthy plant and sterile distilled water should be used in each reaction as negative controls.

The progression of disease symptoms and the rate of CLas multiplication within the host are key indicators of host susceptibility and tolerance. In more susceptible hosts, rapid colonization of the pathogen leads to earlier detection via PCR and an earlier appearance of the HLB symptoms.

The detection time of the bacterium in the phloem of the host plant, along with the appearance time of the disease symptoms, could be an appropriate relative index to evaluate the response of different citrus cultivars to the pathogen and compare them with each other.

A significant correlation was observed between the time of positive PCR detection of CLas after graft inoculation, the disease incubation period, and the disease severity. In highly susceptible cultivars, higher pathogen titers likely facilitated earlier molecular detection and accelerated symptom development. In contrast, those cultivars displaying longer incubation periods and attenuated symptom severity were identified as less susceptible to HLB disease.

According to the investigations, the concentration of the pathogenic bacterium in the plant is not uniform, and it is higher in the symptomatic parts of the infected plant. This demonstrates the enhanced proliferation of the bacterium in the symptomatic parts of the plant (Kunta et al., 2014).

In the surveys in the citrus growing areas in southern Iran, it has been observed that the Asian citrus psyllid (*Diaphorina citri*) has a host preference for Mexican lime and establishes a larger population on it, but the lower severity of the HLB disease has been found on Mexican lime trees. Under natural conditions, the incidence and severity of HLB are considerably higher in orange and mandarin trees than in other citrus species, despite the fact that *D. citri* populations on these trees are much smaller than those on Mexican lime.

Several studies have shown that orange and tangerine cultivars are sensitive, grapefruits and sour orange are semi-sensitive, and lemons are more tolerant to the HLB disease (Folimonova et al., 2009; Halbert & Manjunath, 2004; Miyakawa & Zhao, 1990). Wild genotypes of citrus, such as *Citrus indica* and *Citrus macroptera* in Northeast India, remained asymptomatic despite infected psyllid in the region (Bhagabati, 1993). Poncirus trees and seedlings (*Poncirus trifoliata*) are also very resistant to this disease (Miyakawa, 1980).

Mexican lime had relatively high tolerance against the disease (Safarpour et al., 2022), and uses as a rootstock in different parts of Iran. Persian lime is tolerant to both HLB and lime witches' broom, making it suitable for planting in locations such as southern Iran where both diseases prevail.

The studies suggest that proper tolerance to HLB, with reduced symptoms and probably reduced CLas titer might be usually found in rootstock cultivars and symptomless natural biotypes. These sources can be used as rootstocks, and for breeders to produce resistant cultivars. Due to the lack of natural resistance in citrus species, employing genetic engineering to create HLB-resistant commercial cultivars would have a high chance of success (Bové, 2006).

Over the last few years, research has focused on genome-editing technologies such as CRISPR/Cas9 and single-guide RNA (sgRNA) to enhance crop development. CRISPR/Cas9 is a specific and straightforward technique used to modify the genome of crops to produce disease-resistant/tolerant plants, and it might be the most accepted approach for crop improvement in the future (Ghosh et al., 2023).

Further work is needed to assess investigating the response of more citrus genotypes to CLas. Furthermore, in the citrus genotypes, CLas concentration and multiplication process can be checked in different organs especially root system and compared with each other using quantitative real time-PCR.

## Conclusions

Based on the timing of CLas detection by PCR and the symptom appearance of the disease, Persian lime, and Volkamer lemon placed at the highest tolerance level (lowest susceptibility), and Rough lemon was at the next level (medium susceptibility). The next group included Washington navel orange, Blood orange, Cleopatra mandarin, Clementine mandarin, Bakraei, Alemow, and Rangpur lime which were classified as susceptible to CLas.

In conclusion, all citrus genotypes evaluated, irrespective of their rootstock, were susceptible to the HLB disease. However, disease symptoms varied from cultivar to cultivar, with the most severe symptoms observed in sweet orange and mandarin, whereas symptoms were less severe in Persian lime and Volkamer lemon.

## Acknowledgments

The authors would like to thank Islamic Azad University of Iran, Varamin-Pishva Branch, and Fars Agricultural and Natural Resources Research and Education Center for supporting this work.

## Ethical Approval and Consent

This article does not contain any studies involving human participants or animals performed by any of the authors.

## CRediT author statement

**S. Safarpour Kapourchali:** Writing – original draft, Methodology, Investigation. **M. M. Faghihi:** Writing – review & editing, Conceptualization, Methodology, Investigation, Formal analysis. **N. S. Ebadi:** Methodology, Investigation **A. Alizadeh Aliabadi:** Writing–review & editing. **H. Hasanzadeh Khankahdani:** Software, Methodology, Formal analysis.

## Funding

The author(s) received no financial support for the research.

## Data Availability

All datasets generated during this study are available from the corresponding author upon request.

## Conflict of interest

The authors have no conflicts of interest to declare.

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