

Research Article

## Molecular characterization of aster yellows phytoplasma associated with citrus varieties, using Multiplex PCR

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**Abstract:** A survey was carried out in citrus nurseries in Mazandaran and Kerman provinces as the major citrus growing regions of Iran. Different varieties of orange and tangerine with abnormal symptoms were examined for the occurrence of *Candidatus Liberobacter asiaticus*, *Spiroplasma citri* and phytoplasma as the most important vascular limited pathogens in citrus. By visual inspection and molecular examination of 360 samples for the presence of fastidious prokaryotes only an occasional occurrence of phytoplasma and *Spiroplasma citri* were detected, using phytoplasma universal primer pairs P1/P7 and fU5/rU3 (in single tube nested PCR assay) and *Spiroplasma* specific primers P89-r/f and P58-6f/4r. PCR products of phytoplasmas were digested with *Mse*I, *Alu*I, *Bam*HI and *Taq*I restriction enzymes and the digestion results showed phytoplasma related to aster yellows phytoplasma group. No amplicon was obtained for *Candidatus Liberobacter asiaticus*. The findings suggest when pathogen's titer is low; symptoms of vascular limited diseases are almost confused with disorder and deficiency signs. So for sanitary selection and certification program, use of sensitive detection methods is recommended. Multiplex PCR assay used in this study successfully detected *Candidatus Liberobacter asiaticus*, *Spiroplasma citri* and phytoplasma in one reaction and is suggested for health certification programs.

**Keywords:** vascular limited, citrus, Phytoplasma, Multiplex PCR

### Introduction

Iranian Seed and Plant Certification and Registration Institute (SPCRI) were founded with the objective to improve quality of seed and plant material produced. The main goal of SPCRI is the genetic and phytosanitary certification of plant propagative material. Citrus is one of the most important fruit crops in Iran. That is susceptible to a large number of

pathogens. The laboratories of SPCRI are planning to expand certification program for citrus nuclear stock and mother trees to enhance plant health monitoring.

Lime witches broom caused by *Candidatus phytoplasma aurantifolia* (Bove *et al.*, 2000), Stubborn caused by *Spiroplasma citri* (Bove, 2006) and huanglongbing caused by *Candidatus Liberobacter asiaticus* (Mohkami *et al.*, 2011) are among the most serious citrus diseases facing us today. These diseases have been experimentally transmitted from infected trees to several citrus cultivars by grafting (Salehi *et al.*, 2007, Raju *et al.*, 1981, Bove, 2006, Frison and Taher, 1991, da Graça, 1991;

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OEPP/EPPO, 2006). These phloem-limited agents spread through vegetative propagation and phloem feeding vectors (Seemuller, 2002).

The symptoms of mentioned diseases are not always reliable for diagnosis because they can vary according to environmental conditions and pathogen concentrations.

The early detection of greening, Stubborn and witches broom are vital for revealing their real distribution and for phytosanitary certification. PCR as a sensitive molecular tool has been developed for detection of these diseases (Hung *et al.*, 1999; Heinrich *et al.*, 2001; Yokomi *et al.*, 2008). In view of the increasing interest in plant pathology for the detection of more than one target, such as mixed infection of pathogens in a single reaction (Bertolini *et al.*, 2003), multiplex PCR protocols have been developed. We describe for the first time, a multiplex PCR for the detection of prokaryotic agents frequently infecting citrus trees. Using healthy mother trees and clean budwood could prevent spreading of diseases. Thus a study was conducted to detect the presence of mentioned diseases in Iranian citrus orchards and nurseries.

## Materials and Methods

### Plant samples

A total of 360 samples from different varieties of lime, orange and tangerine (such as Navelate, Okitsu, Fukumoto, Valencia late, Washington navel foyus, Hoshimoto, Washington navel, Clementine clime new less1, Clemantis manitol, Lime Bears, Delta seedless and Navelina) were collected from commercial nurseries in Mazandaran and Kerman provinces of Iran. Samples were placed in labeled plastic bags and stored on ice until taken to the laboratory. leaves' midribs were grinded in liquid nitrogen in a pre-chilled mortar and followed by extraction of nucleic acid with DNA extraction kit according to manufacturer (Fermentas, Vilnius, Lithuania).

Infected citrus samples with *Spiroplasma citri*, *Candidatus* phytoplasma aurantifolia and

*Candidatus* Liberobacter asiaticus were obtained from plant protection organization of Iran and used as positive controls.

### Detection

All of the plants were visually inspected for the presence of phytoplasma related disease in the field. Samples were analyzed with Dienes' staining method. Hand cut tissues were transferred to Dienes' stain for 10 min. The stain was prepared by dissolving 2.5 g methylene blue, 1.25 g azure II, 10 g maltose and 0.25 g sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) in 100 mL distilled water, filtered through filter paper and diluted to 0.2% (v/v) in distilled water (Deeley *et al.*, 1979; Musetti, 2013) The stained sections were then washed in distilled water, mounted in distilled water on a glass slide and examined microscopically.

Molecular detection and identification of phytoplasma was done by direct PCR, using primer pair P1/P7 (Duduk *et al.*, 2013) or by single tube nested PCR in a 50 µl reaction volume containing 50 ng DNA extract, 0.4 mM of each dNTPs, 1x PCR buffer, 3 mM MgCl<sub>2</sub>, 3U Taq DNA polymerase (Fermentas, Vilnius, Lithuania) and 0.1 µM of each primers P1/P7 (Deng and Hiruki, 1991) and 0.3 µM of each primers fU5/rU3 (Lorenz *et al.*, 1995).

The PCR temperature profile was 94 °C (pre-denaturation) for 4 min and consisted of 40 cycles at 94 °C (denaturation) for 35s, 53 °C (annealing) for 35s, 72 °C (extension) for 45s, and final extension at 72 °C for 5 min.

Detection of *Candidatus* Liberobacter asiaticus and *Spiroplasma citri* were performed by specific primers respectively OI1/OI2 and A2/J5 (Hocquellet *et al.*, 1999), P89-r/f and P58-6f/4r (Yokomi *et al.* 2008).

Multiplex PCR was used for detection of phytoplasma, *Candidatus* Liberobacter asiaticus and *Spiroplasma citri* in one reaction. Primers P1/P7, A2/J5 and p58-6f/4r were used in one reaction for amplifying DNA template of mixed positive controls. Multiplex PCR was performed in a volume of

150  $\mu$ l containing 150 ng template DNA, 1 mM of each dNTPs, 1x PCR buffer, 9 mM MgCl<sub>2</sub>, 9 U Taq DNA polymerase (Fermentas, Vilnius, Lithuania) and 0.4  $\mu$ M of each of primers P1/P7 and 0.4  $\mu$ M of each of primers P58-6f/4r and 0.4  $\mu$ M of each primers A2/J5.

The PCR temperature profile was 94 °C (pre-denaturation) for 5 min and consisted of 40 cycles at 94 °C (denaturation) for 45s, 52 °C (annealing) for 45s, 72 °C (extension) for 1 min, and final extension at 72 °C for 10 min.

Approximately 20  $\mu$ l of each reaction mixture was electrophoresed in a 1.5% agarose gel and the products were visualized using UV transillumination after staining by ethidium bromide.

#### RFLP analysis

Ten microliters (approximately 200 ng of DNA) of direct PCR products of P1/P7 from phytoplasma infected samples were separately digested using 2 units of restriction endonucleases *Taq*I, *Alu*I, *Bam*HI and *Mse*I in buffers supplied by the manufacturer (Fermentas, Vilnius, Lithuania). The restriction patterns were electrophoresed in a 2% agarose gel and visualized by staining with ethidium bromide and UV illumination.

#### Results

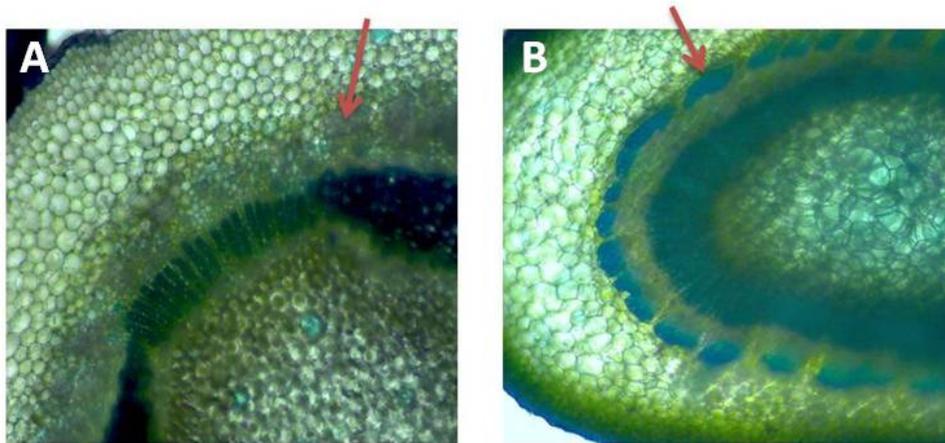
Dienes' staining as preliminary method verified the presence of phytoplasma in plants with deficiency symptoms. The cells infected with Phytoplasma could be observed as purple spots scattered in sieve tubes (Fig. 1).

Primers OI1/OI2, A2/J5, P89-r/f, P58-6f/4r, P1/P7 and fU5/rU3 that were used in this study respectively amplified 1160, 703, 707, 450, 1800 and 876 bp products from genome of *Ca. l. asiaticus*, *S. citri* and *Ca. Ph. aurantifolia* as positive controls (Fig. 2).

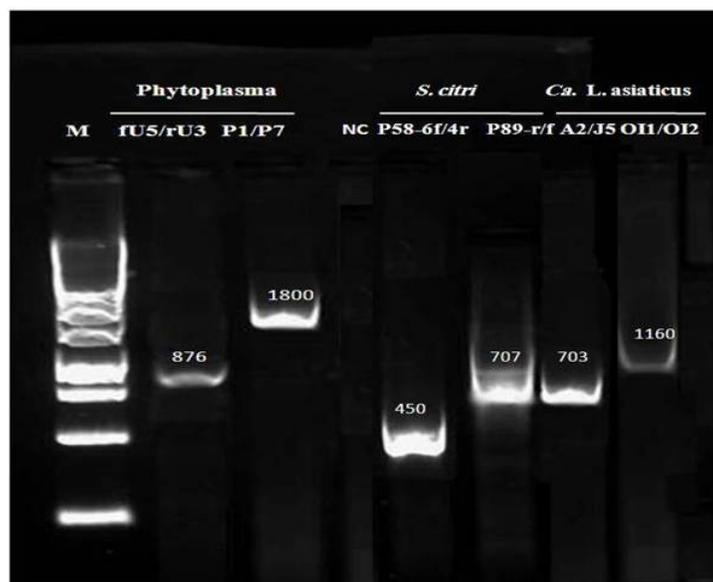
The PCR results indicated that only phytoplasma and *Spiroplasma citri* infection is widespread in some of the citrus varieties. (Fig. 3). No amplicon was obtained for *Ca. L. asiaticus* in 360 samples collected from citrus nurseries.

Multiplex PCR assay successfully detected phytoplasma, *S. citri* and *Ca. L. asiaticus* from mixed positive control samples in one reaction (Fig. 4).

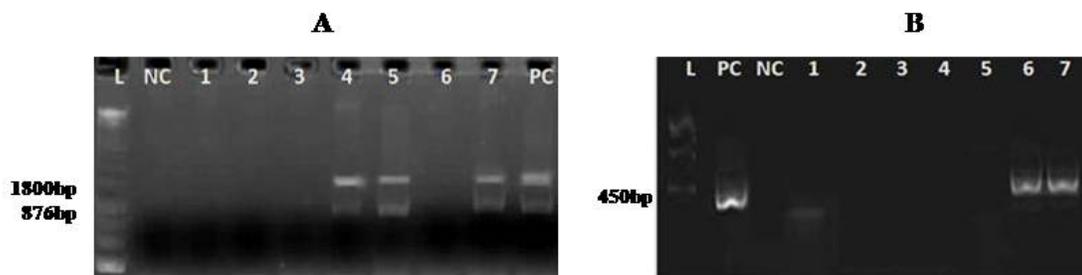
In young trees that exhibited yellowing and deficiency symptoms, the presence of phytoplasma was indicated in 37/360 samples when using single tube nested PCR (Fig. 3). Lime witches broom as positive control and infected samples showed the 1800 and 876 bp fragments using P1/P7 and fU5/rU3 in one reaction, respectively.



**Figure 1** Micrograph of hand cut stems of citrus stained with Dienes' stain. Section of healthy plant with unstained phloem (A), Phloem cells are stained in section obtained from infected plant (B).



**Figure 2** PCR amplification products of *Ca. Liberobacter asiaticus*, *Spiroplasma citri* and *Ca. Phytoplasma aurantifolia* as positive controls with Primers OI1/OI2, A2/J5, P89-r/f, P58-6f/4r, P1/P7 and fU5/rU3, NC: healthy citrus sample, M: 1kb DNA ladder.



**Figure 3** Detection of phytoplasma infection, using primer pairs P1/P7 and fU5/rU3 (1800 and 876 bp) in single tube reaction (A). Detection of *Spiroplasma citri*, using P58-6f/4r (450 bp), from extracted DNA of citrus varieties (B). (1: Okitsu, 2: Hoshimoto, 3: lime bear, 4: Navelate, 5: Delta seedless, 6: Fukumoto, 7: Washington navel). PC: *Candidatus* *Phytoplasma aurantifolia* and *Spiroplasma citri* infected samples used as positive controls, NC: healthy citrus sample, L: 1kb DNA ladder.

Phytoplasma infection was observed in five of Navelate, six of Okitsu, four of Fukumoto, four of Valencia late, three of Washington navel foyus, four of Hoshimoto, four of Washington navel, one of Clementine clime new less1, three of Clemantis manitol, one of Lime Bears, five of Delta seedless and two of Navelina samples.

PCR products of P1/P7 were used in RFLP analysis (Fig 5). Following *Mse*I, *Alu*I,

*Bam*HI and *Taq*I digestion, restriction pattern of phytoplasma isolate showed the same pattern as 16SrI (aster yellows) group (Lee *et al.* 1998).

Stubborn disease was identified in 17/360 citrus samples using PCR method by P89-r/f and P58-6f/4r primer pairs. Infections of *Spiroplasma citri* were observed in eight of Fukumoto, one of Navelina, five of Navelate and three of Valencia late samples.

## Discussion

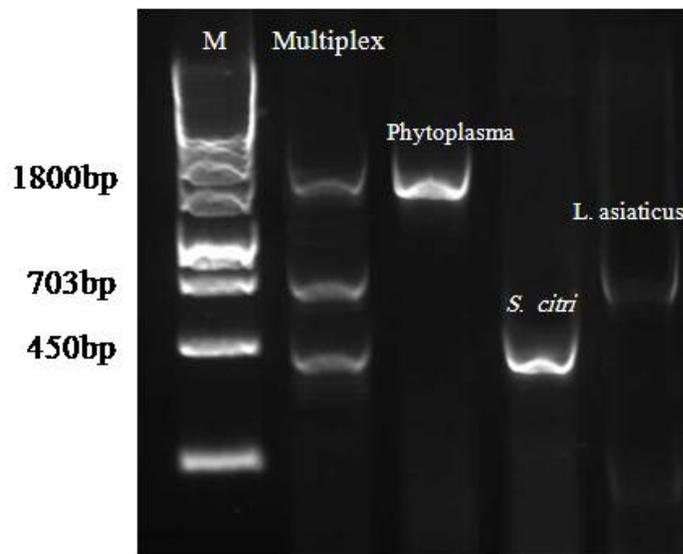
The multiplex PCR assay used in present study, successfully detected *Spiroplasma citri*, *Candidatus Liberobacter asiaticus* and phytoplasma infections in one reaction. The multiplex PCR assay is a faster and cheaper detector of the fastidious prokaryotes in citrus as compared with standard PCR. These results represent an important step towards routine diagnosis of some citrus pathogens in laboratory, and an additional aid for certification with respect to these agents.

PCR-RFLP detected phytoplasma positive samples belonging to Aster yellows group. Typical symptoms of phytoplasma infection with little leaf and proliferation were not observed in infected samples. It may be concluded therefore, that the detected phytoplasmas are mild strains or citrus varieties or that the hosts were not very susceptible to these phytoplasma strains. On the other hand there is evidence that phytoplasma concentration can vary greatly in infected plants (Berges *et al.*, 2000). Sieve tubes in susceptible hosts allows pathogen to multiply and spread

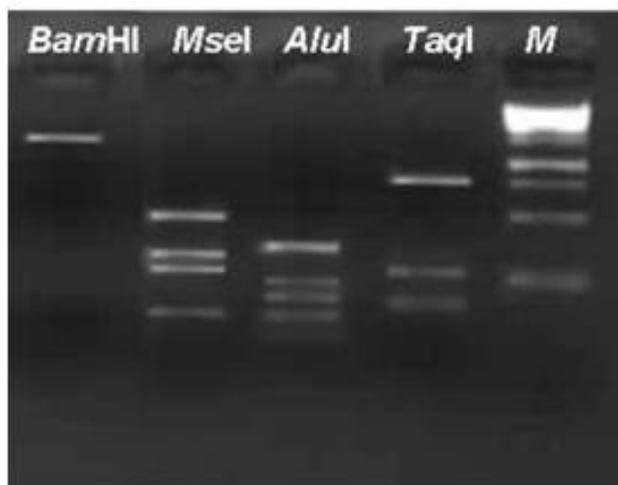
along the tubes. In contrast, in some citrus genotypes or varieties phytoplasma titre is extremely low and only non-specific yellowing symptoms can be developed. (Kartte and Seemuller, 1991). Probably in these citrus varieties due to inability of phytoplasma to increase in concentration, typical symptoms were not observed because phytoplasma concentration is usually regarded as important pathogenicity factor (Marcone, 2010).

The citrus cultivars showing the yellowing and mottling symptoms caused by "phytoplasma" have been observed earlier in citrus orchards of China (Chen *et al.*, 2009), Brazil (Teixeira *et al.*, 2008), Pakistan (Mannan, *et al.*, 2010), and Saudi Arabia (Alhudaib *et al.*, 2009).

Confusion about symptoms being prompted by nutrient deficiencies or vascular limited pathogens such as phytoplasma, *Candidatus Liberobacter sp.* and *Spiroplasma citri* in young trees has been reported (Bove, 2006; Weintraub and Jones 2010; Silva-Stenico *et al.*, 2009). So for sanitary selection and certification program, using sensitive detection methods is recommended.



**Figure 4** Ethidium bromide-stained gel of Multiplex PCR and standard PCR. Detection of phytoplasma. (P1/P7 primers), *Spiroplasma citri* (P58-6f/4r primers) and *Candidatus Liberobacter asiaticus* (A2/J5 primers) by multiplex PCR (lane 1) and in separate reactions (lane 3, 4 and 5). L: 1kb DNA ladder.



**Figure 5** RFLP analysis of phytoplasma isolated from orange. P1/P7 product was digested with *MseI*, *BamHI*, *TaqI* and *AluI*. Lane M, 1kb DNA ladder.

Our investigation determined co-infection of Spiroplasmas and Phytoplasma in different citrus varieties. Infection of many varieties of sweet orange with different range of susceptibility to *S. citri* was reported (Nejat *et al.*, 2007). Since citrus are not the only host plants of *S. citri* and transmission of *S. citri* occurs by vectors and propagative material (Nejat *et al.*, 2011), stubborn disease is widely distributed in citrus culture area in Iran.

## References

- Alhudaib, K., Arocha, Y., Wilson, M. and Jones, P. 2009. Molecular identification, potential vectors and alternative hosts of the phytoplasma associated with a lime decline disease in Saudi Arabia. *Crop Protection*, 28: 13-18.
- Berges, R., Rott, M. and Seemüller, E. 2000. Range of phytoplasma concentrations in various plant hosts as determined by competitive polymerase chain reaction. *Phytopathology*, 90: 1145-1152.
- Bertolini, E., Olmos, A., Lopez, M. M. and Cambra, M. 2003. Multiplex nested reverse transcription-polymerase chain reaction in a single tube for sensitive and simultaneous detection for RNA viruses and *Pseudomonas savastanoi* pv. *savastanoi* in Olive trees, *Phytopathology*, 93: 286-292.
- Bove', J. M., Danet, J. L., Bananej, K., Hassanzadeh, N., Taghizadeh, M., Salehi, M. and Garnier, M. 2000. Witches' broom disease of lime in Iran. Proceedings of the Fourteenth Conference of IOCV. IOCV, Riverside (US), pp. 207-212.
- Bové. J. M. 2006. Huanglongbing: A destructive, newly-emerging, century-old disease of citrus. *Journal of Plant Pathology*, 88: 7-37.
- Chen, J., Pu, X., Deng, X., Liu, S., Li, H. and Civerolo, E. 2009. A phytoplasma related to Candidatus *Phytoplasma asteris* detected in citrus showing Huanglongbing (yellow shoot disease) symptoms in Guangdong, P. R. China. *Phytopathology*, 99: 236-242.
- da Graça, J. V. 1991. Citrus greening disease. *Annual Review of Phytopathology*, 29: 109-136.
- Deeley, J., Stevens, W. A. and Fox, R. T. V. 1979. Use of Diens Stain to detect plant diseases induced by mycoplasma-like organisms. *Phytopathology*, 69: 1169-1171.
- Deng, S. and Hiruki, C. 1991. Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. *Journal Microbiology Methods*, 14: 53-61.

- Duduk, B., Paltrinieri, S., Lee, I-M. and Bertaccini, A., 2013. Nested PCR and RFLP analysis based on the 16S rRNA gene. *Methods in Molecular Biology*, 938: 159-171.
- Frison, E. A. and Taher, M. N. 1991. FAO/IBPGR Technical Guidelines for the safe movement of citrus germplasm. Food and Agriculture Organization of the United Nations, Rome /International Board for Plant Genetic Resources, Rome.
- Heinrich, M., Botti, S., Caprara, L., Arthofer, W., Strommer, S., Hanzer, V., Katinger, H., Bertaccini, A. and Machalo, M. L. C. 2001. Improved detection methods for fruit tree phytoplasmas. *Plant Molecular Biology Reporter*, 19: 169-179.
- Hocquellet, A., Toorawa, P., Bove, J. M. and Garnier, M. 1999. Detection and identification of the two *Candidatus Liberibacter* sp. Associated with citrus huanglongbing by PCR amplification of ribosomal protein genes of beta operon. *Molecular and Cellular Probes*. 13: 373-379.
- Hung, T. H., Wu, M. L. and Su, H. J. 1999. Development of a rapid method for the diagnosis of citrus greening disease using the polymerase chain reaction. *Journal of Phytopathology*, 147: 599-604.
- Karte, S. and Seemüller, E. 1991. Histopathology of apple proliferation in *Malus* taxa and hybrids of different susceptibility. *Journal of Phytopathology*, 131: 149-160.
- Lee, I. M., Gundersen, R. D. E., Davis, R. E. and Bartozyk, I. M. 1998 Revised classification scheme of phytoplasmas based on RFLP analysis of 16S rRNA and ribosomal protein gene sequences. *International Journal of Systematic Bacteriology*, 48: 1153-1169.
- Lorenz, K. H., Schneider, B., Ahrens, U., Seemüller, E. 1995. Detection of the apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and nonribosomal DNA. *Phytopathology*, 85: 771-776
- Mannan, S., Ibrahim, M., Nadeem Chohan, S. and Holford, P. 2010. Detection of phytoplasma in citrus orchards of Pakistan *Journal of pathology*, 2: 49-54.
- Marcone, C. 2010. Movement of Phytoplasmas and the Development of Disease in the Plant In: Weintraub, P. and Jones, P. (Eds.), *Phytoplasmas: genomes, plant hosts and vectors*. Allingford, UK, CABI Publishing.
- Mohkami, A., Satari, R., Lori, Z., Ehsani, A. and Nazemi, A. 2011. First report of citrus huanglongbing in the Orzooiyeh region in Kerman province. *Iranian Journal of Plant Pathology*, 47: 29 (In Persian with English summary).
- Musetti, R. 2013. Phytoplasma. *Methods in Molecular Biology*, 938: 109-113.
- Nejat, N., Salehi, M., Fayyazi, M. and Izadpanah, K. 2007. Survey of sweet orange cultivars for stubborn disease resistance in Iran. *Bulletin of Insectology*, 60: 305-306.
- Nejat, N., Vadamalai, G., Sijam, K. and Dickinson, M. 2011. First report of *Spiroplasma citri* associated with periwinkle lethal yellows in Southeast Asia. *Plant Disease*, 95: 1312-1320.
- OEPP/EPPO, 2006. *Candidatus Phytoplasma aurantifoliae*. OEPP/EPPO Bulletin. 36: 117-119.
- Raju, B. C., Nyland, G., Backus, E. A. and McLean, D. L. 1981. Association of *Spiroplasma* with brittle root of horseradish. *Phytopathology*, 71: 1067-1072.
- 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 leafhopper *Hishimonus phycitis* in Iran. *Plant Disease*, 91: 466.
- Seemüller, E., Garnier, M. and Schneider, B. 2002. Mycoplasmas of plants and insects. In: Razin, S. and Herrmann R. (Eds.), *Molecular Biology and Pathology of Mycoplasmas*. Academic/Plenum Kluwer, London, pp 91-116.
- Silva-Stenico, M. E., Pacheco, F. T. H., Pereira-Filho, E. R., Rodrigues, J. L. M., Souza, A. N., Etchegaray, A. D., Gomes, J. E. and Tsai, S. M. 2009. Nutritional deficiency in citrus with symptoms of citrus variegated

- chlorosis disease. *Brazilian Journal of Biology*, 69 (3): 859-864.
- Teixeira, D. C., Wulff, N. A., Martins, E. C., Kitajima, E. W, Ayres, A. J., Eveillard, S., Saillard, C. and Bove, J. M. 2008. A phytoplasma closely related to the pigeon pea witches' broom phytoplasma (16SrIX) is associated with citrus huanglongbing symptoms in the state of São Paulo, Brazil. *Phytopathology*, 98: 977-984.
- Weintraub, P. and Jones. P, eds, 2010. *Phytoplasmas: genomes, plant hosts and vectors*. Allingford, UK, CABI Publishing.
- Yokomi, R. K., Mello, A. F. S., Saponari, M. and Fletcher, J. 2008. Polymerase chain reaction based detection of *Spiroplasma citri* associated with citrus stubborn disease. *Plant Disease*, 92: 253-260.

## تشخیص مولکولی فیتوپلاسمای گروه زردی مینا در ارقام مختلف مرکبات با استفاده از PCR چندگانه

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**چکیده:** سلامت برخی از نهالستان‌های مرکبات واقع در استان‌های کرمان و مازندران به عنوان مناطق اصلی پرورش مرکبات در ایران ارزیابی گردید. ارقام مختلف با علائم غیرطبیعی از نظر آلودگی به *Candidatus Liberobacter asiaticus*، *Spiroplasma citri* و *phytoplasma* به عنوان مهم‌ترین عوامل بیماری‌های آوندی در مرکبات بررسی شدند. براساس مشاهدات چشمی و آزمایشات مولکولی ۳۶۰ نمونه، تنها آلودگی به عوامل فیتوپلاسمایی و *S. citri* محرز گردید. با استفاده از پرایمرهای P1/P7 و FU5/RU3 (در PCR آشیانه‌ای یک مرحله‌ای) آلودگی فیتوپلاسمایی ردیابی شد. همچنین آلودگی به *S. citri* با استفاده از پرایمرهای P89-R/F و P58-6F/4R مورد آزمون قرار گرفت. نتایج هضم محصول PCR به‌وسیله آنزیم‌های *TaqI*، *BamHI*، *AluI* و *MseI* نشان داد فیتوپلاسمای جدا شده متعلق به گروه زردی مینا است. همچنین با ارزیابی‌های صورت گرفته هیچ نوع آلودگی *Candidatus Liberobacter asiaticus* مشخص نگردید. تمایز علائم بیماری‌های محدود به آوردها خصوصاً زمانی که غلظت پاتوژن اندک است با علائم کمبود بسیار دشوار است لذا درخصوص برنامه‌های گواهی سلامت استفاده از روش‌های تشخیصی دقیق ضروری است. روش PCR چندگانه که در این مطالعه موفق به ردیابی هم‌زمان *Candidatus Liberobacter asiaticus*، *Spiroplasma citri* و *phytoplasma* در یک واکنش گردید به‌منظور استفاده در برنامه‌های گواهی سلامت توصیه می‌گردد.

**واژگان کلیدی:** بیماری، مرکبات، فیتوپلاسمای و PCR چندگانه