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The preservation of lime witches' broom phytoplasma in key lime by tissue culture

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Abstract

Witches' broom disease of small-fruited lime (WBDL) is a severe disease associated with phytoplasmas in the south of Iran. In this study maintenance of WBDL phytoplasma in key lime (*Citrus aurantifolia* Christm) in micropropagation is reported. After the maintenance of tissue cultures in a growth chamber, symptoms of little leaves and shortening of internodes were observed in shoots from infected plants. Micropropagated shoots that developed in tissue culture were tested for the presence of phytoplasmas using the two phytoplasma-specific primers pair P1/P7 and P3/P7. Amplification by polymerase chain reaction confirms the presence of WBDL phytoplasmas in the diseased plants 10 months after the beginning of the *in vitro* propagation. To our knowledge, this is the first report on maintenance of lime witches' broom phytoplasma in key lime shoots through tissue culture in Iran.

Key words: pytoplasma, tissue culture, key lime, PCR.

Introduction

Phytoplasmas are endocellular prokaryotes without cell wall associated with more than 600 diseases in at least 300 plant species (Kirkpatrick, 1992). Lime witches' broom (LWB) associated with '*Candidatus* Phytoplasma aurantifolia' is one of the most destructive diseases of lime in the southern Provinces of Iran (Mirzai *et al.,* 2009). Knowledge about phytoplasmas has been limited by inability to isolate them in pure culture. For scientific investigation, phytoplasmas can be maintained in living hosts (Bertaccini *et al*., 1992, Jarausch *et al.,* 1996). Plant material collected from infected lime fields as phytoplasma source is useful for some studies but does not allow detailed study of the associated phytoplasma. Field collection and nursery or greenhouse propagation of the key lime plants is time and money requiring. Tissue culture has been used for propagation of phytoplasma diseased plants *in vitro* as a more constant source of infected material (Wongkaew and Fletcher, 2004).

The feasibility of maintenance of lime witches' broom phytoplasma in key lime shoots through tissue culture is here reported for the first time in Iran.

Materials and methods

Healthy and witches' broom diseased lime (*Citrus aurantifolia* Christm) plants were collected from key lime gardens of Jiroft in the south of Iran.

Single node shoots were excised from mother plans and surface-sterilized by soaking in a solution containing 6% bleach and 1% tween 20 for 20 min. The explants were rinsed tree times in sterile distilled water.

Sterile explants were placed on agar-solidified MT medium (Murashige and Tucker, 1969) supplemented with 2.2 mg/L N6-benzyladenine, 1 mg/L indole-3-butyric acid and 3% sucrose. The pH was adjusted to 5.8 prior to autoclaving at 121ºC for 15 min. All explants were maintained at a temperature 24±2ºC under 16 h photopriod with light intensity of 3,000 lux. Plantlets were subcultured to fresh medium at intervals of 4 to 8 weeks for 10 months dividing the shoots into 1-cm-long segments.

WBDL phytoplasma was detected using polymerase chain reaction (PCR). Total DNA was extracted from leaves using cetyl trimethyl ammonium bromide (CTAB) extraction method (Zhang *et al.*, 1998). Two primer pairs P3/P7 and P1/P7 were used to amplify part of the 16S rRNA gene, the 16S–23S spacer region, and a portion of the 5-end region of the 23S rRNA gene from phytoplasmas.

Results

The medium successfully supported growth of all explants. Phytoplasma-diseased plants showed significantly reduced shoot height, little leaf and witches' broom symptoms when compared to the healthy control (figure 1).

The best time for subculture was every six weeks. Jar explants grew faster than the tube explants.

Amplification of two pathogen-specific DNA fragments 320 bp and 1,830 bp with two primer pairs P3/P7 and P1/P7, respectively by polymerase chain reaction (PCR) confirm the presence of WBDL phytoplasma in the diseased plants after 10 months of *in vitro* propagation (figure 2).

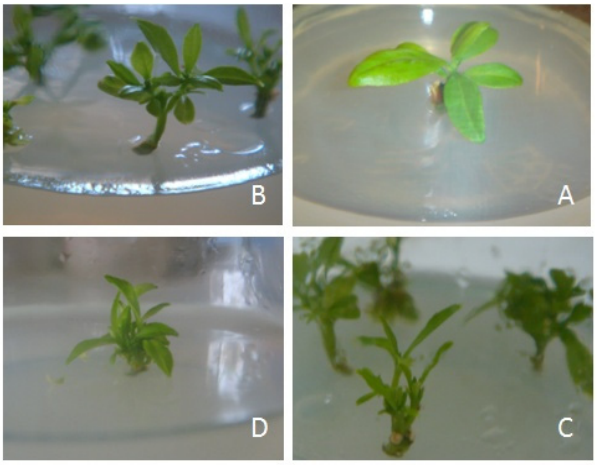

Figure 1. *C. aurantifolia* shoots in micropropagation A. Healthy shoot; B, C, D. Phytoplasma infected explants showing little leaf and witches' broom.
(In colour at www.bulletinofinsectology.org)

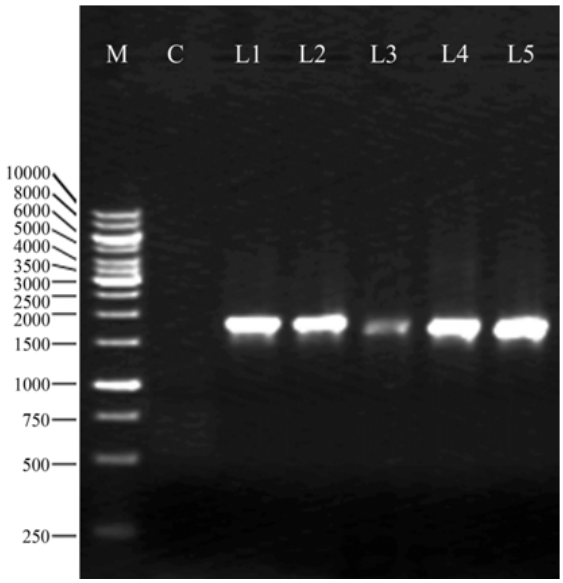

Figure 2. Agarose gel electrophoresis of PCR amplifications of a 1,830 bp fragment from symptomatic key lime shoots. C, negative control; L1, 2-months phytoplasma infected explants; L2, 4-months phytoplasma infected explants; L3, 6-months phytoplasma infected explants; L4, 8-months phytoplasma infected explants; L5, 10-months phytoplasma infected explants.

Discussion

Phytoplasma-diseased explants showed significantly reduced shoot height and leaf width compared to the healthy control. The shoot height and leaf width were reduced by increasing interval subculture until 8 weeks, however the witches' broom symptom seems not to be correlated with phytoplasma concentration.

The maintainance of phytoplasma through tissue culture of diseased key lime can present a useful way to maintain living phytoplasma for studies of phytoplasma-plant interactions toward the understanding of phytoplasma pathogenicity and also to study possibility of their elimination by cryotherapy, or other *in vitro* techniques.

Acknowledgements

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References

BERTACCINI A., DAVIS R. E., LEE I-M., 1992.- *In vitro* micropropagation for maintenance of mycoplasma-like organisms in infected plant tissue.- *HortScience*, 27(9): 1041-1043.

JARAUSCH W., LANSAC M., DOSBA F., 1996.- Long-term maintenance of nonculturable apple-proliferation phytoplasmas in their micropropagated natural host plant.- *Plant Pathology*, 45: 778-786.

KIRKPATRICK B. C., 1992.- Mycoplasma-like-organisms: plant and invertebrate pathogens, pp. 4050-4067. In: *The prokaryotes* (BALOWS A., TRUPER H. G., DWORKIN M., HARDER W., SCHLEIFER K.-H., Eds).- Springer-Verlag, New York, USA.

MIRZAI M., HEYDARNEJAD J., SALEHI M., HOSSEINI POUR A., MASSUMI H., SHAABANIAN M., 2009.- Production of polycolonal antiserum against causal agent of lime witches' broom.- *Iranian Journal of Plant Pathology*, 45(2): 40-41.

MURASHIGE T., TUCKER D. P. H., 1969.- Growth factor requirements of citrus tissue culture, pp. 1155-1161. In: *Proceedings on first international citrus symposium of the international society of citriculture* (ISC) (CHAPMAN H. D., Ed).- Riverside, USA.

WONGKAEW P., FLETCHER J., 2004.- Sugarcane white leaf phytoplasma in tissue culture: long-term maintenance, transmission, and oxytetracycline remission.- *Plant Cell Reporter*, 23: 426-434.

ZHANG Y., UYEMOTO J. K., KIRKPATRICK B. C., 1993.- A small-scale procedure for extracting nucleic acids from woody plants infected with various phytopathogens for PCR assay.- *Journal of Virological Methods*, 71: 45-50.

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