Volume 68, Number 4, May 2019

# Plant Pathology

An International Journal edited by
the British Society for Plant Pathology
**Senior Editor Matt Dickinson**

WILEY ISSN 0032-0862

BSPP

# Plant Pathology

An international journal edited by the British Society for Plant Pathology



*Plant Pathology* is published for the British Society for Plant Pathology which is solely responsible for editorial policy. It has been developed from the journal with this title previously published by Her Majesty's Stationery Office and maintains the sequence of those volumes.

*Plant Pathology* publishes research papers and critical reviews on all aspects of plant pathology. Letters to the Editor are welcome when these comment on matters relevant to plant pathology.

Whilst the Senior Editor takes all possible care to ensure that information contained in the journal is accurate, neither he nor the British Society for Plant Pathology can be held responsible for any loss or damage resulting from reliance upon material published in *Plant Pathology*.

**Instructions to Authors**

For submission instructions, subscription and all other information visit: http://wileyonlinelibrary.com/journal/ppa



**Subscription Information**

*Plant Pathology* is published in nine issues per year. Institutional subscription prices for 2019 are: Print and Online: US$3014 (USA, Canada & Mexico), US$3514 (Rest of World), €2075 (Europe), £1635 (UK). Prices are exclusive of tax. Asia-Pacific GST, Canadian GST/HST and European VAT will be applied at the appropriate rates. For more information on current tax rates, please go to www.wileyonlinelibrary.com/tax-vat. The price includes online access to the current and all online back files to January 1st 2015, where available. For other pricing options, including access information and terms and conditions, please visit www.wileyonlinelibrary.com/access.

**Delivery Terms and Legal Title**

Where the subscription price includes print issues and delivery is to the recipient's address, delivery terms are **Delivered at Place (DAP)**; the recipient is responsible for paying any import duty or taxes. Title to all issues transfers Free of Board (FOB) our shipping point, freight prepaid. We will endeavour to fulfil claims for missing or damaged copies within six months of publication, within our reasonable discretion and subject to availability.

**Backstock**

**Back issues:** Single issues from current and recent volumes are available at the current single issue price from cs-journals@wiley.com. Earlier issues may be obtained from Periodicals Service Company, 351 Fairview Avenue – Ste 300, Hudson, NY 12534, USA. Tel: +1 518 822 9300, Fax: +1 518 822 9305, Email: psc@periodicals.com.


**Despatch**

*PLANT PATHOLOGY* (ISSN: 0032-0862) is published 9 times a year. US mailing agent: Mercury Media Processing, LLC **1850 Elizabeth Avenue, Suite #C, Rahway, NJ 07065 USA**. Periodical postage paid at Rahway, NJ. Postmaster: Send all address changes to *PLANT PATHOLOGY*, John Wiley & Sons Inc., C/O The Sheridan Press, PO Box 465, Hanover, PA 17331 USA.

**Publisher:** *Plant Pathology* is published by John Wiley & Sons Ltd, 9600 Garsington Rd, Oxford OX4 2DQ, UK; Tel: +44 (0)1865 776868; Fax: +44 (0)1865 714591. Blackwell Publishing was acquired by John Wiley & Sons in February 2007. Blackwell's programme has been merged with Wiley's global Scientific, Technical and Medical business to form Wiley-Blackwell.

**Journal Customer Services:** For ordering information, claims and any enquiry concerning your journal subscription please go to www.wiley-customerhelp.com/ask or contact your nearest office.

**Americas:** Email: cs-journals@wiley.com; Tel: +1 781 388 8598 or +1 800 835 6770 (toll free in the USA and Canada).

**Europe, Middle East and Africa:** Email: cs-journals@wiley.com; Tel: +44 (0) 1865 778315.

**Asia Pacific:** Email: cs-journals@wiley.com; Tel: +65 6511 8000.

**Japan:** For Japanese speaking support, Email: cs-japan@wiley.com; **Visit our Online Customer Help** available in 7 languages at www.wileycustomerhelp.com/ask

**Wiley Online Library**

This journal is available online at *Wiley Online Library*. Visit wileyonlinelibrary.com/journal/ppa to search the articles and register for table of contents e-mail alerts.

**HINARI, AGORA and OARE**

Access to this journal is available free online within institutions in the developing world through the AGORA initiative with the FAO, the HINARI initiative with the WHO and the OARE initiative with UNEP. For information, visit www.aginternetwork.org, www.who.int/hinari/en/ and www.oaresciences.org.

**Note to NIH Grantees**

Pursuant to NIH mandate, Wiley will post the accepted version of contributions authored by NIH grant-holders to PubMed Central upon acceptance. This accepted version will be made publicly available 12 months after publication. For further information, see www.wiley.com/go/nihmandate/.

**Abstracting and Indexing Services**

This journal is indexed by *Academic Search, Academic Search Alumni Edition, Academic Search Premier, AgBiotech News & Information, AgBiotechNet, AGRICOLA Database, Agricultural Engineering Abstracts, Agroforestry Abstracts, BIOBASE: Current Awareness in Biological Sciences, Biocontrol News & Information, Biofuels Abstracts, Biological & Agricultural Index Plus, Biological Abstracts, BIOSIS Previews, Botanical Pesticides, CAB Abstracts®, CABDirect, Crop Physiology Abstracts, CSA Biological Sciences Database, CSA Environmental Sciences & Pollution Management Database, CSA Microbiology Databases, Current Contents: Agriculture, Biology & Environmental Sciences, Environment Index, Field Crop Abstracts, Forest Products Abstracts, Forestry Abstracts, Global Health, Grasslands & Forage Abstracts, Horticultural Science Abstracts, InfoTrac, Irrigation & Drainage Abstracts, Journal Citation Reports/Science Edition, Maize Abstracts, Nematological Abstracts, Ornamental Horticulture, Plant Breeding Abstracts, Plant Genetic Resources Abstracts, Plant Growth Regulator Abstracts, Postharvest News & Information, Potato Abstracts, Poultry Abstracts, Review of Agricultural Entomology, Review of Aromatic & Medicinal Plants, Review of Medical & Veterinary Mycology, Review of Plant Pathology, Rice Abstracts, Rural Development Abstracts, Science Citation Index, Science Citation Index Expanded, SCOPUS, Seed Abstracts, Soils & Fertilizers Abstracts, Soybean Abstracts Online, Sugar Industry Abstracts, VINITI, Weed Abstracts, Wheat, Barley & Triticale Abstracts, World Agricultural Economics & Rural Sociology Abstracts, Zoological Record.*

Wiley's Corporate Citizenship initiative seeks to address the environmental, social, economic, and ethical challenges faced in our business and which are important to our diverse stakeholder groups. Since launching the initiative, we have focused on sharing our content with those in need, enhancing community philanthropy, reducing our carbon impact, creating global guidelines and best practices for paper use, establishing a vendor code of ethics, and engaging our colleagues and other stakeholders in our efforts. Follow our progress at www.wiley.com/go/citizenship.

**ISSN:** 0032-0862 (Print)
**ISSN:** 1365-3059 (Online)

# Plant Pathology

**Volume 68, Number 4, May 2019**

## Contents

**New Honorary Member**

619 *Dr Julie Flood*
New Honorary Member of BSPP

**Plant Pathogen Impact - Review**

620 *M. A. H. B. Bhuiyan, N. Vaghefi and P. W. J. Taylor*
Ray blight of pyrethrum in Australia: a review of the current status and future opportunities

**Review**

628 *J. Tahir, M. Rashid and A. J. Afzal*
Post-translational modifications in effectors and plant proteins involved in host–pathogen conflicts

**Original Articles**

645 *E. T. Silva, J. A. Rios, M. U. P. Araujo, P. R. Silveira and F. A. Rodrigues*
Defence responses in flag leaves and spikes of common wheat *Triticum aestivum* cultivars with contrasting levels of basal resistance to blast caused by *Pyricularia oryzae*

659 *G. J. Maree, R. Prins, C. M. Bender, W. H. P. Boshoff, T. G. Negussie and Z. A. Pretorius*
Phenotyping Kariega × Avocet S doubled haploid lines containing individual and combined adult plant stripe rust resistance loci

669 *B. Admassu-Yimer, T. Gordon, J. M. Bonman and K. Esvelt Klos*
Development and validation of a quantitative PCR assay method of assessing relative resistance of oat (*Avena sativa*) to crown rust (*Puccinia coronata* f. sp. *avenae*)

678 *H. C. Wu and W. S. Wu*
Evaluation of virulence and pathogenicity of *Alternaria patula* on French marigold (*Tagetes patula*)

689 *X. Cheng, Y. Lu, D. Chen, C. Luo, M. Li and C. Huang*
Pathogen and disease characteristics of marigold black spot in Beijing and surrounding areas

700 *G. Scott, A. Almasrahi, F. Malekpoor Mansoorkhani, M. Rupar, M. Dickinson and G. Shama*
Hormetic UV-C seed treatments for the control of tomato diseases

*Contents continued on inside back cover*

*Information on this journal can be accessed at: http://wileyonlinelibrary.com/journal/ppa*

This journal is covered by *Academic Search, Academic Search Alumni Edition, Academic Search Premier, AgBiotech News & Information, AgBiotechNet, AGRICOLA Database, Agricultural Engineering Abstracts, Agroforestry Abstracts, BIOBASE: Current Awareness in Biological Sciences, Biocontrol News & Information, Biofuels Abstracts, Biological & Agricultural Index Plus, Biological Abstracts, BIOSIS Previews, Botanical Pesticides, CAB Abstracts®, CABDirect, Crop Physiology Abstracts, CSA Biological Sciences Database, CSA Environmental Sciences & Pollution Management Database, CSA Microbiology Databases, Current Contents: Agriculture, Biology & Environmental Sciences, Environment Index, Field Crop Abstracts, Forest Products Abstracts, Forestry Abstracts, Global Health, Grasslands & Forage Abstracts, Horticultural Science Abstracts, InfoTrac, Irrigation & Drainage Abstracts, Journal Citation Reports/Science Edition, Maize Abstracts, Nematological Abstracts, Ornamental Horticulture, Plant Breeding Abstracts, Plant Genetic Resources Abstracts, Plant Growth Regulator Abstracts, Postharvest News & Information, Potato Abstracts, Poultry Abstracts, Review of Agricultural Entomology, Review of Aromatic & Medicinal Plants, Review of Medical & Veterinary Mycology, Review of Plant Pathology, Rice Abstracts, Rural Development Abstracts, Science Citation Index, Science Citation Index Expanded, SCOPUS, Seed Abstracts, Soils & Fertilizers Abstracts, Soybean Abstracts Online, Sugar Industry Abstracts, VINITI, Weed Abstracts, Wheat, Barley & Triticale Abstracts, World Agricultural Economics & Rural Sociology Abstracts, Zoological Record.*

WILEY

*This journal is available online at Wiley Online Library. Visit wileyonlinelibrary.com to search the articles and register for table of contents e-mail alerts.*

**Front cover:** Severe stripe rust (*Puccinia striiformis* f. sp. *tritici*) infection on a flag leaf of susceptible wheat grown in the annual rust screening nursery of the University of the Free State, South Africa (photograph Z. A. Pretorius). See **68**, [659–668].

Typeset in India by SPS Pvt Ltd.
Printed in Singapore by Markono Print Media Pte Ltd.

*Contents continued from outside back cover*

708 *R. Fredua-Agyeman, S. F. Hwang, S. E. Strelkov, Q. Zhou, V. P. Manolii and D. Feindel*
Identification of *Brassica* accessions resistant to 'old' and 'new' pathotypes of *Plasmodiophora brassicae* from Canada

719 *M. P. Camargo, C. F. Hong, L. Amorim and H. Scherm*
Cryptic species and population genetic structure of *Plasmopara viticola* in São Paulo State, Brazil

727 *A. Pérez-Sierra, P. Romón-Ochoa, C. Gorton, A. Lewis, H. Rees, S. Van Der Linde and J. Webber*
High vegetative compatibility diversity of *Cryphonectria parasitica* infecting sweet chestnut (*Castanea sativa*) in Britain indicates multiple pathogen introductions

738 *R. H. J. Heim, I. J. Wright, A. P. Allen, I. Geedicke and J. Oldeland*
Developing a spectral disease index for myrtle rust (*Austropuccinia psidii*)

746 *C. Nansen, A. N. Stewart, T. A. M. Gutierrez, W. M. Wintermantel, N. McRoberts and R. L. Gilbertson*
Proximal remote sensing to differentiate nonviruliferous and viruliferous insect vectors – proof of concept and importance of input data robustness

755 *S. Teber, A. Ceylan, K. Gürcan, T. Candresse, Ç. Ulubaş Serçe, M. Akbulut, S. Kaymak and B. Akbaş*
Genetic diversity and molecular epidemiology of the T strain of *Plum pox virus*

764 *C. G. Orfanidou, L. C. Papayiannis, P. G. Pappi, N. I. Katis and V. I. Maliogka*
Criniviruses associated with cucurbit yellows disease in Greece and Cyprus: an ever-changing scene

775 *M. Hassan and I. E. Tzanetakis*
Population structure, evolution and detection of blackberry leaf mottle-associated virus, an emerging emaravirus

783 *T. T. Kumssa, J. S. Rupp, M. C. Fellers, J. P. Fellers and G. Zhang*
An isolate of *Wheat streak mosaic virus* from foxtail overcomes *Wsm2* resistance in wheat

790 *I. Opatovsky, M. Elbaz, I. Dori, L. Avraham, S. Mordechai-Lebiush, A. Dombrovsky and L. Tsror (Lahkim)*
Control of lettuce big-vein disease by application of fungicides and crop covers

796 *S. M. Fu, H. W. Liu, Q. H. Liu, C. Y. Zhou and J. S. Hartung*
Detection of '*Candidatus* Liberibacter asiaticus' in citrus by concurrent tissue print-based qPCR and immunoassay

804 *M. Ansari, S. M. Taghavi, S. Zarei, K. Miri, P. Portier and E. Osdaghi*
Pathogenicity and molecular phylogenetic analysis reveal a distinct position of the banana fingertip rot pathogen among the *Burkholderia cenocepacia* genomovars

816 *C. M. L. Lopes, J. E. Cares, F. J. Perina, G. F. Nascimento, J. S. F. Mendonça, A. W. Moita, P. Castagnone-Sereno and R. M. D. G. Carneiro*
Diversity of *Meloidogyne incognita* populations from cotton and aggressiveness to *Gossypium* spp. accessions

# British Society for Plant Pathology (http://www.bspp.org.uk)

The British Society for Plant Pathology (BSPP) was founded in 1981 for the advancement of education by the study of plant pathology in the United Kingdom and worldwide. The BSPP welcomes members from all countries, who are interested in any branch of plant pathology. Its principal activities consist of the organization of scientific meetings and the provision of financial support for members for travel to such meetings and for other types of short-term activities. It publishes three scientific journals, under the titles *Plant Pathology, Molecular Plant Pathology* and *New Disease Reports*. The first is owned and edited by the BSPP, the second jointly owned with Blackwell Publishing and edited by the BSPP and the third publishes short disease reports on the internet.

Meetings are held regularly, often in collaboration with other societies, to cover any discipline relevant to plant pathology. They include paper-reading and poster sessions, discussion forums, workshops on specialist topics and visits to research establishments. Young members are particularly encouraged to contribute. There is one principal residential conference each year.

The Society also fosters the professional interests of plant pathologists and, through its Newsletter, provides an information service and a means of communication between members. Books on plant pathological topics are published and are available to members at reduced prices.

The BSPP is a private company (number 3576579) limited by guarantee and is registered in the United Kingdom as a Charity (registration number 1071465). Its affairs are managed by an elected Board.

Any person interested in the objective of the Society is eligible for membership subject to approval by the BSPP Board. There are several options for membership and members may opt to receive *Plant Pathology* and/or *Molecular Plant Pathology* within their membership subscription for personal use only. Details are available from the Membership Secretary and on the BSPP website. Members can attend the scientific meetings and other functions of the BSPP, and can participate in elections to the Board. Members also receive the Society's Newsletter and news from the International Society for Plant Pathology.

Further information can be obtained from the Secretary. Membership application forms are available from the Membership Secretary. Information about meetings can be obtained from the Programme Secretary.

## Board for 2019

**President:** D. L. ARNOLD
*Department of Applied Sciences, University of the West of England, Bristol, BS16 1QY*

**President-Elect:** N. SPENCE
*Defra, Sand Hutton, York, YO41 1LZ*

**Vice-President:** D. COLLINGE
*Plant & Environmental Sciences, University of Copenhagen, Denmark*

**Secretary:** S. HOLDGATE
*NIAB, Huntingdon Road, Cambridge CB3 0LE*

**Treasurer:** M. TÖR
*Institute of Science and the Environment, University of Worcester, Worcester, WR2 6AJ*

**Programme Secretary:** E. BOA
*Biological Sciences, Aberdeen University, Aberdeen AB24 2TZ*

**Membership Secretary:** W. S. LEE
*Plant Pathology and Microbiology Department, Rothamsted Research, Harpenden AL5 2JQ*

**Elected Member:** (2017–2019) A. BAILEY
*Biological Sciences, University of Bristol, Bristol, BS8 1TQ*

**Elected Member:** (2019–2021) N. CUNNIFFE
*Plant Sciences, University of Cambridge, CB2 3EA*

**Elected Member:** (2019–2021) V. NTOUKAKIS
*Life Sciences, University of Warwick, Coventry, CV4 7AL*

**Elected Member:** (2017–2019) R. HARRISON
*NIAB EMR, East Malling, ME19 6BJ*

**Elected Member:** (2018–2020) P. SPANU
*Life Sciences, Imperial College London, London SW7 2AZ*

**Elected Member:** (2018–2020) P. TAYLOR
*CABI, Egham, TW20 9TY*

## Honorary Members

C. M. Brasier, R. J. Cook, J. G. Ellis, FRS, J. Flood, W. E. Fry, A. Hall, R. Hull, D. S. Ingram, M. J. Jeger, J. A. Lucas, J. W. Mansfield, Lord Sainsbury of Turville, P. R. Scott

**BSPP Funds**

Visit the BSPP website (http://www.bspp.org.uk/) for further details on how to apply.

*BSPP Fellowships*: (Junior, Senior and Grace Waterhouse) intended to stimulate and facilitate studies or training to the benefit of plant pathology by providing funds for travel, consu mables and other necessary costs at another institute.

*BSPP Travel Fund*: to provide financial assistance to members wishing to attend a plant pathology conference in the UK or overseas or make short study visits abroad.

*BSPP Conference Fund*: to support plant pathology related conferences, meetings and workshops.

*BSPP Undergraduate Vacation Bursary Scheme*: to provide support for work on specified research projects for students during the summer vacation (closing date: 1st April each year).

*BSPP MSc Project Bursary Scheme*: to extend the BSPP's commitment to training and education in British plant pathology. This fund supports a limited number of students during the research phase of their MSc projects in a plant pathology related discipline. Selection will be on the merit of both the student and the proposed project (closing date: 1st April each year).

*BSPP Plant Pathology Promotion Fund*: to promote an understanding and awareness of the importance of plant pathology to a wider audience than its membership. Applications for grants up to a maximum of £2000 will be considered for projects that have as their aim the stimulation of interest in, and knowledge and awareness of, plant pathology to people who do not normally come into contact with the subject.

**BSPP World Wide Web Address**

http://www.bspp.org.uk
Web Manager (webmanager@bspp.org.uk).

**Programme News**

Information on all meetings is available on the BSPP web site or by contacting the Programme Secretary (meetings@bspp.org.uk).

**P. H. Gregory Prize**

The P. H. Gregory Competition is open (a) to members of BSPP who have not previously presented an oral paper to a meeting of a learned society and (b) to all registered students. Students need not be members of BSPP and may enter whether or not they have previously presented an oral paper to a meeting of a learned society. Contestants should not have entered the P. H. Gregory competition previously.
Applications to the Programme Secretary (meetings@bspp.org.uk).

**Winners of the P. H. Gregory Prize**

| | |
|---|---|
| 2009 Helen Lovell | 2014 Laura Stevens |
| 2010 (no meeting) | 2015 Fay Newbery |
| 2011 Nichola Hawkins | 2016 Douglas Pyott |
| 2012 Ruth Bryant | 2017 Kathryn Hales |
| 2013 Rebekah Robinson | 2018 Anna González Gil |

**Advantages of publishing in *Plant Pathology***

1 No page charges for text, black and white or half tone figures
2 High standard of refereeing and editing
3 Advance publication *EarlyView*
4 PDF offprints

**Colour illustration for cover of *Plant Pathology***

*Plant Pathology* has a panel for a colour illustration that will change with each issue. If you have a suitable photograph with a short caption please send it to the Senior Editor.

**Instructions to Authors**

These can be found at http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1365-3059/homepage/ForAuthors.html

*Plant Pathology* (2019) **68,** 796–803 Doi: 10.1111/ppa.12998

# Detection of '*Candidatus* Liberibacter asiaticus' in citrus by concurrent tissue print-based qPCR and immunoassay

S. M. Fu[ab], H. W. Liu[b], Q. H. Liu[a], C. Y. Zhou[a] and J. S. Hartung[b]*

[a]*Citrus Research Institute, Southwest University, Chongqing 400715, P. R. China; and* [b]*USDA - ARS Molecular Plant Pathology Laboratory, Beltsville, MD 20705, USA*

'*Candidatus* Liberibacter asiaticus' (CLas) is associated with the most destructive disease of citrus, huanglongbing (HLB). The most widely used methods for detection of CLas are PCR-based and require purification of DNA from plant samples. Elution of DNA from tissue prints made on nitrocellulose membranes followed by qPCR (TPE-qPCR) was compared to DNA extraction of plant tissue followed by PCR (X-PCR) by testing the same tissue samples. The former estimated a higher CLas population in tissue prints than the latter (*t*-test; $P = 0.009$). All extracts prepared for TPE-qPCR throughout the experiment were also tested by conventional PCR and 80.8% were identified as positive. A similar set of stem and petiole tissue samples was tested by TPE-qPCR and immunoassay. Although the detection rate by TPE-qPCR was higher than by immunoassay, about 6% of tissue prints were positive by immunoassay but not by TPE-qPCR. Thus, a higher detection rate would be achieved by combining TPE-qPCR with immunoassay. Significant differences were observed in the performance of nitrocellulose membranes from different manufacturers in these assays. Immunotissue prints showed that the spatial distribution pattern of CLas infection varied widely from one sample to another, but the patterns were highly correlated among serial sections from the same sample, suggesting that CLas preferentially colonizes adjacent phloem cells in a vertical rather than horizontal direction.

*Keywords:* '*Candidatus* Liberibacter asiaticus', citrus greening, huanglongbing, immunotissue printing, qPCR, tissue prints

## Introduction

Huanglongbing (HLB), also known as citrus greening, is the most destructive citrus disease. HLB was first found in Chaoshan, China in 1919 (Bové, 2006) and shortly thereafter was described in India (Nath & Husain, 1927). HLB is now widely distributed in more than 40 countries in Asia, Africa and the Americas (Gottwald *et al.*, 1989; Coletta-Filho *et al.*, 2004; Bové, 2006). Since HLB was reported in Florida in 2005 (Chung & Brlansky, 2005), production of sweet orange and grapefruit has been reduced by 80% or more and production of frozen orange-juice concentrate has been dramatically reduced. '*Candidatus* Liberibacter asiaticus' (CLas), a nonculturable member of the α-proteobacteria, is one of three '*Ca*. Liberibacter' species associated with HLB, but CLas is the only globally important species.

Symptoms of HLB are variable and may be affected by citrus variety and environmental and seasonal effects. Symptoms of HLB that are commonly and easily observed include newly emerged shoots with upright and pale yellow leaves, malformed fruits with colour inversion and aborted seeds (Bové, 2006) and root decline (Graham *et al.*, 2013; Johnson *et al.*, 2014). Collapse and blockage of phloem cells can also be observed microscopically (Folimonova & Achor, 2010). A distinct 'blotchy mottle' of mature leaves is often used for presumptive diagnosis of HLB (Bové, 2006).

A variety of methods have been developed for confirmatory diagnosis of HLB by detection of CLas or by biological indexing. These include graft transmission assays (Garnier & Bové, 1983; Roistacher, 1991), DNA probes (Villechanoux *et al.*, 1992), microscopic examination (Garnier *et al.*, 1984; Folimonova & Achor, 2010), conventional polymerase chain reaction (PCR; Jagoueix *et al.*, 1996; Ding *et al.*, 2005), quantitative PCR (qPCR; Li *et al.*, 2006, 2008; Morgan *et al.*, 2012), loop-mediated isothermal amplification (LAMP; Okuda *et al.*, 2005) and immunoassays (Ding *et al.*, 2015; Pagliaccia *et al.*, 2017).

PCR-based methods are the most commonly used methods for detection of CLas. PCR requires DNA extraction and, generally, purification with organic solvent-based protocols. CLas is restricted to phloem cells, typically sampled from leaf midribs or bark, and, because these tissues are very difficult to grind to homogeneity for optimal recovery of CLas, DNA preparation is time-consuming, costly and problematic; this severely limits the number of samples that can be processed. In addition, the distribution of CLas within the tree is notoriously difficult to predict,

*E-mail: john.hartung@ars.usda.gov

*Published online 6 March 2019*





Plant Pathology

and extensive sampling is required to have confidence in the results of the testing of a given tree (Tatineni *et al.*, 2008; Li *et al.*, 2009; Ding *et al.*, 2015).

In contrast, immunotissue printing is a simple and inexpensive technique and also preserves the cellular locations of macromolecules, such as proteins, enzymes, soluble metabolites and nucleic acids (Cassab & Varner, 1987; Taylor *et al.*, 1993). The method is widely used in both field and laboratory for the detection of other important phloem-limited pathogens of citrus such as citrus tristeza virus (CTV; Garnsey *et al.*, 1993) and *Spiroplasma citri* (Shi *et al.*, 2014). Immunotissue printing has been developed to detect CLas with an anti-OmpA polyclonal antibody (Ding *et al.*, 2015, 2016). Others have demonstrated the amplification by PCR of plum pox virus RNA from nitrocellulose membranes (Olmos *et al.*, 1996; Bertolini *et al.*, 2014). In addition, elution of DNA captured on nitrocellulose membranes in glycine buffer was shown to be useful for the detection of CLas by PCR (Bertolini *et al.*, 2014).

In this study, immuno- and PCR-based methods to detect CLas on nitrocellulose membranes were compared. Tissue prints were processed for immunodetection as described previously in published protocols, or used for sample preparation by simple elution into glycine buffer without tissue grinding or other DNA purification (Bertolini *et al.*, 2014). The eluted DNA was subjected to both conventional PCR and probe hydrolysis-based qPCR. The results of these tests were also compared with PCR and qPCR tests on the same plant samples after conventional tissue homogenization and DNA purification (Li *et al.*, 2006). The results of tissue print elution (TPE)-qPCR were compared with those of immunotissue prints prepared simultaneously from the same samples. The effect of the source of nitrocellulose membranes on the results and the distribution pattern of CLas phloem cells of stem and leaf petioles were also investigated.

## Materials and methods

### Plant materials and pathogens

Plants used throughout the experiments were from either the Exotic Pathogens of Citrus Collection (EPCC) at the USDA Beltsville Agricultural Research Center (BARC) in Beltsville, USA or from the Citrus Research Institute in Chongqing, China. In the USA, trees infected with CLas were maintained and propagated by bud inoculation of sweet orange seedlings (*Citrus sinensis*) as described previously (Li *et al.*, 2009). In China, trees infected with CLas were propagated by bud inoculation of sweet orange cv. Madame Vinous seedlings and maintained as previously described (Fu *et al.*, 2015).

Field-grown plants were also used, including mandarin (*Citrus reticulata* 'Bing-tang') and sweet orange cv. Navel from groves in Hunan, Guangxi and Sichuan, China.

### Sample preparation for TPE-qPCR and X-qPCR

Tissue printing was performed based on a published protocol (Ding *et al.*, 2015). Plants from the greenhouse or field were tested by conventional PCR after DNA extraction (Jagoueix *et al.*, 1996) and confirmed to be infected by CLas; control plants from the greenhouse were also confirmed to be healthy by the same method. For each tissue print, transverse sections, approximately 0.5 mm thick, of petioles or stems were cut and the freshly cut surfaces were pressed onto nitrocellulose membranes (Whatman, 0.45 μm pore size; cat. no. 88018) to give five partially overlapping prints, after which the cut plant tissue disks (*c.* 100 mg) were collected (Fig. 1a,b). The membranes were air dried for 10 min at room temperature and the five partially overlapping prints were cut into pieces and soaked in 20 μL glycine buffer (0.1 M glycine, 0.05 M NaCl, 1 mM EDTA). The five tissue disks collected were pooled for DNA extraction with the DNeasy kit (QIAGEN) and eluted with 20 μL TE buffer (Fig. 1c) for testing by qPCR (X-qPCR) following the published protocol, using a 16S rDNA-based primer probe set (Li *et al.*, 2006). Extracts used to compare TPE-qPCR and X-qPCR were assayed on the same 96-well plates.

To determine the sensitivity of TPE-qPCR to detect a CLas-infected sample among a large number of pooled samples, tissue prints prepared from trees known to be positive for CLas by qPCR were mixed with tissue prints prepared from healthy trees in ratios of 5:0, 4:1, 2:3 and 1:4. Student's *t*-test with unequal variance was used to compare the Cq values after qPCR. Chi-square tests were applied to compare the proportion of samples declared positive for CLas using a cut-off for the 16S rDNA qPCR of quantification cycle (Cq) <38.0. Likewise, a cut-off of Cq <38.0 for the positive internal control of the mitochondrial cytochrome oxidase (*COX*) gene was used to confirm the presence of plant DNA in the extracts throughout. Statistical analyses were carried out with SPSS v. 16.0.

### Sample preparation for TPE-qPCR with membranes from different manufacturers

A test was conducted with three different brands of nitrocellulose membranes to determine whether they were equally suitable for TPE-qPCR. Field-grown plants were identified as CLas-positive or -negative by conventional PCR after extraction of DNA (Jagoueix *et al.*, 1996) and used to prepare tissue prints using stems and leaf petioles. Fifteen serial sections were prepared, and sequential tissue prints were made on nitrocellulose membranes obtained from three suppliers, manufacturers X, Y and Whatman. Each tissue print assayed was made from five serial sections, pressed alternately onto each of the three membranes (Fig. 1). DNA was eluted directly from the tissue prints on the three different nitrocellulose membranes with 20 μL glycine buffer (Bertolini *et al.*, 2014; Fig. 1d,e). qPCR was performed according to Li *et al.* (2006) using 3 μL of template from each sample. A set of 32 samples eluted from each of the three membranes was run on each 96-well plate. qPCR was performed on the eluted extracts in three plates in all, making a total of 96 samples tested for each membrane, of which 86 were from trees known to be positive and 10 from trees known to be negative. A one-way ANOVA analysis was applied for qPCR results from both the 16S rDNA and *COX* genes with SPSS v. 16.0.

### Preparation of TPE-qPCR and immunoassay

In order to directly compare TPE-qPCR and tissue print immunoassay for detection of CLas, samples taken from greenhouse and field-grown trees were identified as CLas positive or CLas negative in advance by conventional PCR with primers OI1/OIc2 (Jagoueix *et al.*, 1996). Tissue prints, consisting of

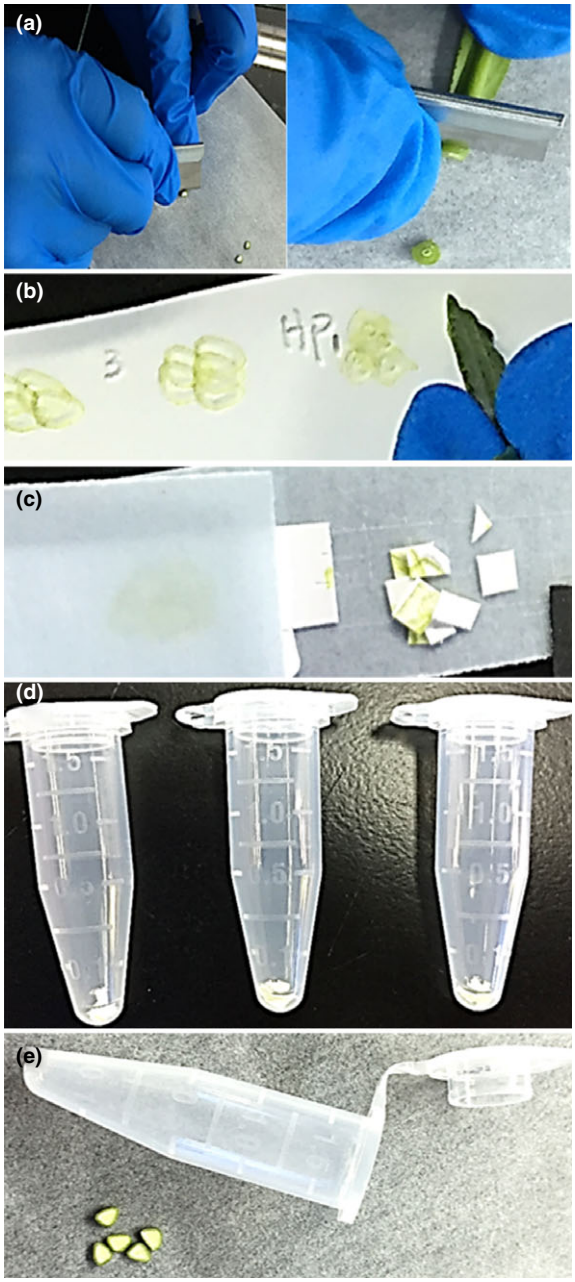


Figure 1 Preparation of samples for tissue print elution (TPE)-qPCR and DNA extraction-qPCR (DNA X-qPCR). (a) Either stem (left) or petiole (right) can be chosen for tissue prints. The freshly cut end of the tissue is pressed to the nitrocellulose membrane and held for 5 s. After each imprint is made, a fresh surface is exposed by removing a 0.5 mm slice. Tissue sections are saved. (b) Five serial imprints are overlapped to create one test sample. (c) The membrane with the tissue print samples is cut and placed in a 1.5 mL centrifuge tube, to which 20 μL glycine buffer (0.1 M glycine, 0.05 M NaCl, 1 mM EDTA; pH 9) is added. (d) Tubes are incubated at room temperature for 30 min or at 4 °C overnight; 3 μL of eluate is used for qPCR testing. (e) The five tissue sections are collected in a tube and total DNA is extracted with a QIAGEN DNeasy kit. DNA is eluted in 20 μL TE and 3 μL is used per qPCR.

one to five sections from known CLas-positive or from CLas-negative field trees and healthy greenhouse trees, were prepared as described above, but alternate sections were pressed onto two different Whatman membranes to make matched tissue imprints (Fig. S1). One set of prints was eluted into 20 μL glycine buffer for TPE-qPCR (Li *et al.*, 2006) and the other was used for immunoassay (Ding *et al.*, 2015).

### Detection of CLas by conventional PCR from samples eluted from tissue prints

All samples prepared for TPE-qPCR throughout the experiments were also tested by conventional PCR (Jagoueix *et al.*, 1996) as follows: reaction mixes (12 μL) were preheated at 95 °C for 3 min; followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 64 °C for 35 s and extension at 72 °C for 80 s; with a final extension at 72 °C for 10 min. Amplification products were evaluated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide.

## Results

### TPE-qPCR and X-qPCR

When the data from the four sets of tissue prints (taken from diseased and healthy samples in ratios of 5:0, 4:1, 2:3 and 1:4) were pooled, the mean Cq of 16S rDNA was 30.8 ± 4.4 ($n$ = 186 positive samples) for TPE-qPCR and 31.5 ± 6.4 ($n$ = 191 positive samples) for X-qPCR ($P$ = 0.009; Table 1; Data S1). The mean Cq of the *COX* assay was 22.2 ± 2.2 for TPE-qPCR and 24.1 ± 6.4 for X-qPCR ($P$ = 0.000; Table 1; Data S1). Thus, the means of both 16S rDNA (CLas) and *COX* (plant mitochondrial control) assays were significantly different and the TPE-qPCR estimated higher populations of CLas in the samples than the X-qPCR tests. This is probably because the TPE-qPCR directly detects CLas DNA on the cut surface of the sample, whereas a portion of the CLas DNA to be tested by X-qPCR from corresponding tissues is diluted and not recovered during the extraction process. However, the magnitude of the difference was not sufficient to change significantly the proportion of tests declared to be positive (76–77% for both assays; $P$ = 0.651; Table 1; Data S2).

### Comparison of different membranes for use in TPE-qPCR

The detection rates of CLas from matched samples were 20.9%, 19.8% and 81.4% for membranes manufactured by companies X, Y and Whatman, respectively (Table 2). The $P$-values of one-way ANOVA tests were 1.000, 0.056 and 0.001 between manufacturers X and Y, Whatman and manufacturer Y, and between Whatman and manufacturer X for the 16S rDNA gene (Data S3 & Data S4). Thus, in the tests presented here, the Whatman membranes produced better results than comparable membranes from manufacturers X and Y for TPE-qPCR. All other data in this report were taken from Whatman membranes.

Table 1 Comparison of TPE-qPCR and X-qPCR for detection of '*Candidatus* Liberibacter asiaticus' (CLas).

| | TPE-qPCR[a] | | | | X-qPCR[b] | | | |
|---|---|---|---|---|---|---|---|---|
| | Cq[c] | | CLas positive | | Cq | | CLas positive | |
| No. prints | 16S rDNA | *COX* | No./tested[d] | % | 16S rDNA | *COX* | No./tested | % |
| 5D + 0H[e] | 30.9 ± 5.2 | 22.6 ± 3.4 | 88/118 | 74.6 | 30.7 ± 8.7 | 23.8 ± 9.6 | 81/118 | 68.6 |
| 4D + 1H | 29.5 ± 0.9 | 21.8 ± 0.4 | 35/35 | 100.0 | 30.7 ± 12.3 | 22.7 ± 3.5 | 17/35 | 48.6 |
| 2D + 3H | 30.8 ± 4.4 | 21.5 ± 0.3 | 47/47 | 100.0 | 32.1 ± 2.2 | 25.6 ± 1.6 | 47/47 | 100.0 |
| 1D + 4H | 33.0 ± 0.2 | 22.4 ± 1.3 | 16/45 | 35.6 | 32.5 ± 1.9 | 24.2 ± 1.8 | 46/46 | 100.0 |
| Combined | 30.8 ± 4.4 | 22.2 ± 2.2 | 186/245 | 75.6 | 31.5 ± 6.4 | 24.1 ± 6.4 | 191/246 | 77.6 |

[a]TPE-qPCR, tissue prints on nitrocellulose membrane were eluted into glycine buffer and used for qPCR.
[b]X-qPCR, DNA was extracted from tissue pieces used to make tissue prints with a QIAGEN kit and used for qPCR.
[c]Cq, quantification cycle.
[d]The number of samples declared CLas positive/the total number of samples tested. Statistical analyses of the data are presented in Data S1 and Data S2.
[e]D, the number of tissue prints tested from diseased samples; H, the number of tissue prints tested from healthy samples.

## Comparison of TPE-qPCR and TPE-PCR

Five datasets with 916 tests in total were compiled based on both qPCR and conventional PCR after DNA was eluted from tissue prints. Each test consisted of one to five tissue prints. Among these, 82.6% and 80.8% of the tests detected CLas by qPCR or PCR, respectively, and 77.4% of tests resulted in the detection of CLas by both (Table 3). The results also showed that there was neither gain nor loss of sensitivity from pooling individual tissue prints prior to elution of DNA (Table 3).

Table 2 Comparison of different brands of nitrocellulose membranes for use in TPE-qPCR.

| | Average Cq[a] | | Detection rate[b] | |
|---|---|---|---|---|
| Brand | 16S rRNA | *COX* | No./tested | % |
| X | 33.6 ± 3.1 | 31.0 ± 3.1 | 18/86 | 20.9 |
| Y | 32.5 ± 3.2 | 30.7 ± 3.4 | 17/86 | 19.8 |
| Whatman | 30.2 ± 3.7 | 22.0 ± 1.5 | 70/86 | 81.4 |

[a]Cq, quantification cycle.
[b]The number of samples testing positive/the total number of samples tested. Statistical analyses are presented in Data S3 and Data S4.

Table 3 Detection of '*Candidatus* Liberibacter asiaticus' (CLas) by tissue print-based qPCR and PCR.

| | qPCR | | PCR | | qPCR & PCR | |
|---|---|---|---|---|---|---|
| No. prints[a] | No./tested[b] | % | No./tested | % | No./tested | % |
| 1P | 134/160 | 83.8 | 134/160 | 83.8 | 130/160 | 81.3 |
| 2P | 134/173 | 77.5 | 140/173 | 80.9 | 126/173 | 72.8 |
| 3P | 161/174 | 92.5 | 156/174 | 89.7 | 155/174 | 89.1 |
| 4P | 113/141 | 80.1 | 110/141 | 78.0 | 107/141 | 75.9 |
| 5P | 215/268 | 80.2 | 200/268 | 74.6 | 191/268 | 71.3 |
| Combined | 757/916 | 82.6 | 740/916 | 80.8 | 709/916 | 77.4 |

[a]Number of tissue prints pooled prior to elution of DNA.
[b]The number of samples declared CLas positive/the total number of tested samples.

## Comparison of TPE-qPCR and immunoassay

Citrus trees previously identified as CLas positive by X-PCR were sampled to prepare tissue prints for both TPE-qPCR and immunodetection with anti-OmpA polyclonal antibody. Among 786 tests, 81.2% and 52.9% were declared CLas positive by TPE-qPCR and immunodetection, respectively (Table 4). Among the 416 tissue samples declared positive by immunodetection, 48 samples were identified as positive by immunodetection but not by TPE-qPCR on the prints made from matched sections (Table 4).

## Visualization of CLas in plant samples

As expected following immunodetection, a dark purple colour was observed in phloem cells of tissue prints prepared from samples collected from trees that had tested positive for CLas by qPCR, but not in tissue prints prepared from healthy plants. CLas showed uneven distribution within the vascular rings observed in prints from individual sections but the distribution patterns were very consistent among tissue prints from serial sections of single stem samples (Fig. 2). The leaves on this stem

Table 4 Detection of '*Candidatus* Liberibacter asiaticus' by tissue print elution (TPE)-qPCR and immunoassay with anti-OmpA antibody.

| | Detection rate | |
|---|---|---|
| Method | No./tested | % |
| TPE-qPCR | 638/786 | 81.2 |
| OmpA | 416/786 | 52.9 |
| TPE-qPCR & OmpA | 368/786 | 46.8 |
| ΔN[a] | 48/786 | 6.1 |

[a]ΔN, samples declared positive by immunoassay with anti-OmpA polyclonal antibody but not by TPE-qPCR.

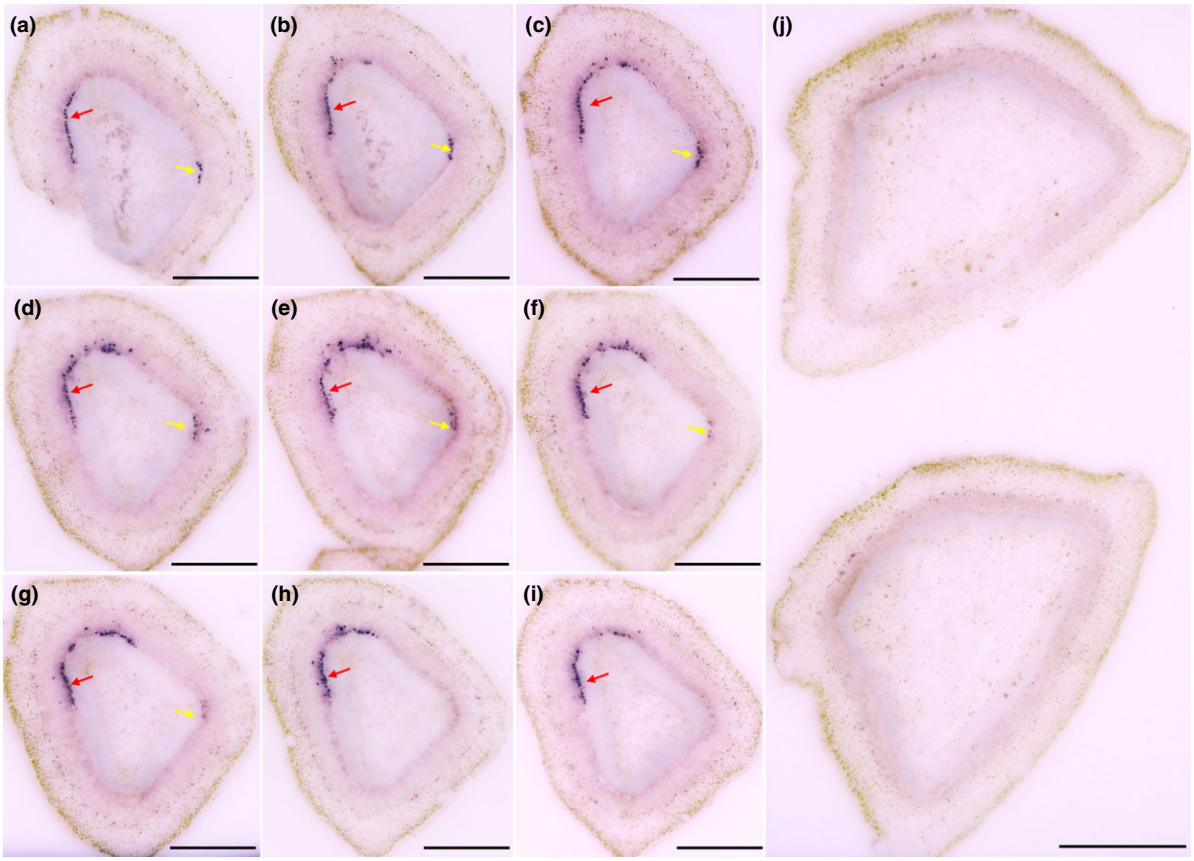


Figure 2 Uneven distribution pattern of '*Candidatus* Liberibacter asiaticus' (CLas) in phloem sieve cells of consecutive stem tissue prints, visualized by immunotissue printing using the anti-OmpA antibody. (a–i) Consecutive stem tissue prints of diseased sweet orange from the greenhouse, without symptoms; (j), stem tissue prints of healthy plant. Red and yellow arrows point to distinct CLas infection foci that were maintained in consecutive serial tissue prints; bars = 1 mm.

did not show any symptoms of HLB, demonstrating the difficulty of diagnosis of HLB and the potential power of immunotissue printing. Similar patterns were also observed in the tissue prints from serial sections of petioles (Fig. 3). DNA eluted from the matched prints made from the same tissues was also positive by TPE-qPCR.

## Discussion

For detection of a high-consequence pathogen like CLas, at least two independent methods should be used to provide mutually confirmatory results. Tissue prints are therefore ideal, because they can be assayed for CLas by both immuno- (Ding *et al.*, 2015, 2016) and PCR-based methods (Bertolini *et al.*, 2014).

In preliminary experiments, positive tissue prints were identified by immunoassay and then the same prints were tested by TPE-qPCR (data not shown). The TPE-qPCR tests were negative, presumably because the CLas DNA was eluted from the nitrocellulose membrane during the development of the immunotissue prints, and subsequently was not available for TPE-qPCR. Based on this observation it was decided to test alternate tissue prints from samples by either immuno- or TPE-qPCR methods.

In the course of these experiments, the supplier of nitrocellulose membranes was changed and it was noticed that results were not comparable. This prompted a systematic comparison of different suppliers of membranes, as described. In this investigation, the Whatman nitrocellulose membrane provided better and more consistent results in both TPE-qPCR and immunoassays, indicating all nitrocellulose membranes may not be equivalent for CLas detection. For this reason, Whatman nitrocellulose membranes were used throughout this study.

The uneven distribution of CLas in citrus plants has been reported in many studies by symptom observation (Bové, 2006) or PCR-based detection (Tatineni *et al.*, 2008; Li *et al.*, 2009). Here, immunodetection with anti-Omp-A antibody has demonstrated that the radially nonuniform distribution of CLas within a vascular ring in cross-section is maintained vertically over substantial distances. CLas showed consistent radial distribution patterns within two to nine consecutive tissue prints with 0.5 mm spacing. In other words, CLas distribution

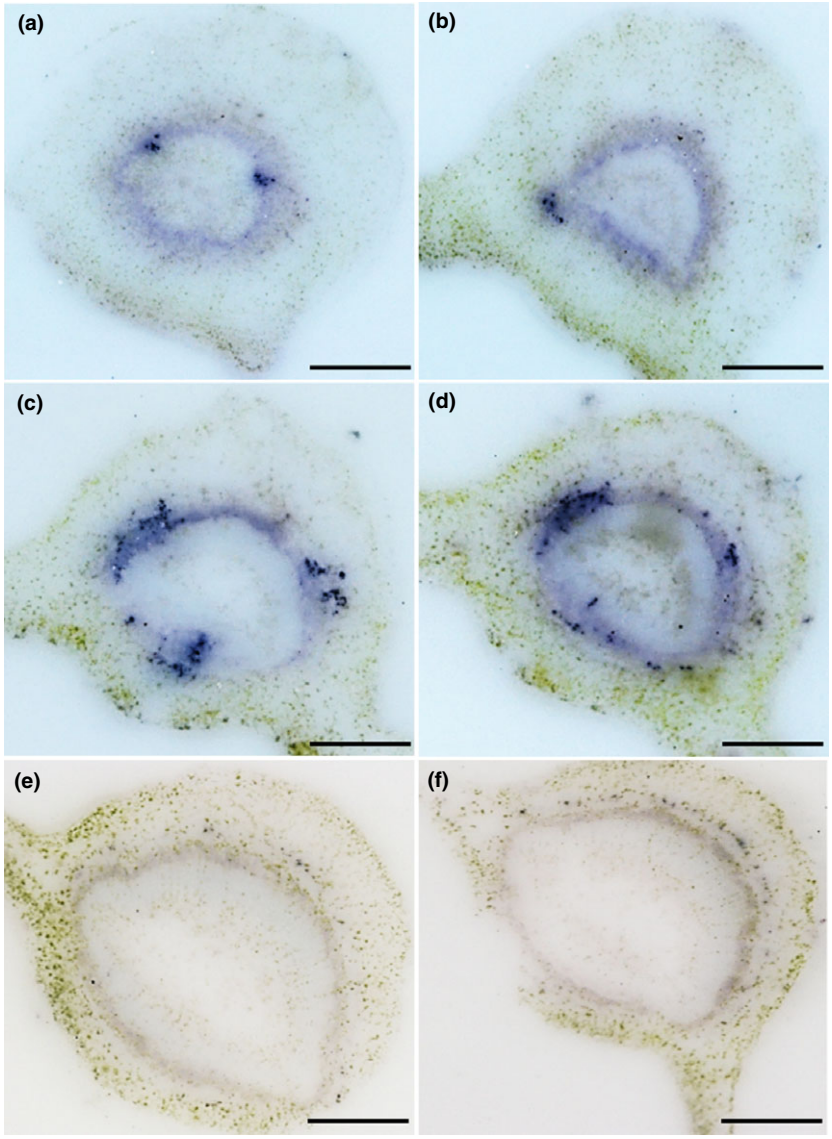


Figure 3 Uneven distribution pattern of '*Candidatus* Liberibacter asiaticus' (CLas) in phloem sieve cells of consecutive tissue prints of petiole, visualized by immunotissue printing using the anti-OmpA antibody. (a–d) Consecutive leaf petiole tissue prints of diseased samples of sweet orange from the greenhouse, without symptoms; (e, f), tissue prints from healthy plants; black bars = 0.5 mm.

among serial sections is correlated within 1–4.5 mm. This enables confirmatory testing by independent methods on consecutive serial sections of samples. The size of CLas is approximately 0.2 × 2 μm but varies substantially (Bové, 2006). The average diameter of phloem sieve pores is approximately 0.411 μm in soybean and 5 μm in cucurbits (Mullendore *et al.*, 2010), while the diameter of plasmodesmata is much smaller, usually less than 0.1 μm. Thus, CLas can move with relative ease with phloem flow in the vertical direction through sieve pores but with much more difficulty through plasmodesmata in the horizontal direction. This would explain why CLas tends to maintain a presence in a vertical column of cells rather than spreading radially. The concentration of CLas changes along the phloem section, as evidenced by a stronger or weaker colour signal during immunodetection. The direct visualization of the uneven distribution patterns of CLas in phloem tissues provides compelling evidence that sampling of trees for PCR-based testing will always be problematic, and the uneven distribution pattern of CLas in different tissues will contribute to false negative results by PCR-based methods, particularly for large trees. Therefore, extensive sampling is minimally required for each tree for field HLB screening. This is very difficult to implement at an adequate level using conventional methods due to expensive and laborious tissue extraction and DNA purification procedures.

TPE-qPCR offers several advantages for detection of CLas. Tissue prints can be prepared with fresh tissue in the field with only razor blades, gloves, nitrocellulose membranes and a cutting surface. This is a particularly good option to preserve DNA and proteins for shipment of samples between distant locations. The elution of DNA directly from nitrocellulose membranes in simple glycine buffer eliminates the need for, and expense of,

DNA purification kits. TPE-qPCR is thus a simple and inexpensive technique and can easily be scaled to large numbers of samples of any plant tissue, including peduncle, petioles, leaf midrib and stem. Peduncle is the best tissue to sample for *S. citri* (Wang *et al.*, 2015) and also produced excellent results by immunotissue printing (Ding *et al.*, 2015) and TPE-qPCR (data not shown), suggesting that peduncles are a good tissue for HLB screening if available. Tissue prints from one to five serial sections combined with probe hydrolysis qPCR works well to determine whether a given sample is positive or negative for CLas, with a large majority identified by conventional PCR as well. As with immunotissue printing, CLas can be detected from a single print by TPE-qPCR. This successful detection of CLas on nitrocellulose membranes by either immuno- or PCR-based methods may be because the concentration of CLas in single phloem cells is higher than an average concentration sampled by DNA extraction of a larger volume of tissue (some of which may be uninfected). qPCR assays in the probe hydrolysis format are less susceptible to the nonspecific amplification seen in the SYBR Green format. In the present study, the cut-off Cq value was conservatively set at 38.0. The results showed that the proportion of samples declared positive for CLas with the rapid and simple TPE-qPCR was not significantly different from the much more laborious and expensive X-qPCR. Although the overall rate of detection was not different, the TPE-qPCR estimated higher CLas populations than X-qPCR for equivalent samples in the study; this is in contrast to the results of Bertolini *et al.* (2014). In addition, the proportion of samples from symptomless leaves that tested positive for CLas in the present study was higher than found by Bertolini *et al.* (2014). Both TPE-qPCR and immunotissue printing detected CLas in symptomless tissues, although the proportion was lower than from leaves with symptoms.

The simplicity of TPE-qPCR also greatly reduces the cost of the assay, the possibility of cross-contamination among samples, and reduces the environmental impact of testing by eliminating hazardous laboratory waste. Of particular interest, sampling by TPE-qPCR can be effectively and rapidly scaled and applied to extensive HLB screening or to exhaustive sampling of individual suspect trees. This could be especially useful if an HLB-suspect tree is identified in an area previously free of the disease and pathogen. The tissue sections cut to expose fresh tissue surfaces for TPE-qPCR can be saved for later DNA extraction for confirmatory testing, limiting laborious extraction to samples with a very high likelihood of being positive.

Although the overall detection rate for CLas by TPE-qPCR was higher than by immunoassay on the same sample set, the results were highly correlated. The immunoassay requires an intact CLas membrane to properly display the OmpA antigen. In contrast, PCR-based methods detect DNA, which is present in HLB-affected trees in both living and dead cells (Hu *et al.*, 2014). This effect may contribute to a higher detection rate for TPE-qPCR than for the immunoassay in these experiments. However, there were about 6% of samples declared positive by immunodetection but not TPE-qPCR, which was probably due to the sporadic distribution of CLas and the fact that the immunoassay performs very well in symptomless tissues (Ding *et al.*, 2017). The results of this study indicate that greater success in detection of CLas will be achieved by using PCR-based and serological methods together.

## Acknowledgements

This research was funded by Fundamental Research Funds for the Central Universities (XDJK2018C032), National Key R&D Program of China (2018YFD0201500), Postdoctoral Fund of China (2018M633307) and National Natural Sciences Foundation of China (31671992). The authors have no conflicts of interest to declare.

## References

Bertolini E, Felipe RTA, Sauer AV *et al.*, 2014. Tissue-print and squash real-time PCR for direct detection of '*Candidatus* Liberibacter' species in citrus plants and psyllid vectors. *Plant Pathology* **63**, 1149–58.

Bové JM, 2006. Huanglongbing: a destructive, newly-emerging, century old disease of citrus. *Journal of Plant Pathology* **88**, 7–37.

Cassab GI, Varner JE, 1987. Immunocytolocalization of extensin in developing soybean seed coats by immunogold-silver staining and by tissue printing on nitrocellulose paper. *Journal of Cell Biology* **105**, 2581–8.

Chung KR, Brlansky R, 2005. *Citrus Diseases Exotic to Florida: Huanglongbing (Citrus Greening)*. Plant Pathology Department Fact Sheet PP-210. Gainesville, FL, USA: Institute of Food and Agricultural Sciences, University of Florida.

Coletta-Filho HD, Targon MLPN, Takita MA, Negri JDD, Pompeu J, Machado MA, 2004. First report of the causal agent of huanglongbing ('*Candidatus* Liberibacter asiaticus') in Brazil. *Plant Disease* **88**, 1382.

Ding F, Wang G, Yi G, Zhong Y, Zeng J, Zhou B, 2005. Infection of wampee and lemon by citrus huanglongbing pathogen ('*Candidatus* Liberibacter asiaticus') in China. *Journal of Plant Pathology* **87**, 207–12.

Ding F, Duan Y, Paul C, Brlansky RH, Hartung JS, 2015. Localization and distribution of '*Candidatus* Liberibacter asiaticus' in citrus and periwinkle by direct tissue blot immuno assay with an anti-OmpA polyclonal antibody. *PLoS ONE* **10**, e0123939.

Ding F, Duan Y, Yuan Q, Shao J, Hartung JS, 2016. Serological detection of '*Candidatus* Liberibacter asiaticus' in citrus, and identification by GeLC-MS/MS of a chaperone protein responding to cellular pathogens. *Scientific Reports* **6**, 29272.

Ding F, Paul C, Brlansky RH, Hartung JS, 2017. Immune tissue print and immune capture-PCR for diagnosis and detection of *Candidatus* Liberibacter asiaticus. *Scientific Reports* **7**, 46467.

Folimonova SY, Achor DS, 2010. Early events of citrus greening (huanglongbing) disease development at the ultrastructural level. *Phytopathology* **100**, 949–58.

Fu SM, Hartung JS, Zhou CY, Su HN, Tan J, Li ZA, 2015. Ultrastructural changes and putative phage particles observed in sweet orange leaves infected with '*Candidatus* Liberibacter asiaticus'. *Plant Disease* **99**, 320–4.

Garnier M, Bové J, 1983. Transmission of the organism associated with citrus greening disease from sweet orange to periwinkle by dodder. *Phytopathology* **73**, 1358–63.

Garnier M, Danel N, Bove JM, 1984. The greening organism is a Gram negative bacterium. In: Garnsey SM, Timmer LW, Dodds JA, eds. *Proceedings of the 9th Conference of the International Organization of Citrus Virologists, 1984*. University of California, Riverside, CA, USA: International Organization of Citrus Virologists, 115–24.

Garnsey SM, Permar TA, Cambra M, Henderson CT, 1993. Direct tissue blot immunoassay (DTBIA) for detection of citrus tristeza virus (CTV). In: Moreno P, Da Graca JV, Timmer LW, eds. *Proceedings of the 12th Conference of the International Organization of Citrus Virologists, 1993*. University of California, Riverside, CA, USA: International Organization of Citrus Virologists, 39–50.

Gottwald TR, Miller C, Brlansky RH, Gabriel DW, Civerolo EL, 1989. Analysis of the spatial distribution of citrus bacterial spot in a Florida citrus nursery. *Plant Disease* **73**, 297–303.

Graham JH, Johnson EG, Gottwald TR, Irey MS, 2013. Presymptomatic fibrous root decline in citrus trees caused by huanglongbing and potential interaction with *Phytophthora* spp. *Plant Disease* **97**, 1195–9.

Hu H, Roy A, Brlansky RH, 2014. Live population dynamics of '*Candidatus* Liberibacter asiaticus', the bacterial agent associated with citrus huanglongbing, in citrus and non-citrus hosts. *Plant Disease* **98**, 876–84.

Jagoueix S, Bové JM, Garnier M, 1996. PCR detection of the two '*Candidatus*' Liberobacter species associated with greening disease of citrus. *Molecular and Cellular Probes* **10**, 43–50.

Johnson EG, Wu J, Bright DB, Graham JH, 2014. Association of '*Candidatus* Liberibacter asiaticus' root infection, but not phloem plugging with root loss on huanglongbing-affected trees prior to appearance of foliar symptoms. *Plant Pathology* **63**, 290–8.

Li W, Hartung JS, Levy LE, 2006. Quantitative real-time PCR for detection and identification of *Candidatus Liberibacter* species associated with citrus huanglongbing. *Journal of Microbiological Methods* **66**, 104–15.

Li W, Li D, Twieg E, Hartung JS, Levy L, 2008. Optimized quantification of unculturable *Candidatus* Liberibacter spp. in host plants using real-time PCR. *Plant Disease* **92**, 854–61.

Li W, Levy L, Hartung JS, 2009. Quantitative distribution of '*Candidatus* Liberibacter asiaticus' in citrus plants with citrus huanglongbing. *Phytopathology* **99**, 139–44.

Morgan JK, Zhou L, Li W, Shatters RG, Keremane M, Duan YP, 2012. Improved real-time PCR detection of '*Candidatus* Liberibacter asiaticus' from citrus and psyllid hosts by targeting the intragenic tandem-repeats of its prophage genes. *Molecular and Cellular Probes* **26**, 90–8.

Mullendore DL, Windt CW, Van As H, Knoblauch M, 2010. Sieve tube geometry in relation to phloem flow. *The Plant Cell* **22**, 579–93.

Nath D, Husain MA, 1927. The citrus psylla (*Diaphorina citri*, Kuw.) [Psyllidae: Homoptera]. *Memoirs of the Department of Agriculture in India* **10**, 1–27.

Okuda M, Matsumoto M, Tanaka Y, Subandiyah S, Iwanami T, 2005. Characterization of the *tufB-secE-nusG-rplKAJL-rpoB* gene cluster of the citrus greening organism and detection by loop-mediated isothermal amplification. *Plant Disease* **89**, 705–11.

Olmos A, Dasí MA, Candresse T, Cambra M, 1996. Print-capture PCR: a simple and highly sensitive method for the detection of *Plum pox virus* (PPV) in plant tissues. *Nucleic Acids Research* **24**, 2192–3.

Pagliaccia D, Shi J, Pang Z *et al.*, 2017. A pathogen secreted protein as a detection marker for citrus huanglongbing. *Frontiers in Microbiology* **8**, 2014.

Roistacher CN, 1991. *Graft Transmissible Diseases of Citrus – Handbook for Detection and Diagnosis*. Rome, Italy: Food and Agriculture Organization of the United Nations.

Shi J, Pagliaccia D, Morgan R *et al.*, 2014. Novel diagnosis for citrus stubborn disease by detection of a *Spiroplasma citri*-secreted protein. *Phytopathology* **104**, 188–95.

Tatineni S, Shankar Sagaram U, Gowda S *et al.*, 2008. *In planta* distribution of '*Candidatus* Liberibacter asiaticus' as revealed by polymerase chain reaction (PCR) and real-time PCR. *Phytopathology* **98**, 592–9.

Taylor R, Inamine G, Anderson JD, 1993. Tissue printing as a tool for observing immunological and protein profiles in young and mature celery petioles. *Plant Physiology* **102**, 1027–31.

Villechanoux S, Garnier M, Renaudin J, Bové JM, 1992. Detection of several strains of the bacterium-like organism of citrus greening disease by DNA probes. *Current Microbiology* **24**, 89–95.

Wang X, Doddapaneni H, Chen J, Yokomi RK, 2015. Improved real-time PCR diagnosis of citrus stubborn disease by targeting prophage genes of *Spiroplasma citri*. *Plant Disease* **99**, 149–54.

## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

**Figure S1**. Preparation of samples for tissue print elution (TPE)-qPCR and immunoassay. (a) Either stem (left) or petiole (right) can be chosen for tissue prints. The freshly cut end of the tissue is pressed to the nitrocellulose membrane and held for 5 s. After each imprint is made, a fresh surface is exposed by removing a 0.5 mm slice. Tissue sections are saved. (b) Ten tissue prints are made alternately onto two membranes. On one membrane, the prints are overlapping and used for TPE-qPCR and on the other membrane, the prints are made separately for immunoassay. (c) Tissue prints for TPE-qPCR are cut and placed in a 1.5 mL centrifuge tube and 20 μL glycine buffer (0.1 M glycine, 0.05 M NaCl, 1 mM EDTA; pH 9) is added. (d) Separate prints on the other membrane are processed with anti-OmpA antibody. (e) The 10 tissue sections are collected in a tube and total DNA is extracted with a QIAGEN DNeasy kit. DNA is eluted in 20 μL TE and 3 μL is used per qPCR.

**Data S1.** Statistical analysis of qPCR assay for '*Candidatus* Liberibacter asiaticus' and plant mitochondrial DNA.

**Data S2.** Chi-square analysis of results of TPE-qPCR and X-qPCR for the detection of '*Candidatus* Liberibacter asiaticus' in infected citrus.

**Data S3.** ANOVA of results of the comparison of different brands of nitrocellulose membranes for the detection of '*Candidatus* Liberibacter asiaticus' by TPE-qPCR in infected citrus.

**Data S4.** ANOVA of results of the comparison of different brands of nitrocellulose membranes for the detection of plant cytochrome oxidase (*COX1*) DNA by TPE-qPCR in infected citrus.