Hort Innovation

Final Report

A Review of Diagnostic Technologies to Benefit the Australian Nursery Industry

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Content

Summary

Recommendations were made for the development, evaluation, validation and adoption of the point-of-care and lab based diagnostic tools, that when used appropriately, will minimize the impact of pathogens and diseases and increase the biosecurity of the Australian nursery industry.

The top ten endemic pathogens or pathogen groups of significance across the broad range of nursery commodities and included: *Pseudomonas* sp., *Xanthomonas* sp*., Calonect*ria sp. (*Cylindrocladium* sp*.), Colletotrichum* sp., *Fusarium* sp., *Phytophthora* sp., *Pythium* sp., *Rhizoctonia* sp., *Potyvirus* sp. and *Orthotospovirus* sp. The methods currently available for the detection of these pathogens include traditional isolation methods such as plating onto media, serology-based tests including enzyme linked immunosorbent assays (ELISA) or dipstick (lateral flow devices; LFD) assays and molecular methods such as polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA).

Current and new smart surveillance, field deployable diagnostic technologies that are recommended for further developed for detection of the top ten endemic plant pathogens include field-based PCR, LAMP and RPA. Exploration of a route to market was recommended for simple tests such as aggregation of functionalized colloidal gold particles that result in a colour change in the presence of a pathogen. Depending upon the gene target, LAMP tests may add greater specificity for detection, compared to RPA, PCR and aggregation, and may be more applicable at the pathogen species, strain or pathovar level.

All field deployable methods could be used in laboratories as a first point of diagnosis and may also hasten diagnostic turnaround times when in-field testing is impracticable. PCR and ELISA will continue to be important for rapid, low and high throughput laboratory-based diagnostics for pathogen detection and development of these tools to support plant pathogen diagnostic testing should continue. Rapid, laboratory-based, metagenomic and metabarcoding next generation sequencing (NGS) tools should be developed to identify broad groups of pathogens or for the specific detection of individual genera, species and strains/pathovars of important to the nursery industry. This technology is becoming be an important tool to resolve diseases of unclear aetiology and for testing samples for a broad range of pathogens. These in-house tests need to be verified as fit for purpose, quality assured and require validation to determine their analytical sensitivity, analytical specificity, repeatability and reproducibility. Nanopore also NGS has the capacity to be deployed in the field. Development and training in techniques to prepare samples for laboratory testing for use by growers, consultants, biosecurity officers and field-based diagnosticians could facilitate laboratory workflows and improve turnaround times, where a laboratory diagnosis is required.

It is recommended that “omics” based approaches (genomic, proteomic, metabolomic) are used to understand pathogen diversity to identify appropriate targets for pathogen detection and ensure reliability of tests.

Field-based PCR, LAMP and RPA tools have a route to market that can be driven by commercial companies that supply off-the-shelf test kits, and further research can build upon current developments in this area. Therefore, they are recommended as a high priority for further development for detection of pathogens significant to the nursery industry. Route to market for some laboratory-based tests such as PCR and NGS technology could be via in-house development, without the need for a commercialisation partner, to facilitate pathogen diagnosis, where commercial tests do not exist, and for complex diagnoses and high throughput diagnostic testing. Continued research to develop and maintain PCR and NGS capability in Australian plant diagnostic laboratories is also recommended. These diagnostic approaches will facilitate the provision of a rapid diagnosis to improve the biosecurity of the Australian nursery industry.

Keywords

Field deployable, surveillance, laboratory, polymerase chain reaction, PCR, loop-mediated isothermal amplification, LAMP, next generation sequencing, NGS, lateral flow device, LFD, serology, molecular, functionalized colloidal gold particles, remote sensing.

Introduction

The nursery industry extends across a broad range of commodities which include monocot and dicot plant species that may be grasses, herbaceous or woody and traded as seed, seedlings, cuttings, tubestock, or mature plants. The industry comprises of the following production nursery types who supply ornamental, fruit, nut, and vegetable species, including introduced and native plant species, for domestic and commercial purposes (Plant Health Australia Ltd, 2006):

A. Fruit and nut tree stock for orchardists
B. Seedling stock for vegetable growers
C. Container stock for ornamental/urban horticulture
D. Landscape stock for domestic and commercial projects
E. Forestry stock for plantation timber
F. Plug and tube stock for cut flower
G. Revegetation stock for farmers, government, landcare and development industry
H. Foliage plants for interior-scapes
I. Mine revegetation for mine site rehabilitation

Each of these nursery commodities have specific endemic and exotic pests including insects, viruses, viroids, bacteria, fungi, oomycetes and nematodes that threaten the biosecurity of nurseries and the biosecurity of their client's production systems through trade and movement of contaminated plants and plant products. Some pests may be restricted to one or a few plant hosts while others, such as *Xylella fastidiosa*, have a broad host range and can impact across many nursery types and horticultural industries.

The Australian nursery industry has developed "guidelines for managing biosecurity in nursery production" (Biosecure HACCP) to manage production risks associated with pest and disease. Biosecure HACCP has been put in place due to pressures associated with intra- and interstate trade, emergency plant pest incursions and requirements to provide evidence of absence of exotic plant pests. It also supports best management practices to ensure profitability, productivity, sustainability and professionalism within the nursery industry. Reliable and affordable diagnostic tools are needed to support Biosecure HACCP and provide early detection of plant pests that threaten nursery production.

Although some pests and diseases can be diagnosed visually, observation of symptoms may mean that the disease is already well established and at greater risk of dissemination. In other cases, symptoms alone are not a reliable indicator of the presence of a pest as multiple factors, both biotic and abiotic, can cause similar symptoms. Some important pests may be symptomless depending upon the plant host species or variety infected or due to environmental conditions. Rapid diagnostics, applied in the laboratory or in-field within the production system, can assist in early control to prevent further spread, even before disease is observed. Recent examples of outbreaks of exotic pathogens, in which nurseries have been negatively impacted, include quarantine pests such as *Cucumber green mottle mosaic virus* and *Potato spindle tuber viroid*. Pathogens like these, which are also seed transmitted, are often symptomless in young nursery stock/seedlings and there is a risk that a low-level contamination within a nursery might remain on surfaces and equipment regardless of the best hygiene practices. Such contamination and symptomless infections can go undetected in the nursery and may result in infection of future stock and further dissemination of the pests beyond the nursery.

Point-of-care diagnostics for routine smart surveillance of nurseries could assist in early detection and prevent movement of these pests onto growers' properties and provide real-time quality assurance of nursery stock to industry.

Smart surveillance tools to facilitate the rapid point-of-care detection in nursery production systems can be used to support nursery industry Biosecure HACCP plans. These diagnostic tools will assist the industry to take greater responsibility and control for the plant health during production and reduce the risk of dissemination of high risk endemic and exotic pests into the wider horticultural community. They can provide an early diagnosis, enabling management strategies to be implemented at an early stage to contain or eradicate a pest, even if detection requires further

verification by an external laboratory. They can also be applied during or after incursion management to monitor the success of the eradication or containment procedures and provide evidence of absence in a previously contaminated production system. Smart surveillance tools can also be applied at the border or during post entry quarantine to facilitate rapid screening of small and large volume plant imports for known quarantine pests such as *X. fastidiosa*. Smart surveillance tools could include techniques that specifically target an individual or group of pests or pathogens. Smart sensor tools that detect and analyse a specified set of plant symptoms could be routinely applied for routine monitoring of plant health and used as an early warning system for disease. Laboratory embedded technologies continue to be developed and improve the capacity to accurately identify known and novel pests and pathogens associated with plant disease.

Although new technologies can improve early detection of a pest, applying a plant pest diagnostic tool with accuracy is dependent on several factors including knowledge of pest biology to assist accurate sampling and an understanding the sensitivity and specificity of the assay. Specific tests are useful when there are a limited number of pests that require surveillance. However, in some situations there may be more than one pest requiring detection, such as the multiple viruses that infect Prunus species in Australia and overseas and which require testing for at the Australian border or within nursery certification programs

This project was undertaken to identify point-of-care smart surveillance and laboratory-based tools for detection of plant pests which can be developed to support efficient and sustainable nursery production and reduce risk of pathogen and disease incursion and spread in Australia. The intention was to find Point of care tools that are user friendly, can be applied and interpreted by personnel with all levels of experience and understanding and laboratory-based tools that can be used to develop a world's best practice diagnostic capability resulting in accurate and rapid diagnosis of plant pests.

Methodology

A small project reference group was established and engaged to provide direction, advice and information to support the project. The composition of the reference group was:

- John Bunker, Greenlife Solutions - member of the Nursery Industry Strategic Investment Advisory Panel
- John MacDonald - the Nursery Industry Biosecurity Manager
- Dr. Brenda Kranz - the Hort Innovation R&D Manager
- Dr. Len Tesoreiro, NSW PDPI - field pathologist with diagnostic experience
- Dr. Victoria Ludowici a Plant Health Australia representative
- Dr. Helen Hayden, Agriculture Victoria - soil/substrate nucleic acid specialist
- Dr Julian Druce, Victorian Infectious Diseases Reference Laboratory, Doherty Institute - medical diagnostics specialist

The committee convened in September 2017 to discuss the program logic provide guidance for the direction of the project. In July 2018 the committee members were presented with a discussion paper, including a summary of the technology and recommendations from the desk top review for ground truthing and broad feedback on the project outputs and findings to ensure that they were feasible, practical and applicable to the nursery industry.

The specific needs for each of the major nursery commodities including the high priority endemic and exotic pests for which tests are required was identified by:

- Engaging with the nursery industry (NGIA), Plant Health Australia, Department of Agriculture and Water Resources and with plant pathologists and diagnosticians in each state.
- Engaging with other project teams (NY15002 Building the resilience and on-farm biosecurity capacity of the Australian production nursery industry; NY15004 National Nursery Industry Biosecurity Program)
- Reviewing previous projects (NY11001 Plant health biosecurity, risk management and capacity building for the nursery industry) and the Nursery Industry Biosecurity Plan.

- Considering the requirements of the National Plant Biosecurity Strategy 2015–2020 Implementation Plan.

A desk top review of scientific literature, including peer reviewed papers, previous diagnostics-based projects (e.g. BS11008; MT12005) and information from commercial scientific companies, was done to identify:

- Current, new and emerging pests of concern to the nursery industry
- Gaps in the understanding of the pests that would otherwise enable improved diagnostic capabilities – especially for on-farm smart surveillance capabilities
- The methodologies currently used for the detection of these pests and the endemic and exotic pests identified by industry as high priority
- Current and new technologies, which may include technology used medical and veterinary diagnostics, that are not used but could be applied for their detection
- A summary of all applicable detection methods for pest and pathogens including:
- Describing the detection methodology including equipment, positive controls, laboratory facilities and sample type, collection and preparation from different substrates such as water, various plant growing mediums, different plant tissues such as seed, tubers, leaves etc
- Accuracy, reliability and sensitivity – risk of false positives and negatives
    - Specificity for strain, species, genera, family of a pest
    - Ease of use – skill level and quality control requirements
    - Results output (yes/no or quantitative) and ease of interpretation
    - Turnaround times from sample collection until a result is achieved and the requirement for further testing for verification of a result
    - Benefits and pitfalls of different technologies
    - Cost of equipment and kits and cost per test/sample
    - At what point can each of the technologies be applied – field or laboratory
- The methodology/technology that is fit-for-purpose for point-of-care and/or laboratory-based detection
- Next generation sequencing (NGS) approaches for screening nursery material will be reviewed and compared, including the diverse approaches to sample preparation and sequence analysis that are used by different laboratories. The most appropriate NGS strategies for routine laboratory-based that can be used for screening of different nursery plant species will be identified.
- Determine how users of point-of-care diagnostic technologies can be supported if the tests don't perform as expected or fail
- "What's next?" – what type of development, verification and validation is required to implement point-of-care and laboratory-based tests for the endemic and exotic pests of interest to the nursery industry.

Outputs

The review (Appendix 1) identified pathogens that affect a broad group of nursery commodities and include: *Peudomonas* sp., *Xanthomonas* sp*., Calonect*ria sp. (*Cylindrocladium* sp.*), Colletotrichum* sp., *Fusarium* sp., *Phytophthora* sp., *Pythium* sp., *Rhizoctonia* sp., *Potyvirus* sp. and *Orthotospovirus* sp. The review identified tools currently used for detection of these pathogens, including visual identification, isolation, serology and molecular methods. The review also identified future development of novel diagnostic laboratory and field-based smart surveillance tools smart for detection of pathogens.

Recommendations were made for the development of and future investment in diagnostic capabilities and tools for field and laboratory-based detection of endemic and exotic pathogens, which are important to the nursery industry.

Activities included:

- Establishment of a reference group & key stakeholders to advise, update and evaluate research
- Development of plan established to guide the project activities and outcomes
- Informing of Stakeholders project aims and objectives via a published preliminary project overview
- Identifying diagnostic needs of major nursery commodities to inform future diagnostic development activities
- Developing a list of priority endemic and exotic (max. 10) pathogens and their biology to assist with biosecurity planning
- An overview of smart surveillance tools that are available or which can be developed for point-of-care and laboratory-based detection
- Informing Stakeholders of project key findings and outputs via a published end of project industry-focused summary

Outcomes

This project provided recommendations to the Australian nursery industry for the future investment in the development, evaluation, validation and adoption of the point-of-care and lab based diagnostic tools, that when used appropriately, will minimize the impact of pests and diseases and increase the biosecurity of the Australian nursery industry.

The project improved knowledge and understanding of biosecurity and diagnostic tools for detection of pathogens associated with diseases of significance to the Australian nursery industry and led to:

- Understanding of the diagnostic needs of major nursery commodities
- Updated knowledge of important endemic and exotic pathogens and their biology
- Improved understanding of point-of-care and laboratory-based smart surveillance tools

Recommendations were made for the future investment in the development, evaluation, validation and adoption of the point-of-care and lab based diagnostic tools, that when used appropriately, will minimize the impact of pathogens and diseases and increase the biosecurity of the Australian nursery industry.

Monitoring and evaluation

A program logic and monitoring and evaluation plan was developed and included linkage to Horticulture Innovation and industry/fund objectives, a project risk register and risk management plan, and a stakeholder engagement/communication plan.

The program logic detailing the relevant SIP outcome(s), end-of-project outcomes, intermediate outcomes, outputs, activities, foundational outputs and foundational activities was developed using the Hort Innovation model. The end of project outcomes will be "Recommendations to the Australian nursery industry for the future investment in the development, evaluation, validation and adoption of the point-of-care and lab based diagnostic tools, that when used appropriately, will minimize the impact of pests and diseases and increase the biosecurity of the Australian nursery industry."

The program logic was discussed in detail at the first project reference group meeting and was updated after clarification and guidance for the direction of the project were provided.

Further evaluation was done by providing a summary of the lab and point-of-care user-friendly smart surveillance tools to the project reference group so that broad feedback on project findings and ground-truthing of the outcomes and recommendations of the project could be sought. This process ensured that the project met the needs of industry levy payers.

The broader industry levy payers were engaged through communications though several online publications (https://yourlevyatwork.com.au/project-puts-pathogen-detection-in-the-palm-of-your-hand/ and http://horticulture.com.au/hortlink-2018-edition-1/nursery/#12).

Ad hoc communication about the project also occurred during various science and industry-based meetings and client interactions. Engagement included commercial nurseries, high health improvement programs, industry bodies and scientists.

Recommendations

Findings

In consultation with industry, diagnosticians, biosecurity agencies and research scientists, this project has:

1. Assessed the biological parameters of the high priority endemic and exotic pathogens (bacteria, fungi, nematodes, viruses and viroids) that threaten nursery production.
2. Identified laboratory-based and field deployable diagnostic tests that are currently used for their detection
3. Identified new point-of-care technologies that could be developed to improve early detection key pests
4. Reviewed the applicability of the smart surveillance tools to the nursery industry.
5. Identified and evaluated new lab-based technologies (e.g. next generation sequencing) to screen nursery material entering Australia at the border and during PEQ.

A review of the specific needs for each of the major nursery commodities identified the top ten endemic pathogens or pathogen groups of significance across the broad range of nursery commodities and included: *Pseudomonas* sp., *Xanthomonas* sp., *Calonect*ria sp. (*Cylindrocladium* sp.), *Colletotrichum* sp., *Fusarium* sp., *Phytophthora* sp., *Pythium* sp., *Rhizoctonia* sp., *Potyvirus* sp. and *Orthotospovirus* sp. The current and new diagnostic technology identified in the review could also be applied to these and other bacterial, fungal, viral, insect and nematode groups.

The methods currently available for detection of the top ten pathogens include traditional isolation methods such as plating onto media, serology-based tests, including enzyme linked immunosorbent assays (ELISA) or lateral flow devices (LFDs; dipsticks), and molecular methods such as polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP) and recombinase

polymerase amplification (RPA).

Serological tests have been developed for many, but not all, of the top ten groups of endemic pathogens of the identified pathogen groups, at the pathovar, species or genus level (Appendix 1). Although ELISA tests may have been developed for some pathogens, serological lateral flow tests (dipsticks; LFD) are not always available. The reason for this is unclear but may be associated with a lack of demand or low sensitivity of serology tests, especially for fungal and bacterial pathogens, and the prohibitive costs in generating "target specific" antibodies. Never-the-less, serology tests, especially LFDs, offer some of the simplest, robust and user friendly diagnostic assays and further consideration to their future development is warranted. The route to market for this technology may be complex due to the difficulty to identify protein targets for test development of specific pathogen groups or species compared to tests designed against DNA or RNA targets.

Many generic and specific PCR tests exist for the pathogens listed in Appendix 1, Table 2, but they are currently in the realm of the laboratory diagnostic testing. There have been some significant advances in PCR based technology with the development of field deployable PCR devices that could use the same well validated laboratory-based tests, and which could be provided as pre-prepared kits that are easy to use. However, the molecular technology may still be too expensive for individual growers to employ. Field-based PCR technology may be useful for biosecurity agencies during an incursion or at ports, or for Agricultural/horticultural consultants supplying pest and disease diagnoses or for large nurseries with the capacity to invest in the technology.

Other molecular tests that could easily be deployed to growers, consultants, biosecurity officers and diagnosticians include isothermal amplification technology (LAMP and RPA). These molecular field deployable diagnostics are a primary focus of Australian plant diagnostic and biosecurity researchers. LAMP technology has been developed locally to detect viruses, viroids, bacteria and insects in Australia. This knowledge and experience could be built upon for the development of tests that are specifically designed to meet the demands of the Australian nursery industry Field-based PCR, LAMP and RPA tools have a route to market through commercial companies who provide off-the-shelf test kits, making them highly desirable for further development.

Laboratory-based PCR and ELISA will remain important for rapid, low and high throughput diagnostics, but field deployable methods could easily be used as frontline, triage diagnostic tools to rule in or out pathogens and determine the next diagnostics steps. They may also hasten laboratory turnaround times and assist when in-field testing is impracticable. Rapid metagenomic and metabarcoding next generation sequencing is emerging as a important tool in plant pathology and can be developed to specifically identify genera, species and strains/pathovars of individual and groups of pathogens important to the nursery industry. To facilitate laboratory workflow, where a laboratory diagnosis is required, development and training in techniques for grower prepared samples could improve laboratory turnaround times. Route to market for some laboratory-based tests such as PCR and NGS could be via in-house development, without the need for a commercialisation partner, facilitating access to fit for purpose tests where commercialisation isn't possible.

A novel test that could be further developed for industry could use aggregation of functionalized colloidal gold particles, which may provide a simple visual detection method for specific pathogens or pathogen groups without the requirement for complex equipment. The route to market is unclear but could be explored.

All technology will require an "omics" based approached (genomic, proteomic, metabolomic) to understand pathogen diversity to identify appropriate targets for pathogen detection. The tests need to be verified as fit for purpose, quality assured and require validation to determine their analytical sensitivity, analytical specificity, repeatability and reproducibility.

Recommendations:

The following recommendations were made to aid industry investment in the future development, evaluation, validation and adoption of the point-of-care and lab based diagnostic tools, that when used appropriately, will minimize the impact of pathogens and diseases and increase the biosecurity of the Australian nursery industry. The pathogens identified for development of diagnostic tests

include *Pseudomonas* sp., *Xanthomonas* sp*., Calonect*ria sp. (*Cylindrocladium* sp*.), Colletotrichum* sp., *Fusarium* sp., *Phytophthora* sp., *Pythium* sp., *Rhizoctonia* sp., *Potyvirus* sp. and *Orthotospovirus* sp.

1. Primary investment should be made in diagnostic tools with that can be developed for all pathogens and samples types, including different plant tissues, planting/growth substrates and water. These tools should have a clear route to market, via commercial partners or in-house through equipped research and diagnostic laboratories that can meet quality assurance and control standards.
2. Research partners should include Australian researchers and diagnostic laboratories who have a good understanding of the biology and epidemiology of the pathogens and their detection under Australian conditions and, where applicable, in collaboration with commercial partners to drive a route to adoption and market for these tests.
3. Investment in development of new diagnostic technology must include development of appropriate and robust sampling and sample preparation strategies:
    a. Reliable detection is based on appropriate samples and there must be investment in research to develop appropriate sampling strategies that are most likely to return a reliable result.
    b. Investment should be in simple and robust sample preparation techniques to support accurate diagnosis, which includes commercially available sample preparation kits as well as crude/manual procedures that may not require a commercial partner for development and availability.
4. Field deployable tools with broad applicability to pathogen and sample type should be the primary area of development as there is good opportunity to develop a route to market through commercial providers of off-the-shelf test kits. These, include:
    a. Investment in loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) technology as a high priority due to its applicability to a broad range of end-users and a higher demand for the product to drive route to market.
    b. Investment in field deployable polymerase chain reaction PCR as there is also a good opportunity to develop a route to market for this technology, although the route to adoption by growers may be lower due to cost. This technology could have greater adoption by consultants, biosecurity agencies and field-based diagnosticians. An advantage of this technology is the ability to translate current and validated laboratory-based real-time PCR assays that are used for detection of pathogens in the top ten pathogen groups to the field-based device. Investment in this area is lower priority as the end -user group may not be broad, and demand for the product may be lower.
    c. A preliminary investigation into the reliability aggregation of functionalized colloidal gold particles to determine if it can be adapted for detection of specific bacteria and oomycetes in nursery water sources. The route to market requires further investigation prior to investment in this area.
5. An investment should be made for the development of current and emerging laboratory-based technology to facilitate faster diagnoses, especially for complex diagnoses, high throughput testing and confirmation of field-based tests. These tests have an obvious route to market through in-house laboratory development and delivery, and which does not require a commercial provider of test kits to drive the route to market. The technology includes:
    a. Investment in next generation sequencing (NGS) based diagnostic tests, especially nanopore technology
    b. Investment in tailored, end-user focused “Targeted diagnostics by sequencing” approaches for specific pathogen groups
    c. PCR and/or PCR array technology for low and high sample throughput testing – PCR technology can be linked to field-deployable PCR for consistency in detection and identification of pathogens in the lab and at pint of care.

6. As a part of test development investment in research using “Omics” approaches (genomics, proteomics, metabolomics) is required to understand the diversity of pathogen groups requiring detection and identify nucleic acid, protein, peptides or other targets for detection of pathogens at a genus, species, pathovar or strain level. This will ensure test reliability.
7. A preliminary project to demonstrate and train a focus group in similar available methods to assess demand and determine a provide a proof of concept that tests are easily deployable to users of all levels of experience.

References

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Intellectual property, commercialisation and confidentiality

Outputs arising from the project including the “Review of the specific needs for each of the major nursery commodities including the high priority endemic and exotic pathogens” are freely available to be shared with relevant nursery industry and state and national research, diagnostic and biosecurity agencies. There are no commercialistion or confidentiality issues to report.

Acknowledgements

The project team thanks industry, diagnostic and researchers for valuable comments about the needs and practicality of tools to support disease diagnosis for the nursery industry.

Appendix 1. A review of the specific needs for each of the major nursery commodities including the high priority endemic and exotic pathogens

1 Background

The nursery industry extends across a broad range of commodities which include monocot and dicot plant species that may be grasses, herbaceous or woody and traded as seed, seedlings, cuttings, tubestock, or mature plants. Each of these nursery commodities have specific endemic and exotic pests including insects, viruses, viroids, bacteria, fungi, oomycetes and nematodes that threaten the biosecurity of nurseries and their clients during trade and movement of contaminated plants and plant products.

The development of smart surveillance tools will facilitate the rapid point-of-care detection of pest and disease in nursery production systems. They will support the Australian nursery industry Biosecure HACCP plans to manage production risks associated with pest and disease. However, it is likely that more complex laboratory-based tools will still be required to ensure an accurate diagnosis of the causal agents of disease and there are evolving technologies that can facilitate pathogen detection, where more than one pathogen requires testing, where discrimination between related species is complex or where disease aetiology is unclear. Development of accurate diagnostic field and laboratory-based tools is dependent upon knowledge of pest biology, to ensure accurate sampling and the sensitivity and specificity of the assay.

2 Aims

This project was undertaken to identify simple, accurate and rapid, point-of-care smart surveillance and laboratory-based tools for detection of plant pests which can be developed to support efficient and sustainable nursery production and reduce risk of pathogen and disease incursion and spread in Australia.

3 Method

A small project reference group was established and engaged to provide direction, advice and information to support the project. The committee convened in September 2017 to discuss the program logic provide guidance for the direction of the project. In July 2018 the committee members were presented with a discussion paper, including a summary of the technology and recommendations from the desk top review for ground truthing and broad feedback on the project outputs and findings to ensure that they were feasible, practical and applicable to the nursery industry.

Industry engagement, discussions with other ongoing projects and team and reviewing previous Hort Innovation funded projects was done to identify the specific needs for each of the major nursery commodities. The National Plant Biosecurity Strategy 2015–2020 Implementation Plan was also consulted. In addition, a desk top review of scientific literature, including peer reviewed papers, previous diagnostics-based projects (e.g. BS11008; MT12005) and information from commercial scientific companies, was done to identify pests of concern and the methodology/technology that is currently used or could be developed for point-of-care and/or laboratory-based detection of pests and pathogens of concern.

4 Results and discussion

Regardless of the breadth of plant species supplied by these production nurseries, a review of a broad range of plant pathology literature, previous research projects and discussions with industry highlighted some disease types and pathogens that are common amongst many Australian nursery commodities. Table 1 lists the commonly mentioned pathogen and pest types (bacteria, fungi, oomycete, nematodes and virus) that impact on nursery production in Australia. Many pathogens and pests in Table 1 are only listed at the genus level because there are a range of species within the genus that may cause disease on the same or different host plant species. For example, there are 160 virus species in the genus *Potyvirus* (Wylie et al 2017), the emergence of which is thought to have coincided with the development agriculture 6,600 years ago (Gibbs et al 2008). Some of the *Potyvirus* species have a broad host range; e.g. *Turnip mosaic virus* primarily infects a broad range of brassicas it can also infect many plant species and is thought to have moved from orchids into brassicas in Germany approximately 700 years ago (Yasaka et al 2017). Other *Potyvirus* species have more specific host ranges e.g. *Zucchini yellow mosaic virus, which* tends to be restricted to cucurbits (Desbiez and Lecoq 1997). For some pathogens listed to species level in Table 1, there are many pathovars, races or strains that may vary in their virulence and host specificity. For example, the *Pseudomonas syringae* complex has more than 60 pathovars (Baltrus et al 2017). *P. syringae* pv. *tomato* is limited to tomato but *P. syringae* pv. *syringae* infects many plant host species including tomato, although *P. s.* pv. *syringae* has pathogenic strains with distinct host specificities and some strains which are non-pathogenic (Monteil et al 2017; Ravindran et al 2015). It is important to note that many of the fungal and bacterial pathogens listed in Table 1 may have species, strains, pathovars or races that are non-pathogenic.

Table 1. The endemic bacteria, fungi, oomycete, nematode and virus genera and or species and the diseases that they are associated with which were identified in this review as being of concern the Australian nursery industry in this review.

Pathogen/pest type	Pathogen	Disease
Bacterium	*Acidovorax citrulli*	Bacterial fruit blotch
Bacterium	*Agrobacterium* sp.	Crown gall
Bacterium	*Brenneria* sp.	Bacterial Canker
Bacterium	*Clavibacter* sp.	Bacterial Canker
Bacterium	*Curtobacterium flaccumfaciens*	Wilt and blight
Bacterium	*Dickeya* sp.	Soft rot
Bacterium	*Erwinia* sp.	Bacterial soft rots and Blight
Bacterium	*Pectobacterium* sp.	Soft rot
Bacterium	*Pseudomonas syringae*	Bacteria canker
Bacterium	*Ralstonia solanacearum*	Bacterial wilt
Bacterium	*Xanthomonas* sp.	Bacterial leaf spot
Fungus/oomycete	*Alternaria* sp.	Leaf spot and stem blight
Fungus/oomycete	*Armillaria luteobubalina*	Root rot
Fungus/oomycete	*Aspergillus* sp.	Fruit rots, systemic infection of onion seedlings
Fungus/oomycete	*Botrytis* sp.	grey mould
Fungus/oomycete	*Bipolaris* sp.	Leaf spot
Fungus/oomycete	*Calonectria* sp. (*Cylindrocladium* sp.)	Leaf spot, crown and root rot
Fungus/oomycete	*Cercospora* sp.	Leaf spot
Fungus/oomycete	*Chalara elegans* (*Thielaviopsis basicola*)	Root rot
Fungus/oomycete	*Cladosporium* sp.	Leaf spot

Pathogen/pest type	Pathogen	Disease
Fungus/oomycete	*Colletotrichum* sp.	Anthracnose
Fungus/oomycete	*Curvularia* sp.	Leaf spot
Fungus/oomycete	*Diplocarpon* sp.	Anthracnose
Fungus/oomycete	*Elsinoë* sp.	Anthracnose
Fungus/oomycete	*Erysiphe* sp.	Powdery mildew
Fungus/oomycete	*Fusarium* sp.	Root rot
Fungus/oomycete	*Leveillula* sp.	Powdery mildew
Fungus/oomycete	*Stagonosporopsis* sp. (inc. Syn. *Didymella bryoniae*)	Gummy stem blight
Fungus/oomycete	*Uredo rangelii*	Myrtle rust
Fungus/oomycete	*Oidium* sp.	Powdery mildew
Fungus/oomycete	*Olpidium* sp.	Virus vectors
Fungus/oomycete	*Pathogenalotiopsis* sp.	Leaf spot
Fungus/oomycete	*Phoma* sp.	Leaf spot and stem canker
Fungus/oomycete	*Phomopsis* sp.	Anthracnose
Fungus/oomycete	*Phyllosticta* sp.	Leaf spot
Fungus/oomycete	*Podosphaera* sp.	Powdery mildew
Fungus/oomycete	*Pseudocercospora* sp.	Downy mildew/ leaf spot
Fungus/oomycete	*Rhizoctonia* sp.	root and hypocotyl rot, damping off
Fungus/oomycete	Order Pucciniales (many family and genera)	Rusts
Fungus/Protist	*Septoria* sp.	Leaf spot
Fungus/Protist	*Sclerotinia* sp.	Root rot
Fungus/Protist	*Sclerotium* sp. (Including *Athelia rolfsii*)	Root rot
Fungus/Protist	*Sphaerotheca* sp.	Powdery mildew
Fungus/Protist	*Stemphylium* sp.	Leaf spot
Fungus/Protist	*Uncinula* sp.	Powdery mildew
Fungus/Protist	*Verticillium dahliae*	Wilt
Fungus/oomycete	*Aphanomyces* sp. (Oomycete)	Root rot
Fungus/oomycete	*Bremia lactucae* (Oomycete)	Downy mildew
Fungus/oomycete	*Peronospora* sp. (Oomycete)	Downy mildew
Fungus/oomycete	*Phytophthora* sp. (Oomycete)	Root rot
Fungus/oomycete	*Plasmopara* sp. (Oomycete)	Downy mildew
Fungus/oomycete	*Pythium* sp. (Oomycete)	Root rot
Nematode	*Aphelenchoides* spp.	Foliar
Nematode	*Meloidogyne* spp.	Root Knot
Nematode	*Pratylenchus* spp	Root lesion
Virus	Species: *Cucumber mosaic virus* (Genus: *Cucumovirus*)	Chlorosis, colour break, distortion, stunting, decline
Virus	Genus: *Crinivirus* (e.g. *Lettuce infectious yellows virus, Beet pseudo yellows virus*)	Chlorosis, colour break, distortion, stunting, decline
Virus	Genus: *Orthotospovirus* (e.g. *Tomato spotted wilt virus, Capsicum chlorosis virus,*	Chlorosis, colour break, distortion, stunting, decline

Pathogen/pest type	Pathogen	Disease
	Impatiens necritic spot virus, Iris yellow spot virus)	
Virus	Genus: *Pospiviroid* (e.g *Potato spindle tuber viroid, Citrus exocortis viroid*)	Chlorosis, colour break, distortion, stunting, decline
Virus	Genus: *Potexvirus* (e.g. *Potato virus X, Cymbidium mosaic virus*)	Chlorosis, colour break, distortion, stunting, decline
Virus	Genus: *Potyvirus* (*Potato virus Y, Turnip mosaic virus, Tulip breaking virus*)	Chlorosis, colour break, distortion, stunting, decline
Virus	Genus: *Tobamovirus* (e.g. *Tobaco mosaic virus, Tomato mosaic virus, Cucumber green mottle mosaic virus*)	Chlorosis, colour break, distortion, stunting, decline

Exotic pathogens of concern include but are not limited to: *'Candidatus* Liberibacter asiaticus', *Erwinia amylovora, Phymatotrichum omnivorum Phytophthora ramorum,. Xanthomonas citri* subsp. *citri, Xylella fastidiosa* and its insect vectors and *Plum pox virus.*

4.1 A review of pathogens of concern and the diagnostic technology used for their detection.

Based on the review of literature and discussions with industry and scientists, Table 2 lists the pathogens that were identified as being the top ten important pathogens that affect the broader nursery industry commodities. Table 2 also lists the availability of tests used for laboratory and field-based detection.

Table 2. The top 10 groups of endemic pathogens of importance to the nursery industry and the laboratory (Lab) and field tests that are currently used for their detection.

Pathogen type	Pathogen	Lab Isolation	Lab: Serology Test	Lab: Molecular test	Field: Serology test	Field: Molecular test	other
Bacteria	*Pseudomonas spp.*	Yes	ELISA: Agdia (www.agdia.com/): *P. savastani pv. phaseolicola* (Psph) Nano Diagnostics, Inc. (http://www.nanodiancs.com) *Acidovorax avena (P. avenae), P. fuscovaginae, P. glumae, P. savastanoi pv. savastanoi, P. syringae pv atrofaciens, P. s. pv glycinea, P. s. pv lachrymans, P. s. pv. phaseolicola, P. s. pv pisi, P. s. pv tomato*	Many specific PCR based tests available for species and pathovar. PCR to detect the *P. syringae* group (Gilbaud et al 2016). PCRs to differentiate amongst *Pseudomonas syringae* phylogroups (Borshinger et al 2015); Differentiation between pathovars using two genes (Lu et al 2017).	No	Isothermal amplification (LAMP and RPA) based assays for specific pathovars (e.g. PBCRC Project 2156: Deployment of validated genome-informed bacterial diagnostics; Lau et al 2016).	IDEXX Pseudalert for *P. aureginosa* (www.idexx.com.au/en-au/) Genome sequencing[b] (e.g. Martínez-García et al 2015 and others)

Pathogen type	Pathogen	Lab Isolation	Lab: Serology Test	Lab: Molecular test	Field: Serology test	Field: Molecular test	other
Bacteria	*Xanthomonas* spp.	Yes	AGDIA: Specific ELISAs: *X. albilineans, X. axonopodis pv. begoniae, X. axonopodis pv. dieffenbachiae, X. axonopodis pv. phaseoli, X. campathogenris pv. armoracieae, X. hortorum pv. pelargonii , X. oryzae pv. oryzae, X. hortorum pv. pelargonii;* Bacterial ELISA Reagent Set for *Xanthomonas* genus specific (Xan) ELISA reagent set[a]; Prime diagnostics specific ELISAS: *X. fragariae , X. axonopodis pv dieffenbachiae, X. hortorum pv. pelargonii* AGDIA, Nano Diagnostics Inc.: Immunoblot assay for *X. campathogenris*[a] .	Many specific PCR tests available for species and pathovar. PCR to detect the Genus (Adriko et al 2014). Prime diagnostics (www.primediagnostics.com): Luminex MagPlex-TAG® kits (van der Vlugt et al 2015;	Loewe *Xanthomonas hortorum* pv. *pelargonii* in suspected cultures and *Xanthomonas axonopodis* pv. *citri* (Xac) in field	Isothermal amplification (LAMP) based assays for specific pathovars (e.g.Bühlmann et al 2013; PBCRC 2156: Deployment of Validated Genome-Informed Bacterial Diagnostics).	Genome sequencing (e.g. Roach et al 2018, and others)
Fungus/oomycete	*Calonectria* sp. (*Cylindrocladium* sp.)	Yes	No	Specific PCR for *C. Pseudonaviculata* (Syn C. buxicola), *C. henricotiae* (Gehesquière et al	No	LAMP for specific species (Katoh et al 2016; Malapi Wright 2016)	LOaf disc bait assays for boxwood (Dart et al. 2014)

Pathogen type	Pathogen	Lab Isolation	Lab: Serology Test	Lab: Molecular test	Field: Serology test	Field: Molecular test	other
				2013, 2016; LeBlanc et al 2018) Generic fungal PCR tests exist, positive results require sequencing of the PCR product (White et al., 1990; O'Donnell & Cigelink, 1997; Crous et al., 2004) Genome sequencing (e.g. Ye et al 2018)			
Fungus/oomycete	*Colletotrichum* sp.	Yes	Nano Diagnostics, Inc.: Genus based ELISA test and a specific test for *C. acutatum*	Many specific PCR tests exist. Generic fungal PCR tests exist, positive results require sequencing of the PCR product. http://www.q-bank.eu/fungi/DefaultInfo.aspx?Page=PrimerList	No	LAMP for specific species (e.g. Wang et al 2017)	Genome sequencing (e.g. Liang et al 2018) Metabarcoding Da Lio et al 2108
Fungus/oomycete	*Fusarium* sp.	Yes	Creative Diagnostics (www.creative-diagnostics.com/): Genus based ELISA	Many specific PCR tests exist - species and race. Genus specific primers (Karlsonn et al 2016). Generic fungal PCR tests could be used. Prime diagnostics: Luminex MagPlex-TAG® kits (van der Vlugt et al 2015; Zhang et al 2016)	No?	Isothermal amplification (LAMP and RPA) based assays for specific pathovars (e.g. Ghosh et al 2015; Lau et al 2016).	Genome sequencing (Ma et al 2010) Amplicon (metabarcoding, single molecule real-time) sequencing using the PacBio® RS II Instrument for community profiling (Walder et al 2017)

Pathogen type	Pathogen	Lab Isolation	Lab: Serology Test	Lab: Molecular test	Field: Serology test	Field: Molecular test	other
Fungus/oomycete	*Phytophthora* sp.	Yes	Nano Diagnostics, AGDIA and Loewe (www.loewe-info.com/): Genus based ELISA	Prime diagnostics: Luminex MagPlex-TAG® kits (Kostov et al 2016). Many specific PCR tests exist - species and race. Generic fungal PCR tests exist, positive results require sequencing of the PCR product. http://www.q-bank.eu/fungi/DefaultInfo.aspx?Page=PrimerList	Lateral flow devices: Agdia, Loewe, Prime diagnostics, and Pocket Diagnostic (www.pocketdiagnostic.com/) Immunostrip genus	Isothermal amplification (LAMP and RPA) based assays for specific pathovars (Miles et al 2015; Khan et al 2017)	Genome sequence (e.g. Tyler et al 2006; Longmuir et al 2017) Metabarcoding (e.g. Sapkota and Nicolaisen 2015, Prigigallo et al 2016
Fungus/oomycete	*Pythium* sp.	Yes	Nano Diagnostics, Loewe: Genus based ELISA	Many specific PCR tests exist. Generic fungal PCR tests exist, positive results require sequencing of the PCR product. Genus based PCR tests exist. (Schroeder et al 2012).	Lateral flow devices: Nano Diagnostics,	Isothermal amplification (LAMP and RPA) based assays for specific species and pathovars (e.g. Fukuta et al 2013; Tsuchida et al 2018)	Genome sequence (e.g. Adhikari et al 2013; Metabarcoding (e.g. Sapkota and Nicolaisen 2015,
Fungus/oomycete	*Rhizoctonia* sp.	Yes	Nano Diagnostics: Genus based ELISA	Many specific tests - species and race. Generic fungal PCR tests could be used.	Lateral flow devices: Nano Diagnostics,	Isothermal amplification (LAMP) based assays for specific species and pathovars (e.g. Lu et al 2015)	Genome sequence (e.g. Hane et al 2014)
Virus	*Potyvirus* sp.	Yes – herbaceous indexing; electron microsc	DSMZ (https://www.dsmz.de/), AGDIA, Nano Diagnostics, Inc : Genus based ELISA	Many species specific PCR tests exist. Genus specific tests (e.g. Zheng et al 2010) Prime diagnostics: Luminex xTAG-based assay or specific species	Lateral flow devices: Agdia Immunostrip for Potyvirus group. Species specific tests available from several companies	Isothermal amplification (LAMP and RPA) based assays for specific species (e.g. Silva et al 2018; Zhao et al 2018)	Genome sequence (Many references)

Pathogen type	Pathogen	Lab Isolation	Lab: Serology Test	Lab: Molecular test	Field: Serology test	Field: Molecular test	other
		opy		(CVMV,KMV, LMV, OYDV, PVA, PVY)			
Virus	*Orthotospovirus* sp.	Yes – herbaceous indexing; electron microscopy	Loewe: Tosposcreen detects TSWV, TCSV, INSV, GRSV, CSNV	Many species specific PCR tests exist. Genus specific primers (e.g. Bald-Blume et al 2017). Prime diagnostics: Luminex xTAG-based assay (Bald-Blume et al 2017; Yu et al 2018)	Lateral flow devices: Loewe three viruses: TSWV, TCSV and GRSV. Species specific tests available from several companies	Isothermal amplification (HDA, LAMP and RPA) based assays for specific species Babu et al 2017; Wu et al 2016; Siu et al 2018)	Genome sequence (Many references) Microarray Ye et al 2017.

[a]The AGDIA *Xanthomonas* genus specific ELISA kit and the ImmunoBlot Kit for *X. campathogenris* detects *Stenotrophomonas maltophilia* (*Xanthomonas maltophilia*), *X. arboricola* pv. *celebensis, X. arboricola* pv. *pruni, X. axonopodis* pv. *begonia, X, X. axonopodis* pv. *citri, X. axonopodis* pv. *dieffenbachia, X. axonopodis* pv. *phaseoli, X. campathogenris* pv. *armoraciae, X. campathogenris* pv. *campathogenris, X. campathogenris* pv. *zinnia, X. citri* pv. *aurantifolii, X. citromelo, X. hortorum* pv. *pelargonii, X. oryzae* pv. *oryzae, X. transluciens* pv. *transluciens*, and *X. vesicatoria*.

[b] Genome sequencing was not used in a diagnostic capacity for bacteria, fungi and oomycetes

4.2 Current diagnostic tools for detection of plant pathogens.

4.2.1 Symptoms

Observation and analysis of symptoms are often the first step in the diagnostic process for a disease, some plant pathogens. Although some pathogens and diseases can be diagnosed visually, observation of symptoms may mean that the disease is already well established and at greater risk of dissemination. In other cases, symptoms alone are not a reliable indicator of the presence of a pathogen as multiple factors, both biotic and abiotic, can cause similar symptoms. In addition, some important pathogens may be symptomless depending upon the plant host species, variety infected or due to environmental conditions. Therefore, some pests and pathogens will require specific tools for their early, rapid and accurate detection.

4.2.2 Isolation

Methods used for plant pathogen detection include isolation techniques, such as plating bacteria or fungi onto general or specific growth media and baiting or inoculation of bacteria, fungi, nematodes and viruses onto susceptible plant hosts. Identification maybe based on morphology of bacterial and fungal colonies or fungal structures or on the symptoms produced on a susceptible indicator plant. These processes can take a few days to several weeks. A delay in diagnosis of a cause of disease prevents an efficient and effective management response by the grower, which could affect production costs and crop loss.

Identification of the specific pathogen can be confounded where multiple organisms are present. For example, mixed virus infections could lead to a different symptom expression in an indicator plant, resulting in misdiagnosis. In some instances, the primary pathogen may be missed if it grows slowly and is overtaken by another organism. In addition, some pathogens, cannot be cultured on media (e.g. phytoplasmas) or easily inoculated to an indicator plant. Misdiagnoses due to the inability to correctly identify the causal pathogen could result in the application of incorrect management practices, also affecting production costs and crop loss.

4.2.3 Serological diagnostic tools

Serology based tests, such as enzyme linked immunosorbent assays (ELISAs) and lateral flow devices (LFDs), are often used for plant pathogen detection, especially for viruses (Boonham et al 2014). Benefits of these tests, compared to molecular methods, include cheaper cost/sample and the simplicity of sample preparation, which simply requires homogenisation of the sample in a basic buffer. ELISA provides an advantage of LFDs because it is high throughput, but LFDs are easy to use at point of care.

Serological tests (ELISAS and LFDs) have been developed for many, but not all, of the top 10 groups of endemic pathogens listed in table 2. Where they do exist, they may be highly specific. It is unclear why serological tests are unavailable for some pathogens, but it may be associated with a lack of demand for tests or low sensitivity, especially for fungal and bacterial pathogens and the prohibitive costs in generating “target specific” antibodies.

The development of antibodies entails purification of specific protein or intact virus particle which requires a high level of skill, therefore production of antibodies to the broad range of pathogens that infect plants may not be achievable due to costs. Purification processes may be tedious, particularly where virus particles are isolated from a plant host and contaminating proteins or the presence of multiple viral particles can lead to a lack of specificity. Serological assays can also lack specificity and may not distinguish between pathovars or strains. Serological tests often lack the sensitivity of molecular tests because they detect only what is present and are limited by the concentration of the target. Never the less, where antibodies are available, serology-based tests provide some of the simplest means to detect a pathogen in the laboratory and at point of care.

Never-the-less, serology tests, especially LFDs, offer some of the simplest, robust and user friendly diagnostic assays and further consideration to their future development is warranted. The route to market for this technology may be complex due to the difficulty to identify protein targets for test development compared to tests designed against DNA or RNA targets. LFDs base on detection of protein tagged (e.g. Biotin) nucleic acids may be easier to develop, because antibodies to proteins such as biotin are more readily available.

4.2.4 Molecular diagnostic tools

Many diagnostic technologies utilise nucleic acids for detection. Tests include polymerase chain reaction (PCR) and various isothermal amplification procedures that make multiple copies of DNA so that it can be easily detected. The benefits of this amplification approach include an increased sensitivity, specificity and greater speed of detection compared to some traditional pathogen detection methods. Many procedures for nucleic acid detection process, from extraction to analysis are also high throughput, enabling many samples to be processed and tested at once. They can also be automated. An alternative molecular method, which is now less frequently used for pathogen detection, includes nucleic acid hybridization or “dot-blots” (Chu et al 1989). Interestingly, ELISA-based nucleic acid hybridization process that was developed in the 1980’s, using antibodies to capture and detect protein labelled nucleic acid probes, form the basis for some of the point of care nucleic acid based lateral flow devices that are currently being developed for field-based plant pathogen detection (Chu et al 1989).

The development of nucleic acid-based detection methods often relies upon knowledge of the genetic makeup of the pathogen – the sequence of nucleotides within the RNA or DNA molecule that is being targeted for detection. As our ability to undertake nucleic acid sequencing has improved the volume of sequence data upon which to base a nucleic acid detection method has increased significantly, especially for viral pathogens. PCR combined with commercialized chain-termination (Sanger) sequencing technology (Sanger et al 1977) has significantly added to the volume of published genetic variants amongst pathogens, especially based on genetic markers such as the bacterial 16S ribosomal RNA (rRNA) subunit, fungal internal transcribed spacer (ITS) regions and viral coat protein genes or gene regions. Next-Generation Sequencing (NGS) technology, through whole genome sequencing (WGS) and amplicon-based sequencing, has also added a large volume of published information about strains, pathovars and genetic variants since it was first developed (Barba and Hadidi 2015). The significant increase in genetic information available allows the continued development of more accurate molecular diagnostic tests utilising PCR.

5 Test specificity

Specific tests are useful when there are a limited number of pathogens that require surveillance. However, in some situations there may be more than one pathogen requiring detection, such as the multiple viruses that infect *Prunus* species overseas (>44 virus species; Rubio et al 2017) and in Australia (>17 virus species; Kinoti and Constable, unpublished) and which require testing for at the Australian border or within nursery certification programs. Testing each pathogen individually can significantly increase test costs and the time taken to report the results.

In some cases, generic tests have been developed which might detect a group of pathogens. Follow-up with an alternative test may be required to identify the specific pathogenic species that was detected. Genus based ELISAs for potyviruses and several fungi are available (Table 1). There are several genera-based PCR tests for viruses e.g. generic reverse transcription (RT)-polymerase chain reaction (PCR) tests that can detect the virus species in the genera *Ilarvirus* or *Potyvirus* (Maliogka et al 2007; Zheng et al 2010). Generic tests can lack specificity and/or reliability e.g. the *Ilarvirus* RT-PCR does not detect some strains of *Apple mosaic virus, Prune dwarf virus* and *Prunus necrotic ringspot virus* in *Prunus* species and can generate non-specific amplicons (Kinoti et al 2014). A more successful example is the generic 16S rRNA gene-based PCR test used to detect phytoplasmas (Gundersen and Lee 1996). Generic PCR tests require an analysis of the PCR amplicon, such as direct or indirect (cloning) sequencing or restriction fragment length polymorphisms analysis, to determine which species might be present. Similar generic molecular tests using conserved and targeted phylogenetic marker genes, are also available for other bacteria (e.g. 16S rRNA gene; Weisberg et al 1991) and fungi (e.g. ITS; White et al 1990). These generic tests for bacteria and fungi are often only applied to an individual isolated bacteria or fungus so that the PCR amplicon can be used for sequencing and identification of the pathogen. However, they can be combined with next generation sequencing (NGS) to study the biodiversity and composition of viral, fungal and bacterial communities (Lindahl et al 2013; Sanschagrin and Yergeau 2014; Hugerth and Andersson 2017; Kinoti et al 2017; Nicolaisen et al 2017). In this metabarcoding approach, DNA is extracted from an environmental sample (e.g soil, water, plant etc.), the gene target is amplified by PCR and the amplicons are subjected to NGS and bioinformatics analysis. Metagenomic NGS as a diagnostic tool is generic procedure that potentially allows the identification of all pathogenic organisms, including fungi, bacteria and viruses in a biological sample (Barba and Hadidi 2015, Chandler et al 2015).

6 Emerging technologies for pathogen detection

6. 1 “Omics” approaches to diagnostic assay development

Increasing capabilities and improved NGS technology is allowing many researchers to use a pan-genomic approach to identify molecular diagnostic markers that can be used for specific detection of a species, strain or pathovar, especially for pathogens with a large genome such as fungi and bacteria (e.g. PBCRC Project 2156: Deployment of validated genome-informed bacterial diagnostics; Klosterman et al 2016). This approach may identify markers that could differentiate at a broader level between disease associated pathogens and closely related epiphytic groups of organisms. The pan-genomic approach relies on whole genome sequencing and comparison of related species to identify core genes, which are present in all individuals and variable genes, which are not present in all individuals (Zheng et al 2010; Mann et al 2013; Feau et al 2018). It may also

identify genetic variability amongst core genes that could be used to differentiate pathogens of interest at a broad level, such as genus, or a finer level, to pathovar, which might be related to one or more single nucleotide polymorphisms (SNPs) within a gene or group of genes that lead to a change in pathogenicity.

Transcriptomics, which uses RNA sequencing (RNA-seq) data often contains a variety of RNA species, such as messenger RNA (mRNA), non-coding RNA, small regulatory RNAs, ribosomal RNA (rRNA) and pathogen RNA etc. that may be expressed differently during infection. Changes in plant-based RNA expression might be used as an early indicator of disease (Byron et al 2016; Lowe et al 2017). Transcriptomics can also be applied to the pathogen, particularly fungi, and is often used to understand the mechanism of pathogenicity but it may be useful for identifying markers, such as effectors, to develop diagnostic tools (Czislowski et al 2018). For example, a PhD project being done at AgriBio in Victoria, is using genome sequencing and transcriptomics to understand effector diversity in Australian *Fusarium oxysporum* species complex, with a focus *on F. oxy* f. sp. *pisi*, which may then be used to improve our diagnostic capability to differentiate pathogenic strains of the fungus (Saidi Achari and Jacky Edwards, pers.comm.).

Various types of mass spectrometry (MS) are used in proteomics and metabolomics to identify molecules as biomarkers in a broad range of plant samples and associated with a range of changing factors, including exposure to pathogens. MS measures the weight of a molecule, including proteins, and can differentiate variants. This approach could be used to identify proteins for the development of improved serological based diagnostic tests. MS is used in medical laboratories as a diagnostic tool to detect biomarkers indicative of disease and this approach could be extended to plant pathology. Although the initial investment in equipment is high, the cost of consumables and simplicity of sample preparation may make this an attractive option for microbial diagnostics (Singhal et al 2015). More recently it was used to differentiate plant infecting *Erwinia* isolates to species and subspecies level (Rezzonico et al 2017).

Nuclear magnetic resonance (NMR) and MS is used in metabolomics, analysing chemical reactions and identifying small or large molecules that may be diagnostic with a change in environment, such as the presence of disease (Fernandez et al 2016, Markley et al 2017). MS and/or NMR may be able to identify early metabolic changes in response to infection, allowing early diagnosis of disease and a rapid management response (Pontes et al 2016a). For example, significant differences in the metabolic profile of Huanglongbing affected citrus trees was observed during the early stages of *Candidatus* Liberibacerter spp. infection (Pontes et al 2016b). Recently, an Enzyme-Linked Aptamer Kissing Complex Assay (ELAKCA) was developed to detect small molecules and this technology could be combined with the results of metabolomics studies to improve pathogen detection/disease sensing based on metabolites in plant samples (Fernandez et al 2016; Chovelon et al 2016). However, ELAKCA is highly novel and requires greater proof of its reliability as a diagnostic tool.

Phenomics measures physical and chemical traits, called phenotypes, associated with the genome and environment. It may use sequencing, MS and other tools to link chemical changes to physical changes in plants. Changes may be measured at the cellular level and up to the broader population. In plant pathology, digital imaging, chlorophyll fluorescence, magnetic resonance, spectral, ultrasound, X-ray and thermal imaging technologies are being used as tools to study changes in phenotypic traits in plants including growth, colour and composition (Simko et al 2017). Volatile organic compounds (VOC) represent another phenotype that can be altered in plants in response to disease.

Integrated approaches or “Pan-omics” that combine genomic, transcriptomic, proteomic, metabolomic and interactome information, are the basis behind customized human healthcare known as “precision medicine”

(Tebani et al 2016;). This systems-based method for disease analysis can assist the understanding of the host-pathogen interaction as well as improving the accuracy of biomarkers for pathogen detection (Slezak et al 2003; Wang and Zhang 2015; Jean Beltran et al 2017). Similarly, "Pan-omics" approaches are being adopted in agricultural research to improve production and sustainability, which includes an improved understanding of disease (AbuQamar et al 2016; VanEmon 2016).

In addition to increasing ability to design new and accurate tests based on the "Pan-omic" information of pathogens and their hosts, detection technology is also transforming. Many laboratory-based assays are becoming more field-deployable and laboratory diagnostic tools are becoming easier, cheaper, automated and high-throughput.

- Omics approaches are required to better understand appropriate diagnostic targets for accurate detection and diagnosis of disease. Targets may include a variety of molecules such as DNA, RNA, Proteins, Peptides, VOCs, other plant or pathogen metabolites etc.

6.2 Field deployable diagnostic tools

6.2.1. Isothermal amplification

Field-based technology is advancing, and assays based on detection of nucleic acids are increasingly being reported in the literature. Most of these tests are based on isothermal amplification, which is like lab-based polymerase chain reaction (PCR) tests except that the enzymes used to copy DNA do not require changing of the reaction temperature to denature the double stranded DNA, to allow annealing of primers and subsequent building of a new DNA strand. Examples of this method include: Loop-mediated isothermal amplification (LAMP), Helicase-dependent amplification (HDA) and Recombinase polymerase amplification (RPA). Citations linked to examples are listed in Table 2. Isothermal methods do not necessarily require specialised equipment, although they may require devices that hold a specific temperature. A positive result may be identified visually associated with turbidity, a colour change or fluorescence of an intercalating dye in the test tube. However, for some methods it may be possible to multiplex by combining multiple primers using probes coupled to a fluorophore in the same reaction tube, but this would require the use of a specialised instrument to read the reactions. Another method of visual detection, which would be highly suitable for growers, is the ability to combine these methods with lateral flow devices (LFDs), somewhat like those used for detection by pathogen targeted antibodies (e.g. Pocketdiagnostic).

Some examples of field-based molecular methods are listed in Table 2 and these may not be commercially available. However commercial companies, such as OptiGene, TwistDX and AGDIA may work with researchers and industry to develop commercially available test ready kits. For example researchers have worked with AGDIA to develop AmplifyRP® Acceler8®Pathogen Specific RPA Kits for seven pathogens, some of which are high priority quarantine pathogens for Australia (e.g. *Candidatus* Liberibacter asiaticus, *Grapevine red blotch virus*, *Tomato chlorotic dwarf viroid* etc; https://orders.agdia.com/pathogen-tests/amplifyrp/acceler8). Opitgene has worked with researchers to develop LAMP kits for *Clavibacter michiganensis* subsp. *sepedonicus, Chalara fraxinea, Botrytis cinerea* etc; http://www.optigene.co.uk/plant-health-pathogens-diagnostics-3/). All these companies supply kits to assist test development.

LAMP and RPA technology is a primary focus of Australian diagnostic and biosecurity researchers and considerable expertise exists in the development of this technology for plant pests and pathogens. The PBCRC Project 2156: Deployment of validated genome-informed bacterial diagnostics demonstrated the ease of deploying these tests in the field using visual analysis or using specific devices to read results. The practical application of LAMP technology was proven for detection of *Potato spindle tuber viroid* (PSTVd) in infected tomatoes grown in Australian glasshouses and wheat fields (Stack 2015; Zheng et al 2018).

- LAMP and RPA technology is developing at a rapid rate and is easy to commercialise, therefore it is recommended for further development. Depending upon the detection method, there may not be a need for expensive equipment. They can be coupled with lateral flow devices to make detection simpler (see 6.2.2). Simplicity makes these devices ideal for field deployable diagnostics, that can be used by experts and non-experts alike.

6.2.2 Nucleic acid lateral flow devices

As mentioned above nucleic acid amplification technologies, especially LAMP and RPA, can be combined with LFDs to easily visualise the results and commercial examples exist. An example is provided in Figure 1.

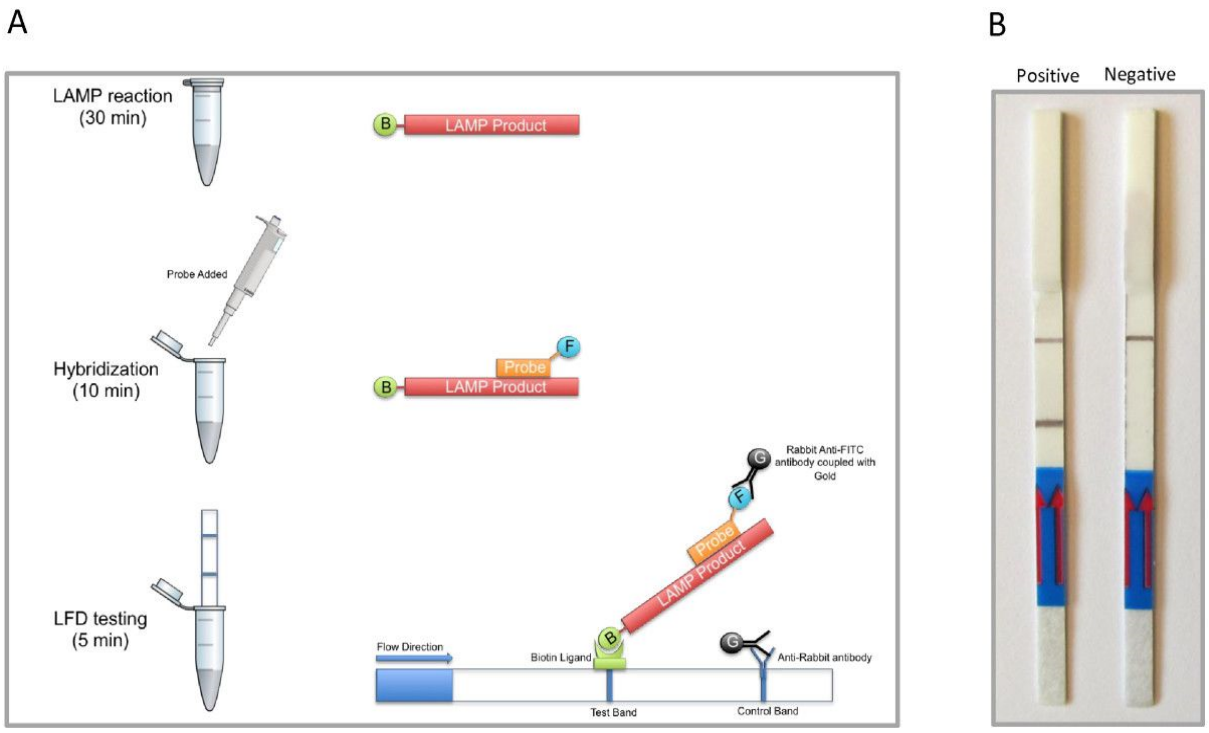

Figure 1. The visualisation of the detection of *Candidatus* Liberibacter asiaticus using a combined LAMP reaction with a lateral flow dipstick (Rigano et al 2014). The biotinylated LAMP product is hybridized to a probe. The LAMP-probe FITC product is captured by a biotin ligand on the test strip and is detected by gold coupled Anti-FICT antibody that leads to the development of a band on the test strip.

LFDs based on detection of protein tagged (e.g. Biotin) nucleic acids may be easier to develop, especially as diagnostics tests become more genomics informed. If a signal could be enhanced it may be possible to directly detect the nucleic acid of a pathogen without amplification. Quantum dots may be one means to increase signal intensity (Yang et al 2010).

6.2.3 Field deployable PCR technology

Several companies are working towards developing field deployable PCR equipment. Examples include the POCKIT™ Micro Series Nucleic Acid Analyzer (http://www.genereach.com) and two3™ Real-Time PCR Thermocycler, which incorporates an iPhone (https://biomeme.com/). Both companies also supply lyophilized reagents that are stable at room temperature and to which the specific primers and probes and the samples can be added and Biomeme provides these in strip tubes. Biomeme also have some test ready kits for detection of human or animal bacterial and viral pathogens and for food quality issues, but so far, no tests for plant pathogens are available. Biomeme may be willing to develop field ready kits for plants pathogens if there is demand. Lyophilized kits could make this technology simple enough for all levels of experience. However, the devices may be too expensive to justify acquisition for commercial nurseries. For example, the two3™ Real-Time PCR Thermocycler is US$3,995.00. The tests using Biomeme PCR devices can be multiplexed so that three pathogens or two pathogens and a quality assurance test can be combined in one tube. It appears that this technology utilizes dual labelled hydrolysis probes (E.g. TaqMan) incorporating FAM/SYBR, Cy5/ATTO647N and TexasRedX fluorophores.

- Field deployable PCR technology can take advantage of available tests that have been validated for pathogen detection, without much further verification. The equipment may yet be too expensive for some growers, but it could be useful for resellers and consultants who support the nursery industry and therefore may be worth pursuing.

6.2.4 Portable next generation sequencing

Oxford Nanopore Technologies (https://nanoporetech.com) has developed miniaturized RNA and DNA sequencing technology for point of care and field deployable use. The smallest commercially available technology is the MinION, which fits in the palm of your hand. It has been applied to detect viral and bacterial plant pathogens directly in plant tissues (Bronzalo Badial et al 2018). It was used in the field in Africa to detect whole plant virus genomes (Boykin et al 2018). The device requires high level computing power for data analysis, however bioinformatics pipelines can be developed to answer specific diagnostic questions and reduce this requirement while increasing time to results. More excitingly Oxford nanopore technologies is developing smaller devices, including the Flongle adapter for smaller samples using the MinION, and the SmidgION, which is designed to connect to a smartphone. All allow for real-time access to sequence data, and sequencing can be stopped once enough information (sequence reads) have been obtained to identify the target. It is possible to obtain sequence data within an hour, with analysis completed in under 24 hours (Lu et al 2016). The down-side is the high error rate of the generated sequences; however, the quality of sequence may be enough to differentiate down to strain or pathovar level depending upon the pathogen that it being tested (Besser et al 2018).

Starter packs that include the MiniION device plus consumables are available for $1000. A replacement flow cell is $900 and replacement sequencing kits cost $450-$600, depending upon purpose. Whilst the initial outlay may seem expensive there are several features that can make this technology cheap for individual sample analysis: enough data can obtained within minutes to make an identification, allowing multiple uses of the flow

cell; analysis and reporting are done in real-time, allowing the device to be stopped as soon as required; and multiple samples can be sequenced one after the other and this can be done with breaks after each sample. Indexing samples, so that they can be differentiated, allows for multiplexing samples and sequencing at the same time, but this will reduce the amount of information obtained for each sample and reduce sensitivity. Nevertheless, the ability to sequence multiple samples reduces the cost per sample. The MinION currently requires a laptop with adequate computing power and appropriate software for primary data analysis. There is a pre-configured computing module (MinIT, $2400) to control the MinION and carries out data acquisition and performs basecalling and which can replace a dedicated laptop for primary data analysis. The MinIT can link to a laptop to facilitate greater control over sequencing, via the laptop. Data analysis can be done in the company's cloud-based platform but, for experienced users, there is also the possibility to do the analysis locally. It may be possible to design specific and simple local data analysis pipelines for less experienced users.

RNA and DNA would need to be extracted from a biological sample prior to sequencing and extraction procedures would need to be evaluated to ensure quality sequencing outputs as long sequence strings are produced by the MinION. To prepare RNA or DNA for sequencing, Oxford Nanopore Technologies have developed an automated library preparation device, VolTRAX ($8000), for hands-free sample preparation. This may assist in quality assuring the input for sequencing. But library preparation can be done manually.

Therefore, whilst this looks promising for the field, currently it would require some technical/science-based expertise to implement the MinION for point of care diagnostics. Some significant developmental work is required to make the technology more user friendly for non-science-based users. Oxford Nanopore have upscaled these devices for higher throughput laboratory analysis – these devices are known as the GridION and the PromethION.

- Portable NGS still requires development and reasonably high level technical expertise. It could be used by experts for infield surveillance. For routine diagnostics, this technology may be better suited to laboratory use (see 6.4.2).

6.2.5 Lab-on-a-chip

Lab-on-chip technology uses miniaturized devices, primarily based on microfluidics, to process and analyse biological samples, primarily for diagnostic purposes. Processes may be serological and /or molecular and might include PCR, LAMP RPA etc. Advantages are typically lower cost, due to less reagents, and faster turn-around to a result (Wu et al 2018).

A review of the literature identified many papers and many reviews about the development and applicability of lab-on-a-chip devices in human, animal and plant diagnostics, yet there are very few commercially available examples. In one commentary (http://news.mit.edu/2017/makerspaces-could-enable-widespread-adoption-of-microfluidics-0421), it was pointed out that these types of devices have largely remained in the realm of research laboratories because "production is not scalable to industry manufacturing". The vision in the article was that such devices could be manufactured in public facilities called "Makerspaces". However, this type of production does not take into consideration the requirement for quality assurance during production for test specificity and reliability. A previous review (McDevitt et all 2015) also identified the following factors that contributed to a lack of update:

- *Integration: Integrating Lab-on-chip systems into Point of care structures that completely replicate the full functionality provided in laboratory settings.*
- *Failure to compete: Current Lab-on-chip systems fail to compete with central and remote laboratories in*

terms of cost and performance.

- *Need for new content: Lab-on-chip systems need to develop new content that adds substantial value to the target application and is currently unavailable at central and remote labs.*
- *Regulatory approval and clinical adoption: Technology developers need to develop strategies for streamlining regulatory approval en route to clinical adoption.*

These four points indicate that novel technologies such as Lab-on-chip/microfluidic devices must provide significantly greater benefits compared to currently used technology before they will be manufactured and adopted (Wu et al 2018). Another review identified that many Lab-on-chip assays still had high level technical requirements that diminished end user adoption in microfluidic paper-based assays (Nilghaz et al 2015). In undertaking this review for the Australian nursery industry, a similar conclusion has been reached: although Lab-on-chip devices are being developed within research programs, technical challenges remain with respect to sample preparation, test operation and results output and analysis and data management, which are often not addressed during assay development. Lab-on-chip assays may still require significant sample preparation, especially for plant samples where quality nucleic acid is required, and additional devices are required to read/ analyse the assay, some of which may not be portable. Assays are often developed using laboratory-based devices to run or read the assay or in the absence of a commercially available devices for point of care use and the practical manufacturing of such a device is often overlooked.

- Commercialization issues and lack of supporting field deployable technology make this type of test less attractive for further development at the current time.

6.2.6 Nucleic acid extraction for molecular detection of pathogens

For molecular methods there is a requirement that nucleic acid is of reasonable quality to prevent false negative results. Isothermal enzymes tend to be more robust and can be used on crude plant samples that have been homogenized in an appropriate buffer. However, purified nucleic acid may be required for PCR and could improve results obtained by isothermal amplification-based tests. Both GeneReach Biotechnology Corp. and Biomeme Inc. have simple nucleic extraction kits for field-based use, which could be used by people with all levels of experience to extract RNA or DNA from plant samples. In our hands (AgriBio), the quality of the nucleic acid produced by the Biomeme RNA and DNA preparation kits was the same or better than methods currently used in the lab, when a small number of different samples were tested. The ability to extract nucleic acids from soil samples using these kits is unknown. Biomeme kits have been used to extract environmental DNA from fresh, marine, or industrial process water samples and water filters used in field collection (Thomas et al 2018).

These nucleic acid isolation methods rely on capturing the nucleic acid and washing away impurities, which can mean multiple steps to complete the process. An alternative has been devised by QuantuMDx (http://quantumdx.com) by which the impurities are captured, and the DNA is left in solution. This protocol could offer a much simpler process for point of care diagnostics, but has not yet been trialed with plant tissues, water or soil.

Another, possibly simpler, method for nucleic acid extraction was recently reported by Zou et al (2017). This method used cellulose-based paper to isolate RNA and DNA from a variety of samples including plant leaves. Its usefulness to extract nucleic acids from soil is also unknown. The method would be very simple to use, but It is unclear if it will be commercialized.

Isolation of nucleic acids from soil is hampered by many factors, especially carryover of inhibitors and binding

of nucleic acid and soil-based compounds, often require complex processes to (Lever et al 2015; Mojarro et al 2017). No simple, field-based extraction procedures are reported. And simple procedures described above would need to be tested.

- Sample preparation will be a critical component in the development of any in field molecular test for industry.

6.2.7 Serological lateral flow devices

A few companies such as AGDIA and Loewe have LFDs available for various plant pathogens (Table 2). Where antibodies are available, but no commercial LFD exists, it may be possible to design an in-house method in a participating laboratory. Companies that offer these solutions include but are not limited to Bioporto (http://www.bioporto.com/Products/Product-by-category/Lateral-flow.aspx) and Cytodiagnostics (http://www.cytodiagnostics.com/store/pc/Rapid-Test-Development-Kits-c307.htm). A company such as Abbingdon Health will undertake contract services to develop and manufacture lateral flow products (https://www.abingdonhealth.com/contract-services/lateral-flow-assay-development/).

LFDs often use gold particles for detection. It may be possible to enhance detection using quantum dots (QD) as biosensors (Hong and Lee 2018). QDs can be functionalised with antibodies or oligonucleotides to enable the specific detection of a pathogen. QDs can be manufactured so that they emit various colours, allowing detection methods to be more easily multiplexed. Detection of QDs relies on availability of an excitation source, which could be an ultraviolet lamp but could also be a simple LED that emits the required wavelength.

TGR BioSciences is an Australian company that is partnering with various life science companies, including plant diagnostics providers (http://www.tgrbio.com), to improve serological detection of various analytes including plant pathogens. This technology replaces typical binding molecules used in other dipstick assays, such as biotin/streptavidin, with a nonbiological peptide attached to the analyte specific antibody and which is captured by a monoclonal antibody that is bound to the assay surface such an ELISA plate, LFD etc. (Figure 2). It has potential for multiplexing so that several pathogens of interest can be detected at once (Figure 2). In 2015, TGR partnered with the German biological company DSMZ to develop “B-Fast ELISA” assays for the detection of ten different plant viruses within two hours, when low numbers of samples are analysed. The advantage of the technology is that it has simplified the ELISA test by removing several incubation and wash steps: all pathogen specific antibodies (primary peptide bound antibody and secondary enzyme conjugated antibody) and the plant homogenate can be combined and applied to the surface to which the peptide specific monoclonal antibody is attached. After one hour of incubation the plate is washed, and a substrate is applied that gives a color reaction in the presence of the pathogen-specific enzyme-bound antibody after 30 minutes. TGR BioSciences claim that their technology also improves test sensitivity and reduces the price of the tests through decreased manufacturing costs. This technology could improve turn around in the laboratory and a multiplexed LFD could be useful where multiple pathogens might be associated with similar symptoms. It would be interesting to understand if this technology could be adapted to molecular probes.

- It may be interesting to pursue serological and molecular LFDs due to ease of use. The development of serological LFDs may require omics-based research to identify relevant targets for detection.

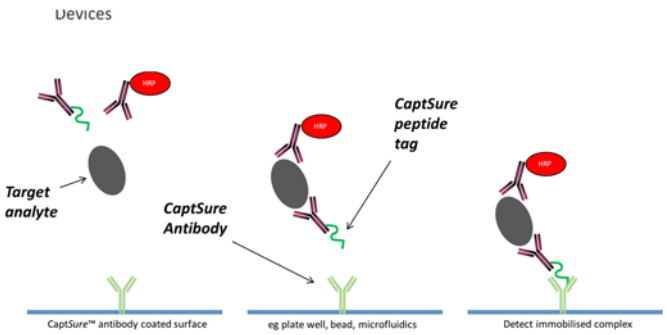

Figure 2. A diagram of the Capt*Sure*™ immunoassay technology: a) the multiplexing ability of the technology, b) the Capt*Sure*™ is composed of the Capt*Sure*™ non-biological peptide is attached to the pathogen primary antibody and the bound peptide is captured by a monoclonal antibody specific for its detection.

6.2.8 Aggregation of functionalized colloidal gold particles

A solution-based test that takes advantage of a colour change when colloidal gold particles aggregate may be a simple test that can be developed for various nursery pathogens.

A test has been developed for microbial (bacterial) contamination of pool water and, although the science behind this specific development is unclear, it is likely to use gold particles. The limit of detection is 100 CFU/ml and it utilizes a small device to measure the colour change. The result is shared to an app on a smartphone where it is presented to the user (http://exactblue.com/; Figure 3). However, it may be possible to visually observe the colour change. The test is commercially available through various sources, but the device is on pre-order through a start-up company (http://exactblue.com/). It appears that this test is non-specific, detecting total bacteria and fungi. However, it is possible to functionalise colloidal particles with antibodies or oligonucleotides to facilitate specific detection of a pathogen and based on reports it appears that the company intend to add specificity for bacterial strains to the test capability. The Exactblue device may enable more accurate interpretation of results, especially where low concentrations of pathogens are present.

Figure 3. Detection of bacterial contamination in water samples using the Genemis AquaVial test kit and the Exactblue device.

The use of colloidal gold particles has recently been used to detect unamplified pathogen DNA and could be easily deployable with a simple nucleic acid extraction method (Baetsen-Young et al 2018).

- Aggregation of functionalized colloidal gold particles combined with a simple analysis device, e.g. Exactblue, could provide grower with a simple test format for the detection of bacteria or fungi in a variety of samples and may be useful for water testing.

6.2.9 Remote sensing and non-invasive detection methods

Phenomics information can be adapted for the development of non-invasive field-deployable diagnostic tools that might "sense" the early stages of disease (Martinelli et al 2015). Digital, chlorophyll fluorescence, magnetic resonance, spectral, ultrasound, X-ray and thermal imaging technologies used in phenomics studies are being adapted for pest and pathogen surveillance. Imagery might be microscopic and up to field level and can be collected using field deployable technology, such as hand-held sensors, drones or satellites (Perez-Sanz et al 2017; Thomas et al 2018). This type of technology is being developed for plant pest surveillance, including phylloxera, in Australian vineyards (Vanegas et al 2018).

Non-invasive field-deployable sensors, called electronic nose (e-nose), can be used for VOC detection (Martinelli et al 2016, Cui et al 2018). E-nose technology is being developed for fruit fly detection in fruit at AgriBio in Victoria (http://agriculture.vic.gov.au/agriculture/pests-diseases-and-weeds/pest-insects-and-

mites/queensland-fruit-fly/research). E-nose technology was successfully applied for the early detection and discrimination between fire blight (*Erwinia amylovora*) and blossom blight (*Pseudmonas syringae* pv. s*yringae*) on apple trees in the field (Cellini et al 2016).

- Remote sensing technology may be useful for larger glasshouse and field-based nurseries

6.2.10 In-field Technology costs

The cost of in-field tests is dependent upon the technology (table 3). Some tests require little or no equipment whilst others may require an initial investment equipment, but ongoing costs may be low. For example, serological LFDs require no hardware, kits are usually supplied with sample bags and homogenization buffer for sample preparation and AmplifyRP® Acceler8 LFDs would require purchase of a heating block (not necessarily from the supplier) in addition to the specific pathogen detection kit. In addition to the costs listed in the table there may be addition consumable costs such as extraction buffers, disposable loops or pipettes, tubes and sample bags. Additional fees may be encountered for import permits required for the importation of diagnostic kits, where those kits are not available onshore (bicon.agriculture.gov.au). In addition to the capability to maintain equipment and store kits in appropriate conditions to ensure test reliability. For example, serological LFDs need to be kept in the refrigerator (4 °C). AmplifyRP pathogen test kits require storage at

Table 3. The estimated price of each available field deployable technology broken down by device (hardware), sample extraction kit and test kit used to detect the pathogen. This does not include labor or other basic infrastructure, equipment and consumables costs, such as power, refrigerators, pipettes, buffers.

Technology	Device cost	Extraction kit cost	Test kit cost*
Serological LFD	N/A	N/A	$250 for 25 tests
Nucleic acid lateral flow (e.g. AGDIA AmplifyRP Acceler8®)	$550 (heating block plus mini pipettes and tips)	Depends upon method	$350 for eight tests
Isothermal amplification	Optigene GenieII (LAMP) $17,696- $21,488 AmplifyRP Acceler8® (RP) $12,600	Plant Material Lysis KIt - 50 extractions $384	Optigene Isothermal Master Mix - user designed primers 400 Reactions $1240, pathogen specific kit unknown AmplifyRP pathogen specific kit with 48 reactions $940
Field deployable PCR (Biomeme)	$6000	$630 (30 extractions)	$250 (10 go-strips10 samples) $140 (60

			reactions)
Portable next generation sequencing - MinION	VolTrax extraction device and library preparation: $8000 MinION: $1000 Replacement flow cell: $900 MinIT: $2400		$600
Aggregation	$650 (ExactBlue)	N/A	$140 (six tests)
Remote sensing	Enose $1100 Drone-based: variable costs: e.g. DJI matrice 600 plus RedEdge-M Professional Multispectral Sensor Kit (www.micasense.com) $20,000	N/A	N/A

*Direct kit costs, additional fees may be encountered associated with additional consumables and import permits

6.3 Laboratory-based methods

6.3.1 Polymerase chain reaction

Laboratory embedded technologies continue to be developed and improve the capacity to accurately identify known and novel pathogens and pathogens associated with plant disease. One of the most widely used laboratory-based technologies includes quantitative polymerase chain reaction (qPCR), which can determine the quantity of a pathogen in a sample. Digital droplet PCR (ddPCR) is newer quantitative technology, which is rarely used for plant pathogen quantification. However, coupled with appropriate sampling, ddPCR has the capacity to more accurately quantify a target within a sample compared to current qPCR techniques and could assist in implementing management strategies informed by pathogen quantification to predict risk.

Many qPCR tests have been developed for RNA and DNA plant pathogens. There is a preference for qPCR tools in many diagnostics labs because they are often more sensitive than endpoint PCR and they reduce the need for post PCR processing because the amplification of the DNA molecule is monitored and measured during the PCR run. qPCR methodologies can allow for multiplexing to detect several pathogens in on sample and in one PCR reaction.

qPCR technologies could be further developed to detect for a broad range of targets in a single test by working with ThermoFisher to develop a TaqMan® Array Card or plate, which run 384 or 96 simultaneous real-time PCR (qPCR) reactions, respectively. The 384-reaction array card can be designed to test 1 sample for 380 targets or up to 8 samples for 24 targets. The 96-reaction plate may also be customizable for multiple samples. These cards or plates would be configured commercially therefore it is likely that they would come with a quality assurance, giving extra confidence to the results. A possible requirement for specific brands of thermocyclers in which to run the test may be a limitation for some laboratories. Another potential downfall is that microarray technology may become obsolete or have significantly reduced demand since they are often used in research to monitor gene expression through measurement of messenger RNA. Microarrays are being replaced in research by a next generation sequencing (NGS) approach, referred to as RNA-seq, because of its sensitivity, high-level detail and ability to identify novel RNA transcripts (Bumgarner 2013). However, microarrays may have a longer term medical/clinical diagnostic application that will maintain some demand

(Pereira et al 2017). Reduced demand in clinical diagnostic or research applications may make microarray technology more difficult to develop for plant pathogens which often experience even lower demand for diagnostic tools.

An exciting approach, which is available for clinical/medical diagnostics, is the FilmArray System from BioFire (https://www.BioFiredx.com/ Figure 4). The system utilizes a pouch in which sample preparation, amplification, detection and analysis are incorporated. It can simultaneously detect numerous pathogens. It could be used to identify the specific genus, species, pathovar, race or strain of a pathogen in a single test, where multiple pathogens are associated with a similar disease. For example, it could be used to identify which fungus or bacterium is associated with non-specific leaf spot symptoms. This could mitigate the need for deductive style diagnostics where tests are done to rule in or out pathogens in a sequential process of elimination.

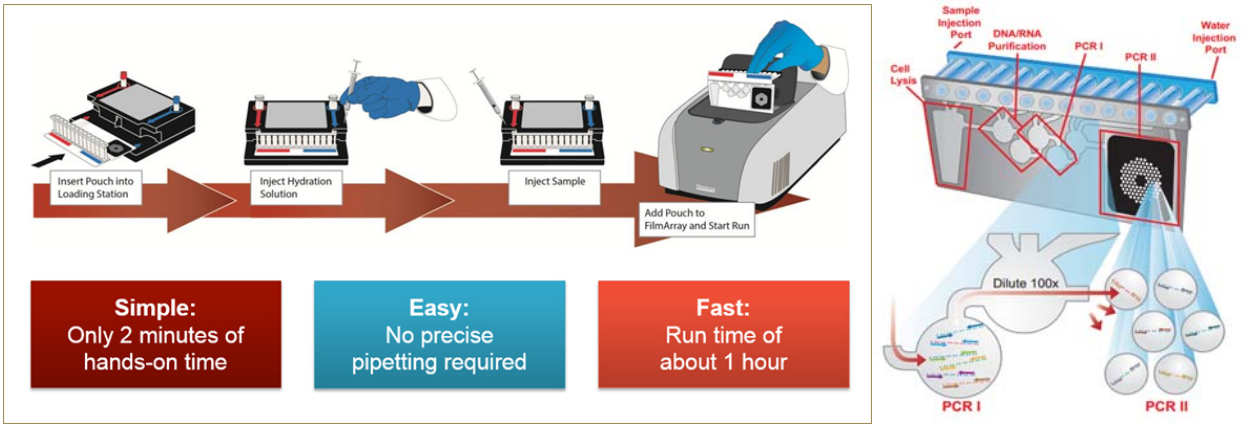

Figure 4. Pathogen detection using the BioFire FilmArray system (https://www.BioFiredx.com/).

PCR based technology is a staple of laboratory diagnostics. Improved diagnostics is facilitated by pangenomic approaches to study diversity and identify genes or gene regions relevant to the target pathogen. Developing a PCR array-based approach could be useful when more than one pathogen, species, strain or pathovar is being tested. It would be useful to approach BioFire and Thermofisher to determine if their technology could be used for plant pathogen diagnostics.

6.3.2 Next generation sequencing as a diagnostic tool

NGS is a rapidly developing and broad-spectrum technology that has the capacity to be used as a diagnostic tool to identify multiple pathogens in a single sample, which may be a plant, soil or water. It can be targeted or non-targeted and identify known, unknown or unexpected pathogens in a single test. However, like other pathogen detection methods, sampling (time, tissue type) might affect the ability to detect certain pathogens due to uneven distribution or titre (Jo et al 2017).

There are various laboratory-based NGS platforms available (e.g. Illumina [iSeq 100, MiniSeq, MiSeq, HiSeq, NextSeq, NovaSeq], SOLiD, Ion Torrent, PacBio, MinION, GridION etc) that can be used for whole genome sequencing, targeted gene (amplicon) sequencing, long range amplicon sequencing, transcriptome sequencing and/or gene expression profiling through sequencing of DNA or RNA. NGS as a plant pathogen detection

method mainly focuses on genome and amplicon sequencing of DNA or RNA. The NGS platforms can vary in the complexity of sample preparation required prior to sequencing, the length of individual sequence reads that are generated, the amount of sequence data that is generated and the accuracy of sequence data. Bioinformatics analysis of some sequence data can be complex and may require significant computational infrastructure, but others are less complex, and both may depend upon the specific diagnostic question that is being asked. Diagnostic questions may be "what's in my sample", which may require more computationally challenging bioinformatics analysis, or "is this pathogen present", requiring specific and less demanding analysis against reference pathogen sequences or databases. These diagnostic questions may also dictate the type and amount of nucleic acid (i.e. total DNA, total RNA, ribosomal depleted RNA, small RNA or double stranded RNA) used for NGS as they affect pathogen detection (Visser et al 2016b; Pecman et al 2017; Maree et al 2018). The degree of complexity associated to the various platforms influences the length of time to a result and can influence their sensitivity and reliability for pathogen detection.

NGS on any platform and for any diagnostic purpose needs to be underpinned by clearly defined quality assurance procedures for all steps in the workflow from nucleic acid extraction, library preparation and to data analysis, to ensure an accurate result. Procedures for routine diagnostics, including NGS, should be verified as fit for purpose and validation may be required to determine analytical sensitivity, analytical specificity, repeatability and reproducibility (EPPO 2017; Olmos et al 2018; Maree et al 2018; Massart et al 2018; Roenhorst et al 2018). Thresholds for the amount of genome information required to consider a pathogen is present in a sample need to be determined as part of determining the analytical sensitivity for genome-based diagnostics and a method to verify a positive result is required (Olmos et al 2018). NGS will sometimes identify unexpected, known pathogens or novel pathogens and it may be necessary to perform additional work to determine its biological significance and the association to or cause of the disease that is being investigated (Olmos et al 2018).

Some laboratories have in-house equipment and capabilities for NGS. There are also many commercial services that offer beginning to end sequencing, including sample preparation and bioinformatics analysis. There are several onshore companies that offer commercial NGS in Australia, including the Australian Genome Research Facility (http://www.agrf.org.au/), Micromon (https://platforms.monash.edu/micromon/) and the Ramaciotti Centre for Genomics (https://www.ramaciotti.unsw.edu.au/). There are also many offshore companies that also offer highly competitive cost effective services. The use of overseas suppliers needs to be considered in the context of biosecurity and market access if an exotic pathogen is detected, especially if they undertake the bioinformatics analysis and with respect to secure and long-term data storage (Olmos et al; 2018). Anecdotally, outputs from commercial providers, can be highly variable and this could lead to a misdiagnosis. Examples include false positives due to contamination from previous NGS runs and assignment of sequences to the wrong samples in multiplexed runs due to index hopping (https://sapac.illumina.com/science/education/minimizing-index-hopping.html). False negatives might be due to poor quality nucleic acid, inappropriate or poor-quality library preparations leading to low depth of data for a low titre target (Pecman et al 2017).

A metagenomic NGS approach can be used to sequence all the DNA or RNA present in a sample to identify the microbes present in a community (Martin et al 2016; Rott et al 2017, Olmos et al 2018; Xu and Wang 2018). Results can be obtained within one week (Higgins et al 2017). This general approach has the capability to detect all or part of pathogen genome and may require additional work to verify the presence of the pathogens that were detected. It is said to be unbiased as it does not rely on pre-designed probes for pathogen detection (e.g. PCR primers etc), which create specificity. It is a very useful method to investigate the possible biotic aetiology of a disease with an unknown cause (Maree et al 2018; Xu and Wang 2018). Bioinformatics analyses using de novo assembly of sequence data into partial or full genomes can be used to identify

previously unknown pathogens, which is typically more computationally complex. Alternatively, analyses can be targeted by assembling the data against reference genomes of known pathogens. These can require significant computational requirements and the different algorithms behind the analysis can influence the efficiency and reliability of data assembly and subsequent pathogen detection. However, bioinformatics pipelines have been developed to simplify and assist analysis of NGS data sets against reference genomes and by de novo assembly into contiguous segments of a genome contig (Ho and Tzanetakis 2014; Chen et al 2016). Reference genome sequencing requires well curated databases in which the deposited viral, bacterial and fungal sequences have been verified (Zhulin 2015; McTaggart et al 2016, Aylward et al 2017). An alternative bioinformatic diagnostic approach is the use of electronic probes (e-probes) to specifically detect pathogens targets within NGS data, (Stobbe et al 2013, 2014; Espindola et al 2015; Visser et al 2016a). This targeted approach could target specific regions in pathogens with large genomes or partial and whole genomes for pathogens such as viruses.

Targeted gene sequencing that enriches and specifically sequences single or multiple gene regions of interest of an organism. This method allows massive multiplexing of samples on Illumina based equipment (Kinoti et al 2017) but has the prospect to be applied to small sample sizes using the nanopore technology, such as the MinION or SmidgION being developed by Oxford Nanopore technologies, which could allow a more rapid identification of a pathogen directly from a sample, in one to two days, rather than following traditional isolation and characterisation methods, that can take longer, and which are currently used in many plant diagnostic laboratories (Kerkhof et al 2017). This type of approach has been used to analyse various microbial communities e.g. the bacterial community in a water sample (Vierheilig et al 2015), fungal communities in strawberry plants (Abdelfattah et al 2016) and *Ilarvirus* species in *Prunus* trees (Kinoti et al 2017). Targeted gene sequencing, using the fungal internal transcribed sequence 1 (ITS1) as a single gene target, was used to examine the diversity of airborne fungal communities and a targeted bioinformatics analysis identified spores of fungal plant pathogens in families, genera and down to species level (Nicolaisen et al 2017). This approach identified the presence of previously unreported ilarviruses in *Prunus* trees in Australia and could differentiate between *Ilarvirus* species and strains in mixed infections a single *Prunus* sample (Kinoti et al 2017).

- NGS technology is a fast-developing technology which is likely to be applied to plant pathology diagnosis as well as research for a range of samples including: ad hoc samples of unclear aetiology and plants being tested during PEQ and entry into high-health certification programs. Amplicon sequencing, using generic primers, linked with nanopore technology could be a useful in-lab approach to facilitate faster diagnosis of suspected bacterial, fungal or viral pathogens

7 Other considerations for development of diagnostic tools

7.1 Sampling and test accuracy

Although new technologies can improve early detection of a pathogen, applying a plant pathogen diagnostic tool with accuracy is dependent on several factors including knowledge of pathogen biology to assist accurate sampling and an understanding of the sensitivity and specificity of the assay. Test accuracy requires knowledge of the location of a pathogen within or on its host, pathogen concentration, which can change with season or other environmental factors such as temperature, to support accurate sampling. For example, phytoplasmas can be unevenly distributed in grapevines and testing is most accurate in late spring and up until harvest using symptomatic tissues (Constable et al 2003). Further work may be required for some tests to validate or

improve on current sampling strategies to assist accurate detection

Most of the nucleic acid and serological based assays discussed in this review are applicable to different substrates such as water, various plant growing mediums, different plant tissues such as seed, tubers, flowers, pollen, leaves and stems as well as green and woody tissues, if the test target can be adequately isolated from the sample.

7.2 Improving diagnostic lab workflows

All the laboratory methods described above can be done in the laboratory. Although they seem rapid, the may be delayed due to sample volumes and surges experienced by laboratories. However, there are some other approaches to diagnostics that could be developed to hasten lab-based testing. One approach could be to provide industry with simple tools to partially prepare samples before submitting them for testing. This approach could be as simple as providing tools for homogenisation and stabilisation of a sample in a buffer to extraction of nucleic acids, proteins or metabolites that are targeted in laboratory-based tests. For example, some of the simple nucleic acid methods described in 2.3.6 could be done by the grower and a nucleic acid sample as well as a plant sample could be supplied. The nucleic acid sample could facilitate the use of an initial molecular test while the plant sample could provide a backup if further identification methods are required such as isolation.

7.3 Route to development, adoption and market

There are several steps in the route to market and adoption of diagnostics assays, for point of care and laboratory testing (Figure 5, Derda et al 2015). The commissioning of this review indicates a demand for improved Point of care and laboratory diagnostics for the nursery industry, especially for tests that reduce time to diagnosis and are easy to use. This review is second step along the pathway, it has identified tools that could be developed and are fit for purpose for detection of various pathogens of concern to the broader nursery industry (Table 1; Table 2).

As a part of the initial assessment in step 2 (Figure 5), the ability to commercialize a test should also be considered prior to research and development. Potential commercialization partners and manufacturers were not contacted as a part of this review. An economic analysis will need to be undertaken by commercial partners and manufacturers based on industry demand for a test (volume) and the cost of production for off the shelf tests, such as LFDs, Lab-on-chip, aggregation of functionalized colloidal gold particles (e.g. ExactBlue) and the BioFire array. Some of these technologies are readily available and developed for clinical (human health) applications, but the volume of testing, and therefore demand for these tests, is likely to be far greater than some agricultural /horticultural applications.

Many tools described in this review are being developed for other pathogens and a route to market exists, especially for LAMP, RPA and field-based PCR. Optigene collaborated with Fera Science Ltd, a UK research agency, to develop LAMP kits for specific detection of several plant pests and diseases. Agriculture Victoria Research (DEDJTR) is in discussions with a commercial provider to manufacture LAMP test kits that can also be bought off the shelf. AGDIA and BioMeme have a developer programs for researchers to enable global

availability of field deployable RPA and PCR tests, respectively, using their platforms. LAMP and RPA could be developed into nucleic acid LFDs. Simpler equipment requirements, ease of interpretation and an obvious route to market make LAMP and RPA an obvious choice for future field deployable test development. Remote sensing technology, such as hyperspectral imaging, for disease detection is fast evolving. There is a potential route to market with several technology companies collaborating with researchers to develop this technology for a broad range of surveillance and monitoring applications, including disease diagnostics.

The route to market is often simpler for laboratory-based tools such as PCR and next generation sequencing, especially tests that are developed in-house by larger laboratories that have the capability to maintain and update them, without the need to engage a commercialization partner. Continued research and development of laboratory-based diagnostics, especially to improve and hasten workflows, will improve diagnosis delivery. Improving workflows could include development of point of care sample preparation. However rapid workflow will be limited by the resources available in some laboratories. Less resourced laboratories may also benefit from development of in field technology that has a low requirement for equipment to run and interpret tests.

This review has also identified some tools that could be simple field deployable tests or facilitate faster laboratory turnaround to diagnosis. However, they have a less obvious route to market, such as colloidal gold aggregation, (e.g. ExactBlue) and the BioFire array. The simplicity of the ExactBlue technology makes it highly desirable, but the development of the test could be complex, especially in ensuring accuracy to the target. It is also unclear if the company is viable with respect to providing commercialization and longer term support and availability. BioFire array equipment could require significant investment by laboratories (approx. $70000). BioFire array test panels could cost around $200, but careful thought to the design of panels is required as they may lack flexibility for updating panels as new information about genetic diversity of known pests is discovered and novel pathogens are found. Volume of off the shelf test panels for agriculture/horticultural pests to make this technology commercially viable also needs to be explored.

Once a test has been developed, it needs to be evaluated for analytical sensitivity, analytical specificity, repeatability and reproducibility (EPPO 2017). This could include ring testing by end-users. Tests supplied commercially or within laboratories need to be quality assured, quality controlled and supported to ensure their ongoing accuracy and reliability (Figure 5.). All tests are at risk of delivering false positive and negative results and all will require adequate development to ensures their accuracy, reliability and sensitivity for the targets to which they would be developed.

The diagnostic tools identified in the project have varying skill levels for their application. LFDs (serological and nucleic acid), LAMP, RPA and field deployable PCR can be developed so they easy to use by minimizing the steps involved in performing the assay and providing clear and simple guidelines to facilitate interpretation, with or without devices (Zheng et al 2018). LAMP tests are likely to be more pathogen specific compared to PCR and RPA and may be better suited to detection of species, strains ad pathovars. Commercial kits can be developed that include quality controls to ensure accuracy and to assist interpretation. The ExactBlue technology is very user friendly and the device provides confidence in interpretation of results. Other tools presented in this review are more complex and could require significant training and skill. However, end to end workflows could be developed to simplify the nanopore sequencing technologies, such as MinION, to make this technology useful in a field-based system, especially for field-based diagnosticians and consultants.

Field-based diagnostic testing is not typically high-throughput, for many samples or many pathogens, and can be difficult to apply to emerging diseases. PCR, ELISA are and NGS will become the backbone of rapid laboratory diagnostic testing and continued development of these tools is critical in ensuring the biosecurity of the nursery industry.

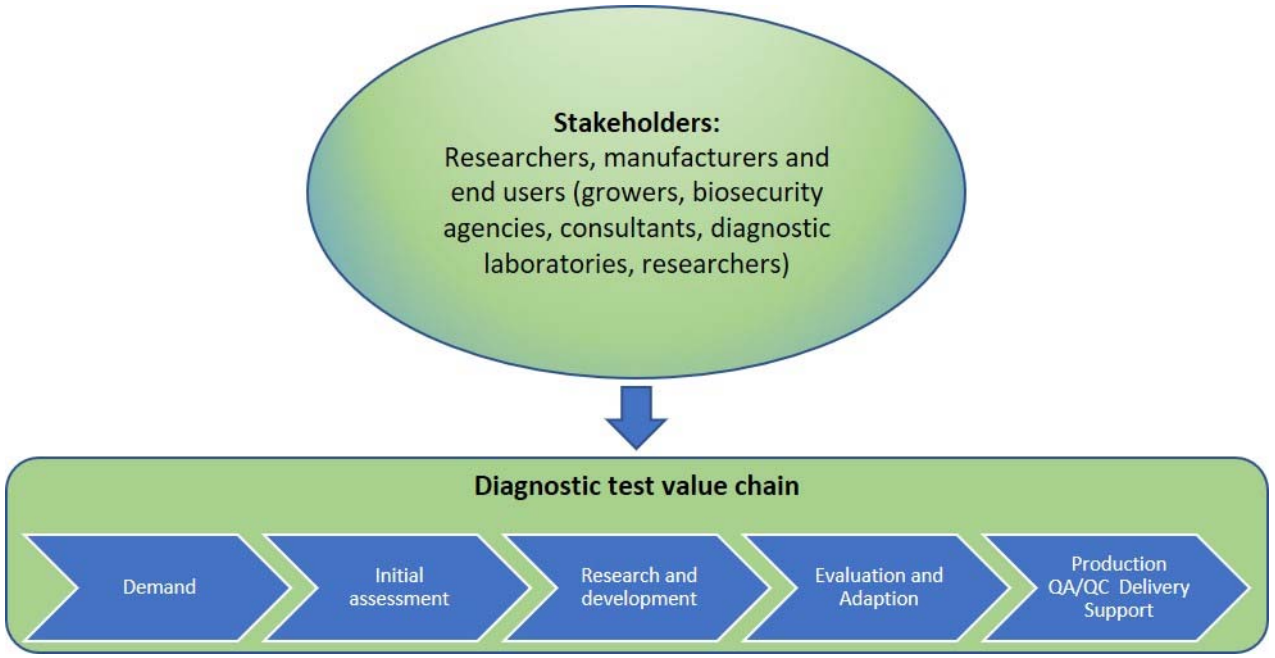

Figure 5. Value chain of diagnostics tests, which indicates all stakeholders as well as the delivery process for of an accurate and rapid test for plant pathogen detection (after Derda et al 2015).

8 Conclusions

The top ten endemic pathogens or pathogen groups of significance across the broad range of nursery commodities and included: *Pseudomonas* sp., *Xanthomonas* sp., *Calonect*ria sp. (*Cylindrocladium* sp.), *Colletotrichum* sp., *Fusarium* sp., *Phytophthora* sp., *Pythium* sp., *Rhizoctonia* sp., *Potyvirus* sp. and *Orthotospovirus* sp. The methods currently available for detection of some or all of these pathogens includes traditional isolation methods such as plating onto media, serology-based tests including enzyme linked immunosorbent assays (ELISA) or lateral flow devices (LFDs; dipsticks) and molecular methods such as polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA).

This review identified field deployable technology that could prioritised for further development for detection of these pathogens to support the biosecurity needs of the Australian nursery industry. There is significant experience amongst the Australian plant pathology research community to facilitate development of serological based LFDs as well as field-based PCR, LAMP and RPA tools. These tools have a clear route to market through commercial companies who provide off-the-shelf test kits and should be priority for

immediate development.

Other technology such as tests based on the aggregation of functionalized colloidal gold particles could provide a simple visual detection method for specific pathogens or pathogen groups without the requirement for complex equipment. However, a preliminary investigation is required determine the diagnostic potential of this novel technology.

PCR and ELISA continue to be the mainstay of many laboratories and continued investment in the development and validation of this technology for plant pathogen detection is warranted, especially for rapid, high throughput testing during surveillance and certification testing.

Rapid metagenomic and metabarcoding NGS are emerging technologies that could be applied as a single test to identify multiple pathogen in a single sample, aid in the diagnosis of causal agents for diseases of unclear aetiology or to specifically identify genera, species and strains/pathovars of individual and groups of pathogens important to the nursery industry. PCR and NGS can be developed in-house by experienced researchers for laboratory use and in-field by plant pathology diagnosticians, facilitating access to fit for purpose tests and investment in this technology is recommended.

Development of simple tools that allow growers to prepare samples for laboratory-based diagnosis will assist laboratory workflows and improve turnaround times for results.

All technology will require an “omics” based approached to understand pathogen diversity and identify appropriate genomic, proteomic or metabolomic targets for pathogen detection to ensure test reliability. The tests need to be quality assured and require validation to determine their analytical sensitivity, analytical specificity, repeatability and reproducibility to ensure they are fit for purpose.

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