FINAL REPORT

GRDC
GRAINS RESEARCH & DEVELOPMENT CORPORATION

PHA00016

Biosecurity preparedness for the grains industry - High-throughput diagnostic for Karnal bunt

PROJECT DETAILS

PROJECT CODE:	PHA00016
PROJECT TITLE:	BIOSECURITY PREPAREDNESS FOR THE GRAINS INDUSTRY - HIGH-THROUGHPUT DIAGNOSTIC FOR KARNAL BUNT
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Summary

Karnal bunt (KB) is caused by a fungus (*Tilletia indica*) which affects bread wheat, durum wheat and triticale. While KB causes little yield loss, it can be difficult to manage in rotations and detection of spores in grain shipments is a quality issue and can result in consignment rejection to current grain export markets. This scoping study identified the development of DNA amplification (real-time polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) tests) as the most effective, lowest risk research and development (R&D) pathway to achieving the goals of stakeholders. For ongoing surveillance for area freedom, use of high speed optical sorters could be developed as a less sensitive, but more cost effective technology for detection of bunted grain.

Conclusions

Following a detection or suspected detection of KB, it is anticipated market closure would occur. Trade would resume, however, but the length of time taken to re-open markets would depend on where the detection occurred and the speed at which grain growing regions or individual consignments of grain could be declared free from the fungus. For domestic trade, governments could consider implementing a complete standstill of grain consignments until delimiting surveillance has been undertaken to determine and contain the extent of an incursion. This would have a significant impact on grain handling and shipment, an individual grower's ability to deliver grain and the supply of grain to intensive livestock industries (particularly pigs and poultry). The more quickly surveillance can be undertaken, the faster a return to trade for both the domestic and international markets could be negotiated.

An efficient, robust diagnostic test that can handle high numbers of grain samples is therefore a critical element of preparedness for a potential incursion of KB. The current international diagnostic standard (largely developed by Australian researchers) comprises both morphological assessment of spores and a PCR test. The morphological test is time consuming and the PCR test is not reliable, meaning both tests are required for full validation. While the PCR test can be used for initial screening of samples, it has not been adapted as a high-throughput (HT) test.

A key focus of attempts to refine the current diagnostic tests for KB should be the removal of the need for significant numbers of samples to be germinated because the required time (1-2 weeks) precludes meaningful surveillance. The most common reason that germination is required is to produce material for DNA extraction to confirm a diagnosis using PCR and LAMP tests. All DNA amplification tests are generally amenable to commercially-available fluorescent platforms that would provide HT for a surveillance program. Therefore, the most cost effective way to remove the need for significant germination and to provide HT, is to improve the accuracy of existing multiplex real-time PCR and/or LAMP tests.

The current multiplex PCR shows promise, but has a relatively low diagnostic specificity and sensitivity (both of approx. 48%) and therefore cannot be used in its current form as the primary screening test to significantly reduce reliance on morphological diagnosis. The recently published KB LAMP test also shows significant promise, but similarly requires further testing, optimisation and adaptation to a HT platform.

Inadequate specificity and sensitivity of these tests are technical issues that should be possible to overcome, for example by changing primer combinations or reducing the degree of multiplexing in each reaction. There is a high chance that DNA amplification will provide the capabilities required and with a definable research pathway and timeframe.

Another option for HT that has a high chance of success and could be deployed for analysis of KB, as well as other contaminant or quality issues, is the use of optical sensing. This technique is used in the United States for routine surveillance,

however it would need to be developed and used in conjunction with other assessment methods such as morphological and/or DNA amplification tests.

Recommendations

This study identified that there are currently four main areas that require addressing in developing a robust, HT test for KB. These areas relate to the following difficulties in sampling and removing bottlenecks to existing tests:
- Determination of representative sampling that is achievable and statistically robust.
- Extraction systems for KB spores from grain samples.
- Extraction systems for DNA having requirement to germinate spores of KB.
- Specificity and sensitivity of the current PCR test.

The following recommendations are provided from the technical assessment of options for a HT test for KB to address these issues:
1) Review of the current Draft National Contingency Plan for KB to provide realistic, achievable and statistically robust sampling targets for confirming consignment or area freedom from KB.
2) Assessment of methods to improve spore and DNA extraction techniques to remove the existing substantial bottlenecks within the current diagnostic test for KB.
3) Tandem development of real-time PCR and LAMP tests for detection of KB. These technologies were considered to be the lowest risk approach for development of the HT test as both have undergone significant preliminary development, have good potential to be optimised, and utilise similar equipment and expertise to deliver cost efficiencies.
4) Redesign and optimisation of a real-time PCR test to provide HT capacity and be consistent with existing capability present in many laboratories in Australia that operates on 'off-the-shelf' HT platforms.
5) Use of optical sensing technology to deliver ongoing HT testing for area freedom. This technology is considered to be a low risk approach as it has been proven to work for KB and can be used to test for other grain quality or contamination issues. It is likely calibration of optical sensing machines for KB would need to be undertaken off-shore, as importing bunted grain into Australia will be unlikely even if spores were denatured.
6) Establishment of ongoing sampling and assessment of grain samples to ensure that Australia remains free of KB and that area freedom data are available. The National Residue Survey (NRS) sample collection system should be investigated for provision of samples that are representative across Australia, however engagement with bulk handling companies and government would be required to identify mechanisms to use samples for this purpose.

Outcomes

Australia is free from many of the diseases, pests and weeds that cause a significant impact in other grain growing regions of the world. With the increase in global trade and movement of people, equipment and produce, the risk of accidental importation of an exotic plant pest (insect, pathogen or weed) continues to increase. The Biosecurity Plan for the Grains Industry (July 2015) identified 54 high priority pests, with KB ranked as the only pest considered an extreme risk, due to its potential impact on trade. A recent Australian Bureau of Agricultural and Resource Economics (ABARES) report (ABARES project 43482, Hafi et al. 2015) estimated that an incursion of KB in Australia would cause demand shock that would reduce the average price for wheat on the domestic market by 12% as a result of export market closure. Annual profits for farms were estimated to be reduced by 7%.

While tests for KB are currently available that can identify and confirm a detection of the fungus, they are slow, costly, labour intensive and require specialised skill sets. Given the potential impact of a suspected or actual detection of KB on trade, this scoping study has identified that research investment would be required for the deployment of a testing regime that can screen large numbers of grain samples (i.e. HT) with high accuracy. Development of such a test could be utilised for ongoing surveillance of the grain supply chain to provide evidence for area freedom and for delimiting surveillance if an incursion was detected. For delimiting surveillance, it is of particular importance to screen as many samples as quickly as possible.

This scoping study has assessed and compared current and developing technology and processes that could be used to deliver this capacity and to achieve outcomes of being able to provide the capability to delimit an incursion and also to provide reliable evidence of absence for area freedom. Development of DNA amplification (real-time PCR or LAMP tests) is recommended as the most effective, lowest risk R&D pathway to achieving the goals of stakeholders. If DNA amplification techniques were selected to deliver a HT test, spore and DNA isolation remains the primary bottleneck in the diagnostic

process. For ongoing surveillance for area freedom, use of high-speed optical sorters could be developed as a cost effective, though less sensitive technology for detection of bunted grain.

Achievements/Benefits

Key considerations for assessing priorities for development of a KB surveillance program are:
o The aim(s) of applying the surveillance.
o Sensitivity that is required to meet these aims to the satisfaction of stakeholders.

While tests for KB are currently available that can identify and confirm a detection of the fungus, they are slow, costly, labour intensive and require specialised skill sets. The current international diagnostic standard, (largely developed by Australian researchers), comprises both morphological assessment of spores and a PCR test. The morphological test is time consuming and the PCR test is not reliable, meaning both tests are required for full validation. In addition, while the PCR test can be used for initial screening of samples, it has not been adapted as a HT test. Given the potential impact of KB, an efficient, robust diagnostic test that can handle high numbers of grain samples is a critical element to support surveillance for KB.

Routine surveillance could be applied with either the aim of detecting an initial KB incursion early enough to eradicate it, or detecting it at a point where it could be contained to a small area. A subsequent aim would be to undertake delimiting surveillance to monitor presence or absence of KB if an incursion occurred. It should be noted that the USA has an ongoing national screening program in place due to incursion and establishment of KB, the distribution of which is currently accepted as being limited to Arizona. This program is based on detection of bunted grains by high speed optical sorting and has been sufficient to prove area freedom outside of Arizona and retain access in most markets. However, the approach of detecting bunted grains in harvested grain samples is insensitive for a delimiting surveillance program. In addition, given the smaller market size of Australian grain and the sensitivity of the main markets to KB, it is doubtful this approach would be accepted by trading partners. It is, therefore, recommended that a more sensitive test for both delimiting surveillance and area freedom may be required.

It is proposed that if a highly sensitive HT test was developed and it facilitated eradication or containment of a KB incursion, then it would be economically advantageous because of the huge cost of KB becoming established in Australia. However, development of such a HT test would require a greater strategic research investment to provide an industry relevant technology. To provide options for the best type of HT test to implement, this project undertook a scoping study to evaluate current methodologies, different types of tests available, and the needs and capacity of government and industry stakeholder.

The following range of diagnostic test options were considered within the scoping study:
- DNA amplification tests such as real-time PCR and LAMP.
- Optical sensor technology.
- Specific detection targets (e.g. high performance liquid chromatography-mass spectrometry (HPLC-MS) and matrix assisted laser desorption/ionization-time-of-flight (MALDI-TOF) assays).
- Use of specific immuno-detection methods (e.g. enzyme linked immunosorbent assay (ELISA)).
- Transduction assays (e.g. fluorescent PCR).
- Use of volatile molecules (e.g. sniffer dogs or electronic nose technology).

Of these, the tests considered to be most appropriate for HT and also the lowest risk R&D pathway were the DNA amplification tests. This recommendation was provided given the current real-time PCR test is an established technology, and there would need to be significant identifiable benefits to invest in development of novel technologies. It was recognised, however, that there are still major challenges to the development and deployment of real-time PCR and LAMP as HT tests. Optical sensing was also considered a viable option for HT, as this type of test has been developed and deployed in the United States and represents an option that can be used to screen high numbers of samples. Advantages and disadvantages of DNA amplification and optical sensing are as follows:

Advantages to LAMP technology are that it has proven to be robust and easy to use for other pathogens and can be used in the field. It could easily be deployed 'up-country', i.e. at regional receival points before grain was aggregated into bulk receivals, providing improved opportunity for tracing and containment if a potential detection occurred. Preliminary development of LAMP tests for KB are showing promising results (Gao et al. 2016) and if LAMP technology was developed and deployed, it could be useful for ongoing surveillance for area freedom.

Disadvantages with LAMP technology are that it is limited to approximately 30 samples per hour (per machine) and additional work will be required to take this from proof of concept to deployment, including the isolation of KB spores for testing from bulk grain samples.

The advantages to real-time PCR are that once developed, tests can be deployed at relatively low cost, and can be multiplexed with tests for other pathogens associated with grain. Providing spore isolation and DNA extraction issues can be resolved, a large number of samples can be processed each day. Disadvantages to real-time PCR are that it will be relatively expensive to develop tests to become HT as significant R&D challenges exist with the bottlenecks associated with spore and DNA extraction from bulk grain samples and also with improving the specificity of the current test. Compared with LAMP and optical sensing, specific expertise will be required to conduct tests.

The advantages to optical sensing are that this is a proven low cost, HT method used in the United States so has a very high likelihood of success. Large numbers of samples can be processed each day and the technology can be calibrated to incorporate assessment of other quality or biotic issues, including identification of weed seeds. The disadvantages of optical sensing are it has very low specificity and sensitivity, and any potential suspected positive samples would require assessment using PCR and morphological methods. In Australia, optical sensing is only being used routinely within CBH (the EyeFoss system), and R&D will be required to ensure systems can be 'trained' to detect bunted grain. Testing and development may have to occur overseas as importing bunted grain into Australia (even as irradiated or sterilised samples) is likely to be difficult.

Stakeholder analysis
To determine the capacity requirements of a HT test for KB, interviews were conducted with staff from CBH, Department of Agriculture and Food Western Australia (DAFWA), Primary Industries and Resources South Australia (PIRSA), the South Australian Research and Development Institute (SARDI), NRS and Grains Industry Market Access Forum (GIMAF). Information was also obtained from these stakeholders, as well as Viterra and Agriculture Victoria (VIC) as part of Exercise Haryana, the Karnal bunt Simulation Exercise conducted in 2015/2016. Exercise Haryana has focused on activities associated with tracing and sampling a simulated detection of KB in SA. This simulation exercise was based on the existing diagnostic tests (morphological and PCR amplification) and provided significant additional input from stakeholders for issues associated with these current tests, as well as the difficulties of sampling and tracing grain parcels.

From the stakeholder consultations, information was obtained on the volumes of grain samples that could be likely in the event of a KB detection. For SA, it was determined that if the current Draft Contingency Plan for KB is used as the basis for a sampling strategy, and each cell in bulk storage holds approx. 2,000 tonnes, this equates to approx. 2,850 samples required per cell (at a sampling rate of 1kg/700 kg). For SA, it was estimated that if sampling was required across all bulk aggregation sites, the average number of samples to be considered was calculated to be approx. 42 million samples (given there are 100 sites, 50 cells/site, and 0.3 of these cells have susceptible grain). This level of sampling is clearly unachievable and information from Exercise Haryana recommends an urgent update to the Contingency Plan to re-evaluate realistic targets based on statistical confidence of detection for sampling strategies, especially given International Seed Testing Association (ISTA) seed testing guidelines refer to a sample of every 300t of grain for phytosanitary purposes. If this level of testing was used, the average number of samples to be assessed in SA would still be in the vicinity of 10,500.

For WA, CBH provided information that approx. 20,000 'audit' samples are taken each harvest, equating to approx. 8% of total grain. If sampling could be undertaken 'up-country' i.e. before grain was aggregated at large receival sites such as Kwinana, at worst case this could equate to a total 240,000 to 300,000 samples for a harvest period. It is unlikely, however, that these high volumes of samples would be experienced as composite bulking of samples would be undertaken and CBH indicated it would be capable of collecting, labelling and storing samples and providing whatever composite is needed, depending on the level of detection required.

If information for area freedom was required on a national basis, a potential method proposed for representative sample collection could be use of samples collected under the NRS. In this instance, it is estimated that processing of approx. 3,500 samples would be required per annum and it is suggested that this volume of samples is a reasonable target to consider for a HT testing system.

Other research

Information obtained from Exercise Haryana indicates the current Draft National Contingency Plan for KB requires updating as the numbers of samples to be collected for delimiting surveillance are unachievable, it does not clearly articulate what to do with a suspect property, and there were inconsistencies with the Plan and the AUSVET Plan, making movement of staff between types of biosecurity incidents more difficult.

Intellectual property summary

In the first instance, the end users of these recommendations are the GRDC and government stakeholders. If investment proceeds with development of a HT test, an option for delivery of a HT test could be under a fee-for-service model, however it is considered unlikely that there would be sufficient market to develop a fully commercial test.

End users of this scoping study would also be the Sub-Committee on Plant Health Diagnostics (SPHD). Opportunities within this scoping study for development of recommendations for converting diagnostic protocols (for confirmation of pest and disease incursions) to methods suitable for HT diagnostics for surveillance for area freedom, have been identified as part of national planning processes.

No confidential information has been included in the technical report on assessment of methods for HT detection of KB and no identification of specific restrictions on intellectual property (IP) have been identified. Should development of a HT test proceed, it is anticipated IP will be generated associated with processing large volumes of samples.

Additional information

Gao Y, MK Tan and YG Zhu (2016). Rapid and specific detection of *T. indica* using loop-mediated isothermal DNA amplification. Australasian Plant Pathology 45: 361-367.