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ORIGINAL RESEARCH ARTICLE

Evaluating molecular diagnostic techniques for seed detection of *Pseudomonas savastanoi* pv. *phaseolicola*, causal agent of halo blight disease in mungbean (*Vigna radiata*)

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Abstract

Halo blight of mungbean (*Vigna radiata* var. *radiata*) is caused by the bacterium *Pseudomonas savastanoi* pv. *phaseolicola*. This pathogen is transmitted via infected seed, facilitating the spread of the disease into new cultivated areas. Prospective mungbean seed crops are currently subjected to visual inspection as a means of determining disease status, however, this is a poor method that relies on visible symptoms and does not account for latent infections. A range of molecular diagnostics targeting *P. savastanoi* pv. *phaseolicola* have been developed, but these have not been deployed on seeds. Quantitative PCR (qPCR) SYBR assay, hydrolysis probe, and conventional PCR, using the same primers were optimised against a plate-truthed dilution series of *P. savastanoi* pv. *phaseolicola*. The detection limit of the conventional PCR assay was approximately 9,000 CFU μl^{-1}, while both qPCR assays could detect 9 CFU μl^{-1}. These tests were then used to screen DNA extracted from 200 g allotments of 38 seed lots comprising six mungbean cultivars representing the primary Australian production area, and two seed lots of known infection status. Of these, the pathogen was detected in six seed lots by conventional PCR. The SYBR assay and hydrolysis probe methods detected 20 and 24 infected seed lots respectively. This shows that the hydrolysis probe method was the most effective at diagnosing the presence of *P. savastanoi* pv. *phaseolicola* in mungbean seed, providing a valuable molecular diagnostic to aid in integrated disease management and seed certification, substantially mitigating losses to halo blight disease.

Keywords qPCR · PCR · Diagnostic assay · Halo blight · Mungbean

Introduction

Mungbean (*Vigna radiata* var. *radiata*) is a vital source of food and income for smallholder farmers globally and is of increasing importance to Australia's agricultural exports. It is the most valuable pulse crop and the main broadleaf rotation for dryland farmers in eastern Australia, and is worth over $100 M in exports each year. A major biotic production constraint to global mungbean production is halo blight, caused by the bacterium *Pseudomonas savastanoi* pv. *phaseolicola*. Halo blight is a devastating seed-borne disease that is responsible for extensive yield and economic losses in mungbean and tropical legumes such as common bean, lima bean, pigeon pea, pea and runner bean (Taylor et al. 1996; Ryley et al. 2010; Sun et al. 2016; Noble et al. 2018). It was first identified as an issue in Australian commercial mungbean production during the mid-1980s (Ryley

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et al. 2010) and has also been reported in Pakistan (Akhtar 1988), Nigeria (Umunna et al. 2014) and China (Sun et al. 2016). The pathogen thrives in cool, wet and windy conditions, where it is spread by water-splash and enters through wounds and stomata, in addition to seed transmission (Taylor et al. 1979). *Pseudomonas savastanoi* pv. *phaseolicola* does not survive long in the soil (Wimalajeewa and Nancarrow 1980), but has been reported to survive for ten years in controlled grain storage of common bean and caused epidemics with less than 0.01% infected seed (Taylor et al. 1979; Taylor 1970). Therefore, detection of the pathogen in seed is critical to the integrated disease management of halo blight.

Australian mungbean crops intended for seed distribution are visually inspected for symptoms of halo blight to minimise the risk of spreading the disease. However, as symptom expression is dependent on environmental conditions, there is a high likelihood that latent infections result in infected seed harvested from asymptomatic crops. To date, no molecular diagnostic assay has been reported to test mungbean seed for the presence of *P. savastanoi* pv. *phaseolicola.* The absence of a rigorous, molecular-based, seed certification scheme is likely to be a contributing factor to continuing outbreaks of halo blight throughout sub-tropical growing regions of Australia, in Queensland and northern New South Wales.

There is limited resistance to halo blight in current commercial mungbean varieties. Opal-AU released 2020 (Douglas 2020) and Celera II-AU released 2015 (Douglas 2014) are both rated as moderately resistant to a strain of *P. savastanoi* pv. *phaseolicola* in Australia (accession T11544). The presence of other *P. savastanoi* pv. *phaseolicola* strains may also impact the utility of genetic resistance (Noble et al. 2018). Therefore, a key part of integrated disease management to limit the risk of epidemics and transmission is through identifying latent infections in seed prior to planting.

Published PCR protocols targeting *P. savastanoi* pv. *phaseolicola* were tested to determine their capacity to amplify DNA fragments from the pathogen. This was conducted on aqueous suspensions of pure cultures of the pathogen, in addition to DNA extracted from mungbean seeds. This study reports the accuracy and sensitivity of conventional and quantitative PCR protocols to identify the halo blight pathogen associated with mungbean seed samples and aims to facilitate the adoption of these molecular diagnostic techniques for ongoing detection, surveillance and monitoring of halo blight by mungbean researchers and industries.

Table 1 Primer and probe targets and sequences used in this study

Assay	Primer	Sequence 5'-3'	Locus	Product (bp)	Reference
PCR	P5.1	AGCTTCTCCT-CAAAACACC	Phaseolotoxin gene cluster	502	Schaad et al. 1995
	P3.1	TGTTCGCCAGAG-GCAGTCATG			
qPCR	Psy_cyoII-pb	GCCAAGTACACGC-CGGACTGGTC	Cytochrome o ubiquinol oxidase subunit II	176	Xu et al. 2011
	Psy_cyoII-F	TCGAGCAGCG-GAACCTGATC			
	Psy_cyoII-R	TGGGTAC-GCCCCAGACTGCGA			

Materials and methods

Sample collection

During the summer-autumn growing season (Jan-Apr) of 2017 and 2018, a total of 35 one-kilogram mungbean grain samples were collected from processors representative of the primary Australian cultivated area. Additionally, three samples of seed were harvested from artificially inoculated halo blight disease nurseries located at the Queensland Department of Agriculture and Fisheries, Hermitage Research Facility, Warwick, Queensland, Australia (28°12′S, 152°5′E), to be used as positive controls. Six commercial cultivars were represented among the 35 samples, all with unknown halo blight disease status. DNA was extracted from 200 g of seed from each sample using the SARDI proprietary protocol that has been developed for soil DNA extraction and has been successfully applied to a range of environmental samples, including grain (D. Giblot-Ducray unpublished data, Giblot-Ducray et al. 2016; Haling et al. 2011). Seeds were not washed prior to extraction. These DNA extractions were used as the template for the PCR assays in this study (Table 1).

Assay sensitivity

A serial dilution of *P. savastanoi* pv. *phaseolicola* isolate T11544 was performed to assess the sensitivity of detection for conventional and quantitative PCR. Isolate T11544 has been the source of inoculum for field screening in the Australian's National Mungbean Improvement Program (an investment of the Queensland government and the Grains Research and Development Corporation) for the past fifteen

Table 2 Mean viable plate counts of serially diluted cultures of *P. savastanoi* pv. *phaseolicola* isolate T11544 and detection thresholds for each assay, + refers to a positive reaction and – a negative reaction

Dilution factor	Ave. colony count[a]	Projected CFU μl^{-1}	Mean C_q Hydrolysis probe	Mean C_q SYBR	Conventional PCR
10^{-3}	TMTC	9,000	29.56	25.26	+
10^{-4}	TMTC	900	32.47	28.63	-
10^{-5}	TMTC	90	34.42	31.03	-
10^{-6}	TMTC	9	36.94	34.23	-
10^{-7}	89.33	0.9	n/a	n/a	-
10^{-8}	9	0.09	n/a	n/a	-

a. Average colony counts on triplicate plates after 100 ul of bacterial suspension from each dilution was plated onto KB medium. TMTC - too many to count

years (Noble et al. 2018; Ryley et al. 2010). Isolate T11544 was grown on King's medium B (KB) (King et al. 1954) for two days then transferred using a sterile loop of the culture into 1 ml of sterile water in a 1.5 ml centrifuge tube. This was mixed by vortexing before being used to seed a series of eight 1:10 dilutions by successively adding 100 µl of suspension to 900 µl water, mixing and then repeating the process. Once the dilution series was prepared, three 100 µl aliquots of each suspension were placed onto each of three KB plates and spread using a flame-sterilized glass spreader. All plates were sealed with Parafilm and incubated at 28 °C for approximately 2 days, and colony counts were conducted to calculate the *P. savastanoi* pv. *phaseolicola* titres in the dilution series (Table 2). Projected CFU ml^{-1} were calculated based on the average of counts for the – 1.E + 07 dilution. The remaining suspensions were stored at -20 °C and used as the template for conventional and quantitative PCR assays.

Conventional PCR conditions and gel electrophoresis

Conventional PCR primers (Schaad et al. 1995) were used to amplify a 502 bp fragment of the tox gene cluster unique to *P. savastanoi* pv. *phaseolicola* from DNA extracted from seed and a dilution series of cultured T11544 cells. Reactions were conducted in 10 ul volumes, using 5 µl of GoTaq master mix (Promega), 0.5 µl of 10 µM each primer, 3 µl of water and 1 µl of template. Thermal cycling was conducted using an Applied Biosystems, Life Technologies Proflex cycler, using the following profile: 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 60 °C for 30 s, 72 °C for 30 s; then a final extension of 72 °C for 5 min. Products (10 µl) were electrophoresed on 1% agarose gels cast and run in 0.5 x TBE buffer containing 1% SYBR safe (Invitrogen). Bands were visualized using the G-Box Syngene gel documentation system.

Hydrolysis probe assay

Primers developed by Xu and Tambong 2011 were designed to amplify a 176 bp section of the single copy cytochrome o ubiquinol oxidase subunit II gene of *P. savastanoi* pv. *phaseolicola.* Quantitative PCR was conducted on DNA extractions from seeds and the dilution series of T11544 cells. Reactions were performed using three technical replicates of each sample analysed on a CFX 384 instrument (Bio-Rad). Reaction mixtures contained 2 µl of template, 5 µl of SensiFAST (Bioline); 0.25 µl of 20 µM forward/reverse primers and probe, and pure water to a final volume of 10 µl. No-template controls contained 2 µl of pure water in place of the suspension. Positive controls included 2 µl of a 1:100 dilution of an aqueous suspension of cultured T11544 cells and DNA extracts from known infected grain samples. Thermal cycling conditions consisted of an initial denaturation step for 15 min at 95 °C followed by 55 cycles at 95 °C for 10 s and 68 °C for 1 min. Representative PCR products were cloned and sequenced to confirm target-specific amplification via the pGEM T-Easy (Promega) cloning system according to the manufacturer's protocol.

SYBR assay

Each hydrolysis probe assay was also performed using the SYBR protocol, but without the addition of the internal probe. Reaction mixtures contained 2 µl of template, 5 µl of 2x GoTaq qPCR master mix (Promega), 0.25 µl each of 20 µM forward and reverse primers, and pure water to a final volume of 10 µl. Thermal cycling conditions consisted of 5 min at 95 °C followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. To facilitate the scoring of results based on melt-curve analysis of PCR amplicons, a melting step followed real-time amplification consisting of linear temperature ramping from 60 to 95 °C, rising by 1 °C per step, with 90 s of pre-melting on the first step and 5 s for subsequent steps. Samples with melt peaks specific to *P. savastanoi* pv. *phaseolicola* in comparison with positive controls for two or three of the technical replicates were deemed positive.

Results

Analytical sensitivity of diagnostic assay

Conventional PCR on a dilution series of cultured *P. savastanoi* pv. *phaseolicola* amplified the target at a dilution of -1.E + 03, which represented approximately 9,000 copies μl^{-1} (Table 2). Both qPCR chemistries (SYBR and hydrolysis probe) amplified the target at a dilution of -1.E + 06,

Table 3 Comparison of diagnostic assays detecting *Pseudomonas savastanoi* pv. *phaseolicola* in mungbean seed

Sample	Cultivar	Location	Year Grown	Hydrolysis probe qPCR	Hydrolysis probeCq Value	SYBR Green qPCR	SYBR Green Mean Cq Value	Conventional PCR
B001	Mix	Warwick, Qld, Disease nursery	2017	+	24.15	+	23.51	+
B017	Crystal	Southern Toowoomba, Qld	2017	+	27.58	+	26.86	+
B019	Jade	Clifton, Qld	2017	+	28.85	+	27.83	+
B022	Crystal	Bowenville, Qld	2017	+	24.80	+	23.71	+
B024	Jade	Clifton, Qld	2017	+	25.87	+	24.71	+
B028	Mix	Warwick, Qld, Disease nursery	2017	+	22.94	+	21.45	+
B004	Mix	Warwick, Qld, Disease nursery	2017	+	31.82	+	30.82	-
B007	Unknown	Unknown	2017	+	28.86	+	28.16	-
B010	Unknown	Unknown	2017	+	33.71	+	34.37	-
B011	Unknown	Unknown	2017	+	30.65	+	29.57	-
B012	Unknown	Unknown	2017	+	29.86	+	28.86	-
B013	Unknown	Unknown	2017	+	34.60	+	34.28	-
B014	Unknown	Broadmead, Qld	2017	+	32.44	+	30.79	-
B018	Crystal	Jondaryan, Qld	2017	+	29.32	+	28.08	-
B020	Jade	Clifton, Qld	2017	+	34.73	+	32.74	-
B026	Celera	Brookstead, Qld	2017	+	35.73	+	33.81	-
B029	Unknown	Unknown	2018	+	35.22	+	34.54	-
B037	Crystal	Unknown	2018	+	32.91	+	29.68	-
B040	Jade	Unknown	2018	+	35.36	+	33.06	-
B043	Jade	Unknown	2018	+	36.52	+	33.75	-
B016	Jade	Pilton Valley, Qld	2017	+	31.99	-	0	-
B009	Unknown	Chandler	2017	+	34.20	-	0	-
B025	Jade	Cambooya, Qld	2017	+	34.43	-	0	-
B021	Crystal	Killarney, Qld	2017	+	35.00	-	0	-
B008	Unknown	Unknown	2017	-	0	-	0	-
B015	Unknown	Chandler, Qld	2017	-	0	-	0	-
B023	Crystal	Pilton Valley, Qld	2017	-	0	-	0	-
B027	Regur	Formartin, Qld	2017	-	0	-	0	-
B030	Unknown	Unknown	2018	-	0	-	0	-
B031	Jade	Unknown	2018	-	0	-	0	-
B032	Onyx	Unknown	2018	-	0	-	0	-
B033	Onyx	Unknown	2018	-	0	-	0	-
B034	Onyx	Unknown	2018	-	0	-	0	-
B036	Jade	Unknown	2018	-	0	-	0	-
B038	Crystal	Unknown	2018	-	0	-	0	-
B039	Regur	Unknown	2018	-	0	-	0	-
B041	Unknown	Unknown	2018	-	0	-	0	-
B042	Jade	Unknown	2018	-	0	-	0	-

representing approximately 9 CFU μl^{-1} correlating to a 1,000 times higher sensitivity than conventional PCR.

Diagnostic assay comparison

The conventional PCR primers tested in this study reliably detected six infected seed samples. These included 2 out of the 3 known infected positive control samples. SYBR identified 20 infected samples, including the six detected by conventional PCR, while the hydrolysis probe method detected all samples detected via SYBR, in addition to four more. A total of 14 samples were considered infection-free by all methods (Table 3). Both qPCR protocols detected *P. savastanoi* pv. *savastanoi* in all three positive control seedlots, and all qPCR amplicons were confirmed via Sanger sequencing.

There were significant differences in infection rates between the two sampling years. Of the 24 samples collected in 2017, 20 were found to be positive using the hydrolysis probe method, 14 using SYBR and four via conventional PCR. In 2018, of the 14 samples collected, 4 showed positive results for both the hydrolysis probe and SYBR assays,

but no samples had sufficient bacterial load to be detected by conventional PCR. The differences in infection between the years was statistically significant at the 1% level using the Chi-squared test ($p = 0.0007$).

Discussion

In this study, we compared conventional and quantitative PCR chemistries targeting *P. savastanoi* pv. *phaseolicola*. For many years the Australian mungbean industry has been limited to in-crop inspections by professional seed inspectors to reduce the incidence of bacterial seed-borne diseases. To deem a paddock disease free, inspectors walk through fields visually checking plants for symptoms of halo blight. While this method represents a step towards limiting disease spread, it is costly, time-consuming and can be prone to misdiagnosis. For example, symptomatic plants may be missed and foliar symptoms may be misdiagnosed as infected. Furthermore, *P. savastanoi* pv. *phaseolicola* can be present as an asymptomatic epiphyte pending environmental conditions favourable for symptom development. Therefore, fields of mungbean that look visually disease-free may have high pathogen loads but appear asymptomatic. Further to this, symptoms late in the mungbean lifecycle may be masked by features of plant senescence as the plant deteriorates with age. Molecular diagnostics offer great promise for the detection and quantification of pathogens in seed samples prior to distribution.

Conventional PCR has been available since the early 1980s, with the first PCR targeting *P. savastanoi.* pv. *phaseolicola* reported in 1993 (Prosen 1993). Today there are a range of PCR assays reported to target unique segments of the *P. savastanoi* pv. *phaseolicola* genome (Noble et al. 2018). Schaad et al. 1995 reported an assay sensitivity of 3–15 cells per reaction using a nested PCR. The sensitivity of the hydrolysis probe qPCR used here was stated to be 4.5×10^3 CFU ml^{-1}, or approximately 4 cells per reaction if using 1 µl of bacterial suspension as template (Xu and Tambong 2011). There is a one-to-one relationship between amplification and bacterial number as there is only a single copy for the cytochrome o ubiquinol oxidase subunit II gene for *P. savastanoi* pv. *phaseolicola*, meaning that a single amplicon equates to a single bacterium. The same assay could detect *P. savastanoi* pv. *phaseolicola* from the 100 fg dilution of extracted DNA, presumably also from 1 µl template per reaction, although this was not stated. With a 6 Mbp genome with 58% G + C (Noble et al. 2020), 100 fg DNA represents 15.5 copies of the genome, which suggests a lower sensitivity for qPCR on extracted DNA versus direct bacterial suspensions used as template. However, as Xu and Tambong (2011) failed to state the template volumes or the concentrations, it is not possible to determine whether extracting DNA from cultures represents any improvement on amplifying from suspensions directly. In the current study, both the hydrolysis probe and SYBR assay were 1,000 times more sensitive than conventional PCR.

It should be noted that the current study did not determine the absolute level of detection (LOD). This could be achieved by conducting multiple replicates at a range spanning the limit of detection and employing probit analyses. However, our objective was not to determine exactly how many target bacteria could be detected with the assays tested, but rather whether any of those assays were more sensitive than others. Additionally, as we were not conducting an epidemiological analysis of Australian mungbean crops, we report the analytical sensitivity of the tests rather than their diagnostic sensitivities. Having demonstrated that the halo blight pathogen can be detected in DNA extracted from mungbean seeds, we have provided a pathway by which mungbean yields can be improved through safer selection of seed.

There was a substantial difference in the number of infected samples between those collected in 2017 and 2018. In 2017 up to 86% of samples were found to have some level of *P. savastanoi* pv. *phaseolicola* present compared to 2018 with a reduction to 22%. The considerable decrease in infected samples in 2018 may be explained by the smaller sample size of 14 compared to 21 in 2017, and the inclusion of 4 *Vigna mungo* (black gram) samples known to be more resistant halo blight. The three positive control samples of seed collected from deliberately infected fields tested positive by both qPCR protocols, but only two were positive via conventional PCR. It is noted that the sample that was negative via conventional PCR had Cq values higher than 30, suggesting that the target template was too low for positive identification with the less sensitive PCR technique.

This study has shown the efficacy of using different PCR assays to identify Australian isolates of *P. savastanoi* pv. *phaseolicola* infecting mungbean seed, the causal agent of halo blight disease. Although conventional PCR does amplify highly infected samples, our results show that qPCR is significantly more sensitive. The lack of sensitivity of conventional PCR is due to it requiring enough amplicon to be visible on an agarose gel which is also dependent on the amount of DNA visualisation reagent added. Using the methods presented here, halo blight can be detected accurately at very low levels in mungbean seed (Table 3). Considering the severity and frequency of halo blight outbreaks and the length of time the pathogen can persist in seed, there was a concern all samples of mungbean seed would have some degree of infection. In total 39% of the 35 field samples tested were deemed negative for the presence of the halo blight pathogen using the most sensitive

qPCR hydrolysis probe method. This high level of infection is likely influenced by the seed consignments received, as collaborators were made aware that a seed test for the disease was being developed. Furthermore, location data was not supplied for 57% of samples as collaborators were unwilling to link crop disease-status to growers. It should be noted that over 50% of seed samples tested negative for the disease. It is not yet known whether higher bacterial loads detected in seeds lead to greater disease in subsequent crops, but it is clearly optimal to plant from seed where the pathogen has not been detected.

The implementation of molecular testing for the presence of halo blight in mungbean seed has enormous potential economic benefits for farmers and seed producers. In the absence of cost-effective management interventions, the key control method for halo blight is keeping it out of the field in the first place. Collecting metadata associated with samples tested would provide a reservoir of data to further assist in answering industry-relevant research questions. For example, determining whether specific cultivars are more prone to higher levels of infection, whether there are regional differences in infection loads, and whether infection levels can be correlated with prevailing weather conditions. The metadata required to address these research questions would include documentation of each sample with year and location grown (GPS coordinates), new or retained seed, crop rotation for the previous four years, size of crop and the number of samples taken per kg of the seed lot. Additionally, consistent tracking of material and quantification data could provide a threshold for the number colony forming units based on environmental conditions which could be used to develop an application to assess the risk of using a particular seed lot.

Conclusions

In conclusion, *Pseudomonas savastanoi* pv. *phaseolicola* is highly transmissible seed-borne disease that is spreading rapidly throughout the Australian and global mungbean growing regions, causing immense economic loss. The current assessment method in Australia, relying on visual inspection for symptomatic plants is not sufficient to identify infected crops in order that they can be excluded as sources of planting seed. The transition towards molecular diagnostics is paramount to combating the disease and could be done in a short timeframe. Here we have shown that molecular diagnostics, especially qPCR, are highly effective at detecting the pathogen of interest and can quantify the bacterial loads in seed lots. In the longer term, with careful monitoring and recording of environmental conditions and time of planting, the bacterial loads could be utilised to develop a risk assessment of a seed lot's potential for disease outbreak and severity. Once implemented, molecular diagnosis could be further leveraged to include other diseases such as tan spot in a dual diagnostic qPCR to transform the way clean seed is delivered to growers, leading to cleaner planting seed, higher yields and economic gains for growers and the industry.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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