

Detecting and quantifying pathogens causing potato tuber decay using real-time quantitative PCR, to predict storage potential

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Abstract

We present a methodology to detect and quantify *Phytophthora infestans* (late blight), *Phytophthora erythroseptica* (pink rot), *Pythium ultimum* (leak), *Fusarium sambucinum* (dry rot), and *Erwinia carotovora* subsp. *carotovora* and subsp. *atroseptica* (soft rot) in whole potato tubers, using real-time quantitative-PCR (Q-PCR). Amplification efficiencies ranged between 95 and 100% over a five-log dilution series. The detection level of the primers reached 0.5 pg of target DNA. Pathogens were detected four days after inoculation in 100 pg of total tuber DNA in the absence of symptoms. We also compared pre-harvest tuber sampling strategies from potato fields. A collection of 1,650 tubers was carried out in six fields prior to harvest. Sampling sites were pre-identified from aerial photographs and geo-referenced. Q-PCR amplifications were conducted on the collected tubers to detect and quantify the five pathogens. This study suggests that the most informative sampling scheme to identify the risk of tuber decay prior to harvest should be stratified along the distance from the center of the pivot. The same scheme could be applied in various locations regardless of the soil type and planted cultivar.

Introduction

Post-harvest losses in potato storages reach ~10% every year, and in Wisconsin, this amounts to US\$16 million. Five decay pathogens cause the majority of disease-related losses: *Phytophthora infestans* (late blight), *Phytophthora erythroseptica* (pink rot), *Pythium ultimum* (leak), *Fusarium sambucinum* (dry rot) and *Erwinia carotovora* subsp. *carotovora* (syn. *Pectobacterium carotovorum*) and subsp. *atroseptica* (syn. *Pectobacterium atrosepticum*) (Fig. 1).

Potato tubers intended for processing (French fry, chip or dehydration) are stored in piles of 4-7 m in height at a relative humidity exceeding 95%, and temperatures of 7-13°C. Such temperatures are used to prevent the transformation of starch into reducing sugars at low temperature, and then caramelize during processing. High relative humidity is needed to reduce weight loss by evapo-transpiration. Such conditions are highly favorable for the infection of tubers. Additionally, decaying tubers release large amounts of water, which disperses the pathogenic propagules to neighboring tubers.

The objectives of this study were to provide stakeholders with a methodology to direct potato crops to storage or immediate processing. This is achieved by i) detecting and quantifying decaying pathogens prior to harvest and ii) establishing a field level sampling methodology representative of the pathogen distribution.

Materials and Methods

Primer design. Real-time quantitative PCR primers were designed from the sequences of single copy protein coding genes. Primer efficiencies were established on five-point ten fold serial dilutions of pure culture DNA (Fig. 2). The primers were tested on tubers inoculated with each of the five pathogens four days after inoculation, and on field collected samples showing disease symptoms.

Field sampling. We selected four fields in Wisconsin (2 muck and 2 sand fields), one field in Minnesota (MN) and one in North Dakota (ND). No late blight has been observed in Wisconsin since 2002. All fields were center pivot irrigated and 100-130 acres. Aerial photographs of each individual field were anchored in the StreetMap software in ArcGIS and used to generate geo-referenced coordinates for each sampling location within a field (Fig. 3). Each field was divided into three strata based on the distance from the center of the pivot (Fig. 3). The first stratum extended to the second wheel line (~100 m), the second stratum reached the fourth wheel line (~100 m) and the third extended to the edge of the field (~150-200 m). Because of the large size of the outer two strata, they were each divided into four sub-strata (Fig. 3). Within each substratum, two sampling schemes were used, one random and one systematic. The systematic sampling was in a W-shape with 50 m between sampling locations. The coordinates of each location were used in a sub-foot accuracy differential GPS unit. Three healthy looking tubers (170-250 g) were collected at each location. A total of 1,650 tubers were collected and subjected to Q-PCR.

Q-PCR amplification. 100 pg DNA were amplified in triplicates of 20 µl Q-PCR reactions. Reactions included 200 nM of each primer and RealMasterMix with SYBR Green (Eppendorf, Hamburg, Germany). Amplifications, PCR efficiency calculations and quantifications were conducted in IQ iCycler real-time PCR detection system (Bio-Rad, Hercules, CA). Ct-values (cycle threshold) were used in the statistical analyses.

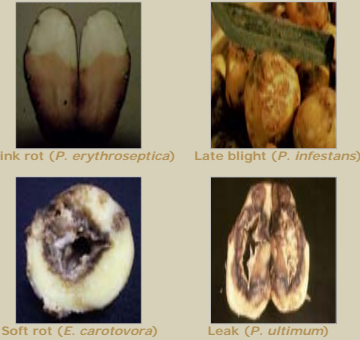


Figure 1. Typical symptoms of late blight, pink rot, soft rot and leak on tubers collected in the field.

Table 1. P-values ($\alpha = 0.05$) for analysis of variance of the impact of hierarchicalization on representative sampling of potato tubers from a field, to predict the storability of the crop. Notice the significant impact of subdividing field into substrata

| Disease | Random | Systematic | |
|----------|--------------------------------------|----------------|--------------------|
| Pink rot | Type ^a | 0.9031 | 0.5846 |
| | Site>Type ^b | 0.3907 | 0.1301 |
| | Substrat>Type*Site ^c | 0.0006* | 0.0008* |
| | Samp>Type*Site*Substrat ^d | 0.6314 | 0.0172* |
| Leak | Type | 0.9589 | 0.4028 |
| | Site>Type | 0.2961 | 0.6939 |
| | Substrat>Type*Site | 0.0014* | 0.0013* |
| | Samp>Type*Site*Substrat | 0.2922 | 0.9382 |
| Dry rot | Type | 0.7173 | 0.5248 |
| | Site>Type | 0.3221 | 0.3669 |
| | Substrat>Type*Site | 0.0132* | 0.0001* |
| | Samp>Type*Site*Substrat | 0.3358 | 0.945 |
| Soft rot | Type | 0.6121 | 0.767 |
| | Site>Type | 0.0076* | 0.013* |
| | Substrat>Type*Site | 0.0003* | <0.0001* |
| | Samp>Type*Site*Substrat | 0.0006* | 0.4525 |

Table 2. Comparison of P-values ($\alpha = 0.05$) between random and systematic sampling by disease and in each of the fields. No significant differences were found between the two sampling schemes, if performed within the described substrata

| Site | Pink rot | Leak | Dry rot | Soft rot |
|-------|----------|-------|---------|----------|
| Gnz | 0.743 | 0.583 | 0.997 | 0.141 |
| Kin | 0.528 | 0.157 | 0.169 | 0.483 |
| MN | 0.967 | 0.520 | 0.468 | 0.205 |
| NVrom | 0.368 | 0.760 | 0.413 | 0.523 |
| OW | 0.228 | 0.519 | 0.296 | 0.804 |
| WMT | 0.068 | 0.987 | 0.047 | 0.835 |

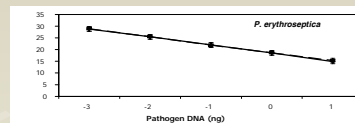


Figure 2. Example of Q-PCR primer efficiency for a five point ten-fold serial dilution of pure culture DNA of *Phytophthora erythroseptica*. The solid line shows the pure culture DNA alone, and the dashed line shows the same efficiency curve with the addition of 25ng of potato DNA. A 100% efficient PCR reaction would yield a slope of 3.323. No significant difference was found from the addition of excessive amounts of potato DNA.

Results

Primers specificity, sensitivity and efficiency. All primer pairs amplified target organisms and failed to amplify related organisms. The only exceptions are the primers designed to amplify *P. infestans* and *E. carotovora*, which amplified closely related organisms not associated with disease on potato. Primer efficiencies were greater than 95% regardless of the presence of potato DNA, and primer sensitivity reached 0.5 pg DNA. Such amounts of DNA are equivalent to fewer than 10 nuclei of each of the five organisms tested.

Field sampling. No late blight was detected in any sample. All four other pathogens were detected in tubers. Infection coefficient values ($C_{t_{host}}/C_{t_{pathogen}}$) were computed for each tuber by averaging the mean Ct value of the two DNA samples extracted from each tuber (total six Q-PCR reactions per tuber).

No statistical differences were observed among types of fields (muck, sand, ND, MN) fields, fields, or sampling sites for pink rot, dry rot and leak (Table 1). Fields and sampling sites were significant for soft rot, which is expected based on the water source and refuse from previous crops (Table 1). Most importantly, the division of fields into nine substrata had a significant impact. A totally random scheme in a potato field will not provide adequate prediction power unless taking into consideration the variability in irrigation by center pivot systems. No significant differences were observed between random and systematic sampling schemes within each of the sub-strata (Table 2). Further geo-statistical analyses are being conducted using semi-variogram analyses to understand the correlations between sampling locations at set distances. So far no such correlations could be observed at 50 m transects.

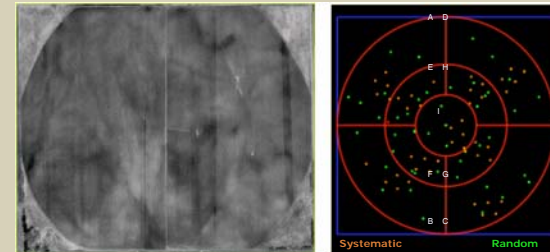


Figure 3. Aerial photograph of a center pivot irrigated potato field in Wisconsin and the sampling scheme applied to collect potato tuber samples. The field (53 ha) was divided into three concentric rings (strata), with the outer two split into 4 substrata each. The 9 substrata are indicated with the white letters. A W-shape systematic sampling was applied in each substratum, in addition to a random sampling.

Conclusions

This study produced a methodology to detect fewer than ten nuclei of *P. infestans*, *P. erythroseptica*, *P. ultimum*, *F. sambucinum* and *E. carotovora*. This methodology showed high efficiency (Fig. 2) regardless of the presence of potato DNA. Whole tubers are used, which preempts potential problems from a tuber sampling.

The field sampling study we conducted showed that it is essential to sample all field strata using distance from the field center as reference. Furthermore, dividing a field into substrata is critical to a representative sampling. No significant differences were found among fields or types of fields for pink rot, leak or dry rot. Alternatively, significant differences were found among fields for soft rot. Such differences are expected because of the source of water used for irrigation or the availability of infested refuse from previous crops. Random or systematic samplings are equally effective, as long as they are conducted within the substrata described in this study.

We were able to detect and quantify pathogens from tubers prior to harvest, which will allow the direction of tubers from whole fields, or portions of fields to the appropriate destination. Such destinations include immediate processing of crops, long- or short-term storage, and the separation of crops based on pathogenic load. This study will also allow storage managers to tailor the management of stored crops based on the infestation levels of specific pathogens.

The authors apologize for not being able to attend the annual meeting. Please refer any questions, comments or suggestions to Z. Atallah: 1-608-262-6291 or atiala@plantpath.wisc.edu

