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Methodology

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Posted Date: July 22nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-723240/v1>

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RESEARCH

Optical Coherence Tomography Applied to Non-Destructive Seed Coat Thickness Measurement

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Abstract

Background: Seed coat thickness is a parameter of interest in lentil breeding and processing. Methods described in the literature are destructive and time consuming, limiting the usefulness of this seed characteristic in breeding or grading. In this study, a low-cost optical coherence tomography (OCT) system (OQ Labscope 2.0 from Lumedica, Inc., Durham, NC USA) was used to non-destructively measure lentil seed coat thicknesses. These measurements were compared to destructive measurements obtained via optical microscopy of cross-sectioned seeds.

Results: Measurements from the two methods were comparable with consistent trends in thickness and population separability among the five lentil accessions used. The average microscope-based measurements for each accession were higher than those from the OCT, with a linear relationship between the two sets of measurements. The discrepancy in absolute thicknesses is attributed to differences in instrument calibration and in the definition of seed coat boundaries in the two methods.

Based on data collected from a larger population, OCT measurements took a mean time of 30 seconds/seed, and a median time of 16 seconds/seed, with no sample preparation required.

Conclusions: Optical coherence tomography is a viable method for acquiring comparative data on seed coat thickness of lentil seeds, and presumably other species with similar seed characteristics. The method is non-destructive, and fast enough to be practical for studying large breeding populations. Opportunities for greater efficiencies as part of an automated imaging system are being explored.

Keywords: OCT; seed coat; lentil; thickness measurement

Introduction

The seed coat thickness of pulse crops impacts a variety of seed characteristics, such as moisture control and milling qualities. Generally, seeds with thicker coats have an improved survival rate in the wild due to the fact that they are less water permeable [1][2]. However, this trait is undesirable when it comes to seed processing. Reduced water imbibition (absorption) results in extended germination, cooking and de-hulling times. Therefore, information on seed coat thickness can provide plant breeders, farmers and processors with insight on the quality of a particular seedlot or variety.

Studies of seed coats have used several methods to measure thickness. Imaging methods require cross-sectioning of samples, sample mounting and measurement using either optical [1][3] or electron microscope [2][3][4] imagery, with electron microscope techniques typically requiring a conductive coating on the sample. Alternatively, seed coats may be removed from the seed using a number of approaches and measured with a micrometer [5]. These methods are able to acquire high-quality measurements, but accuracy is dependent on the skill of the operator and quality of sometimes extensive sample preparation. The destructive nature of these methods also limits their application to small, expendable seed samples, and the amount of time required for sample preparation makes them impractical for high-throughput phenotyping of large breeding populations.

Optical coherence tomography (OCT) is a non-invasive imaging technology that uses low-coherence light to obtain cross-sectional images within biological samples. It is widely used in the field of ophthalmology to measure retinal thickness, for instance. The images generated are similar to ultrasound images in appearance. Recently, OCT imaging has been applied in the plant sciences with examples being the monitoring of seed germination [6], and examination of leaf cross-sections [7][8].

The objective of this study was to determine the feasibility of using a low-cost OCT system to non-destructively and efficiently measure lentil (*Lens* genus) seed coat thickness for purposes of genetic studies and crop breeding. Samples of five accessions from the *Lens* genus were used. Seed coat thickness measurements taken using the OCT system (OQ Labscope 2.0, Lumedica Inc., Durham, NC USA) were compared to those acquired via destructive seed cross-sectioning and imaging using calibrated optical microscope images. The absolute accuracy of either measurement method is dependant on a number of factors and calibrations outside the scope of this project; for this reason the intention was not to directly compare the measurements in an absolute sense, but rather to compare the trends in seed coat thickness with respect to accession for the two methods. This would be sufficient for monitoring relative seed coat thickness in a high-throughput setting.

Results

The OQ Labscope 2.0 OCT and microscope data display similar trends for seed coat thickness (Fig. 1), with accessions being ranked in the same order of mean seed coat thickness for both methods of measurement. A systematic difference was observed between the methods, with the OCT measurements being lower than those acquired with the microscope. The relationships between the means is highly linear ($R^2 = 0.98$). The coefficients of variation (CV, standard deviation divided by the mean) were higher in the microscope data for all varieties (Table 1).

Thickness data for most samples were found to be normally distributed. Exceptions in the microscope data were Lupa and CDC Greenstar which failed all tests ($\alpha = 0.05$). With the OCT data, only data from CDC Greenstar were determined to be non-normal, failing all but the Lilliefors test. Based on these results, the non-parametric Wilcoxon rank-sum test was used to determine whether the OCT and microscope methods distinguished among the same groups of lentil varieties (Table 2). Based on the results of this test all pairs of lentil varieties, except for Lupa and CDC Greenstar, were distinguishable regardless of the method of measurement.

Discussion

Comparing the optical microscope-based measurements to those acquired using the OCT system, it was clear that the mean OCT thickness measurements were consistently smaller than the microscope measurements. The relationship between the means was linear, however, and had a high coefficient of determination. There are several possible explanations for this systematic bias. First, if the microscope calibration was incorrect, this would introduce a systematic error consistent with the bias observed. Second, the distance calculation of an OCT system is dependent on the refractive index of the material being measured. This is not well-quantified for the seed coats. In the absence of a known value, the refractive index of a glass slide ($n = 1.5$) was used, and assumed to be higher than that of the seed coat. As the speed of light through a medium is inversely related to its refractive index, this would result in an underestimation of seed coat thickness proportional to the ratio of glass and seed coat refractive indices. Third, given the fundamental differences in the image formation methods, there is no guarantee that the same boundaries were being used in both methods to define the inner and outer seed coat edges. Recognizing these potential sources of difference in thickness between the two methods, the more significant question was whether the two methods produced similar results when comparing between lentil accessions. Examining the overall trends in seed coat thickness for the two measurement techniques, both methods produced identical results in terms of ordering, and similar variability trends as indicated by CVs. Furthermore, the patterns of separability determined by the pairwise Wilcoxon rank-sum test based on mean seed coat thickness were the same for both measurement methods.

The primary benefits of using OCT over traditional methods are that it is non-destructive and much faster. In the context of a breeding program, where there are hundreds or thousands of candidate accessions, with a precious few seeds each, the benefits of the OCT method make seed coat thickness a viable phenotype to incorporate into a breeding program. An preliminary example of applying the OQ Labscope OCT system to measuring seed coat thickness of a recombinant inbred line (RIL) population (117 lines) is provided in Fig. 2. Parental lines are indicated with a dashed line. In this population, most RIL seed coat thickness values lie between those of the parental lines. However, a number are either thinner or thicker, indicating transgressive segregation may be occurring. These data include measurements from nearly 1200 seeds. With a simple manual alignment tool to aid with seed positioning, the average elapsed manual measurement time per seed was 30.5 seconds and the median time was 16 seconds for these OCT measurements. This includes time for changing samples and seeds, and instructing the software to save the average image for each seed. This is a small amount of time compared to that required for destructive sample preparation and measurement using either cross-section + microscopy or de-hulling + micrometer methods. A further benefit is that no consumables are required; costs are reduced to a one-time investment in the OCT equipment itself and operator time. Because sample preparation is limited to inserting a seed into a slot in the alignment tool, it also requires less skill than that required to do the destructive measurements.

Automated extraction of the seed coat thickness from OCT images was robust, with the standard deviation of the between-curve distances being an effective metric

for detecting poor fits. In subsequent data analysis the amount of curvature in the image was found to have an impact on the mean thickness. The amount of curvature is a function of the seed (roundness and size) and the width of the OCT scan, with a narrower scan resulting in less image curvature, all else being equal. As the absolute value of the line slope increases, the image geometry results in the upper and lower seed coat boundaries appearing closer together. Horizontally scaling images acquired with a wider scan to a narrow equivalent reduced the effective curvature, mitigating this effect. Subsequent to this proof-of-concept work, this function has been added to the analysis software, and only the center 30% of the image (35% left- and right-margins) has been used to calculate the thickness, excluding the portions of the seed coat most susceptible to curvature effects. Lentil seed coats generally exhibit a smoothly curved surface; the performance of this method would need further evaluation if applied to species such as pea with more wrinkly seed coats.

Conclusions

Seed coat thickness measurements from five lentil accessions were successfully acquired using an optical coherence tomography system and compared to measurements using cross-sectioned samples and 40X microscope imagery. The microscope mean thickness measurements were slightly larger than the OCT measurements, but with a strong ($R^2 = 0.98$) linear relationship between them. This suggests a calibration error, possibly related to using the default index of refraction for the OCT measurements. More important for the intended purpose, trends in the two measurement methods were very similar; accession thicknesses were ranked in the same order, demonstrated similar trends in variability, and had the same pairwise separability for both measurement methods. It was concluded that this low-cost OCT system had sufficient performance to find meaningful differences in lentil seed coat thickness. It is assumed that similar results can be expected for other species with similar seed coat characteristics (beans, soybean, pea, maize).

Image analysis to automatically extract seed coat thickness from OCT images was implemented using an iterative curve fitting and clustering method. The combination of curve fitting and ability to average many measurements per seed resulted in an effective measurement resolution exceeding the resolution of the raw instrument measurements. While this method worked well, further work could be done on mitigating the impact of seed coat curvature on the mean.

The speed at which the non-destructive OCT measurements can be taken makes seed coat thickness a viable phenotype to use in breeding or plant physiology studies having a large number of samples, a small number of individual seeds per sample, or both. While manual measurement with this technique is quite fast, incorporating OCT measurement into an automated individual seed-imaging system[9] would further accelerate the process.

Methods

Five accessions representing the *Lens* genus were studied: CDC Greenstar (*L. culinaris*), Eston (*L. culinaris*), Lupa (*L. culinaris*), BGE 016688 (*L. orientalis*), and IG 72623 (*L. odemensis*). Seeds were bulk samples from single plots of field-grown plants. Separate subsamples of each accession were used for the destructive,

microscope-based seed coat thickness measurements and the non-destructive OCT measurements.

Optical Microscope Measurements

Samples for the optical measurements were prepared by embedding the lentil seeds in cold-cure epoxy and cross-sectioning them by sanding with progressively finer sandpaper to 2000 grit. Each epoxy block contained nine seeds of one lentil accession (Fig. 3). The epoxy was allowed to cure at room temperature for four days to reach sufficient hardness for sanding. Sanding took 5 to 10 minutes per block of 9 seeds.

Eighteen seeds per lentil variety were measured for a total of 90 seeds over the measurement period (June 17, 2020 to July 20, 2020). A compound microscope was used to acquire cross-sectional images of both seed coat halves of each seed at 40X magnification. Spatial calibration was done using a calibration slide. Seed coat thickness measurements were collected manually using AmScope image processing software (United Scope LLC, Irvine, CA, USA). Five seed coat thickness measurements were taken in each image and reported as an average for the image. An example microscope image is shown in Fig. 4 with annotated measurements.

The boundaries for the seed coat thickness measurements were assumed to span from the outside surface of the seed coat to the inner wall before the cotyledon. Seed coats that were heavily damaged during preparation were deemed un-measurable and removed from the sample populations. Lentil cross-sections with minor blemishes were selectively measured in areas without obvious flaws. These assumptions were made to minimize any measurement error produced by seed coat defects and unclear layering in the seed or damage that may have occurred during the cross-sectioning process.

Optical Coherence Tomography Measurements

Optical coherence tomography measurements were taken with an OQ LabScope 2.0 system (Lumedica Systems, Durham, NC, USA). Vertical resolution was set at 5 μm per pixel. Thirty seeds each of Lupa, Eston, IG 72623, and BGE 016688 lentil varieties, and 36 seeds of CDC Greenstar, were measured. A series of 30 scan lines (B-scans) were acquired for each seed and the average image recorded. Fig. 5A is an example averaged image.

Relative to other methods, OCT-based thickness measurements were hypothesized to be fast. To evaluate this assumption, the time intervals between seed measurements were examined for a single operator measuring nearly 1200 seeds from 117 accessions in a separate study. Intervals larger than 5 minutes were attributed to breaks taken by the operator and excluded.

Seed coat thickness measurements were extracted from the OCT images using a regression clustering algorithm. In the initial pass, a 7th-order polynomial was fit to the return pixels in the OCT image to establish a baseline curve that generally followed the seed coat. This initial curve tended to locate closest to the interface between the cotyledon and the seed coat, and was used as the starting point for the "lower" seed coat-cotyledon boundary cluster. The starting point for the "upper" cluster (i.e. the air-seed coat interface) was defined as the initial fit with an offset of 12 pixels. Once the starting polynomial coefficients were set for the upper and lower

boundary clusters points were given membership in the closest cluster, provided that distance was below a threshold distance. The boundary coefficients were re-fit on their membership, weighted by pixel value, and the process repeated. With each iteration, the maximum distance threshold for a pixel to belong to a cluster was reduced. This gradually drove the upper and lower polynomials toward the areas of highest point density and intensity. This process continued until the root sum of squares change in the R^2 values of the fits was below an exit threshold (0.00001), or until 10 iterations had completed. Fig. 5B shows a normal fit case for the upper and lower seed-coat boundaries.

Based on the upper and lower boundary polynomials, the perpendicular distance between the curves was calculated at every second column across the image, scaled to micrometers, and descriptive statistics calculated (mean, median, standard deviation (SD), quartiles, maximum and minimum distances). A 51-pixel margin (10% of image width) on the image sides was excluded from these calculations to avoid edge effects that distorted the fits.

A few cases were observed to have poor curve fits (e.g. Fig. 6) The standard deviation was found to be effective at identifying these cases and used as a filter when combining single-seed results, with seeds having between-curve distances with a $SD \geq 5 \mu m$ being excluded from the sample statistics.

Statistical Analysis

Data from the OCT and microscope measurements were compiled in R [10] and the mean seed coat thicknesses and standard deviations for each lentil accession were calculated. Normality of the data for each accession was tested using several tests (Lilliefors, Anderson-Darling, and Shapiro-Wilks using `lille.test`, `ad.test`, and `shapiro.test`, respectively, from the R base package [10], and Jarque-Bera using `jarque.bera.test` from the `tseries` package [11]) on each data set. Seed coat thickness measurements for CDC Greenstar (microscope and OCT) and Lupa (microscope) failed the majority of the tests for normality. Based on this result, a non-parametric, pairwise test (Wilcoxon rank-sum test) was applied to both sets of data to compare the separability of the seed coat thickness by accession. P-values were adjusted using the false discovery rate (fdr) method [10].

Declarations

Acknowledgements

The authors would like to thank Dr. William Brown and Lumedica, Inc. (Durham, NC, USA) for their assistance and for providing a OQ Labscope 2.0 demonstration unit for this proof of concept study.

Funding

This work was supported by the 'Enhancing the Value Of Lentil Variation for Ecosystem Survival (EVOLVES)' project funded by Genome Canada and managed by Genome Prairie. We are grateful for the matching financial support from the Saskatchewan Pulse Growers, Western Grains Research Foundation, the Government of Saskatchewan, and the University of Saskatchewan. BDN was supported by a matching MITACS Research Training Award. Funding agencies did not have a role in conducting this study.

Abbreviations

CV coefficient of variation OCT optical coherence tomography RIL recombinant inbred line SD standard deviation

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Authors' contributions

BDN prepared samples, collected microscope and OCT data, conducted statistical analysis, and prepared the initial manuscript draft. KEB supplied lentil samples, contributed to experimental design and manuscript review. SDN supervised BDN, developed sample preparation and OCT image analysis methods, and prepared the final manuscript.

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Figures

Tables

Table 1 Mean, standard deviation (SD) and coefficient of variation (CV) of each line for both microscope and OCT measurements.

Line	Microscope			OCT		
	Mean [μm]	SD [μm]	CV	Mean [μm]	SD [μm]	CV
Eston	42.74	2.96	0.07	39.25	2.02	0.05
Lupa	46.31	6.37	0.14	40.03	2.35	0.06
CDC Greenstar	47.13	5.67	0.12	40.81	1.65	0.04
BGE 016688	56.76	7.76	0.14	49.50	2.66	0.05
IG 72623	60.36	5.09	0.08	51.47	2.75	0.05

Figure 1 Boxplots comparing seed coat thickness measurement distributions for five varieties using OCT and optical microscopy techniques.

Table 2 Adjusted pairwise P-values for Wilcoxon rank-sum test results for OCT and microscope data sets.

	Lupa	CDC Greenstar	BGE 016688	IG 72623
	OCT			
Eston	0.043	0.001	0.000	0.000
Lupa		0.367	0.000	0.000
CDC Greenstar			0.000	0.000
BGE 016688				0.010
	Microscope			
Eston	0.000	0.000	0.000	0.000
Lupa		0.199	0.000	0.000
CDC Greenstar			0.000	0.000
BGE 016688				0.005

Figure 4 Examples microscope image of lentil cross-section (BGE 016688) at 40X magnification, annotated with seed coat thickness measurements taken using the AMScope software.

Figure 5 (A) 30-scan line average image of a lentil OCT cross section image. (B) Image of (A) with polynomial fit lines on upper and lower seed coat boundaries.

Figure 6 Examples of poor line fits and associated standard deviations (SD) of distance between the curves.