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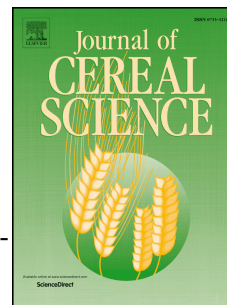


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A wheat grain quantitative evaluation of vitreousness by light transmission analysis

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Abstract

Light transmission through wheat (*T. aestivum* L.) grain longitudinal cross sections of different thickness was used to study the endosperm microstructure and was shown to strictly follow a Beer-Lambert law allowing a non ambiguous quantification of the endosperm vitreousness. Therefore similar samples obtained from near-isogenic lines differing by hardness and grown in two distinct environments affecting their vitreousness were analyzed and confirmed the relationship between light transmission and the endosperm microstructure. In each sample, moreover analysis of light transmission within the different grain parts highlighted the greater compactness of the central endosperm cheeks in comparison with the distal and the proximal regions. These results help a better understanding of the endosperm microstructure.

Keywords: endosperm, hardness, microstructure, puroindoline, *Triticum*

1 **1. Introduction**

2 The protein-starch adhesion and the microstructure of the common wheat
3 (*Triticum aestivum* L.) starchy endosperm were found to differ depending on
4 genetic and environmental factors [1]. These factors are both found to af-
5 fect the grain mechanical resistance i.e. endosperm hardness [2] and thus
6 play a key role in grain milling behavior. Recently, an effort was made to
7 experimentally clarify the role of these factors using genetically well-defined
8 grains grown under different cultural conditions [3, 4]. The main genetic
9 locus controlling the starchy endosperm texture, called *Ha*, was located on
10 the short arm of chromosome 5D [5] where two important genes encoding
11 specific proteins called puroindoline A (PINA) and puroindoline B (PINB),
12 were found [6, 7]. Presence of the wild-type alleles of both puroindoline genes
13 (*Pina-D1a/Pinb-D1a*) leads to both functional PINA and PINB and results
14 in a soft mechanical behavior, whereas mutation or deletion of one or both
15 of the puroindoline genes was found to lead to a hard texture [7]. Moreover,
16 translocation of the wild type puroindoline genes in a durum background [8],
17 which lacks D genome, leads to mechanical resistance and similar character-
18 istics to soft common wheat *i.e.* a higher production of the finest particles
19 with low starch damage after milling [9, 10, 11]. Conversely, removal of the
20 chromosome 5D distal part (which carries the puroindolines genes) in a soft
21 hexaploid wheat led to hard vitreous grains [12].

22 Differences at the interface between protein and starch granules are sug-
23 gested to explain the mechanical differences between soft and hard cultivars
24 [13] and puroindolines are believed to affect the starch-protein adhesion. In-
25 deed recent studies showed mechanical changes at the interface using near-

26 isogenic lines differing only by the wild-type or mutated allele of the gene
27 encoding PIN B [14].

28 Besides genetic factors, environmental conditions were found to affect the
29 starchy endosperm appearance the so-called vitreousness [15] which is an op-
30 tical property attributed to differences in endosperm porosity [16]. Grains are
31 classified as mealy when the starchy endosperm is porous and appears white
32 and floury, or as vitreous when it is translucent and glassy. Vitreousness is
33 generally estimated through examination of a number of grain cross-sections
34 made with a Pohl grain cutter. However, this method is operator-dependent
35 and time consuming. Therefore, other methods were recently developed for
36 rapid classification of wheat grains depending on their vitreousness level.
37 Transmitted light images were found allowing correct classification accord-
38 ing to vitreousness even if percentages of accuracy relative to the visual
39 inspection differ between authors [17, 18]. Soft X-ray, dual energy X-ray or
40 light reflectance coupled with image analysis were also found to be poten-
41 tially efficient in the differentiation between vitreous and non-vitreous grains
42 [17, 19, 20]. Near infrared hyper-spectral imaging was also used to satisfac-
43 tory classified vitreous from non-vitreous grains [21, 22]. These classification
44 methods are rapid and non-destructive. However, they are based on refer-
45 ence wheat samples which need to be well characterized and are only valid
46 for grains which share the same characteristics as the references (grain geo-
47 metric characteristics, tissue thickness, colors, hardness, etc.). These recent
48 methods are hardly used to quantify the level of vitreousness as a function
49 of the microstructure.

50 In the present work, we chose grains from two near-isogenic lines with a

51 known profile of puroindoline alleles, encoding either native or mutated PINB
52 in order to fix hardness due to the genetic background, respectively soft or
53 hard, and selected two different environments from a previous study [15] al-
54 lowing obtaining contrasted levels of vitreousness for the starchy endosperm.
55 Then, locally distinct regions within the endosperm were explored thanks
56 to an original experimental assembly allowing measurement of transmitted
57 light through grain cuts. It was thus possible to objectively compare the
58 endosperm microstructure between the four wheat grain samples depending
59 on genetic or environmental conditions, as well as within each type of sample
60 depending on intra-grain location.

61 2. Material and Methods

62 2.1. Wheat grains

63 Near-isogenic lines (NIL) of *Triticum aestivum* L. were produced by Insti-
64 tut National de la Recherche Agronomique (INRA) and displayed either the
65 wild-type *Pinb-D1a* or the mutated *Pinb-D1b* allele (leading to single amino
66 acid change in PINB, *Gly46Ser*), which respectively conferred to grains the
67 soft or the hard phenotype. They were derived from a cross after selection
68 of the two allelic forms at the F6:F7 selfing generation (F7 siblings issued
69 from the same F6 parent plant, construction and genetic similarity testing
70 detailed in [3]). Grains from the two lines were cultivated in different loca-
71 tions in France, collected and cleaned to remove broken kernels or impurities
72 and stored at 4°C before analysis. Percentage of vitreousness in each col-
73 lected location was determined on grain cross sections (n=500) obtained with
74 a Pohl grain cutter (Versuchs and Lehranstalt, Brauerei, Berlin, Germany)

75 depending on the proportion of glassy (translucent) area observed on the
76 surface after visual analysis as described in [23]. Briefly, grains were classi-
77 fied into five groups according to the percentage of vitreous surface in the
78 analyzed grains (i.e., grains that displayed less than 25% vitreousness were
79 placed in the first group; those with around 25% vitreousness constituted the
80 second group; and those with around 50, 75 or 100% vitreousness were placed
81 in the third, fourth, and fifth groups, respectively). The number of grains
82 in each class (N_1 represents number of grains in class 1, for example) was
83 multiplied by the corresponding factor of vitreousness for that class, and the
84 percentage of vitreousness in the grain sample was calculated according to
85 the following equation: $\frac{N_1 \times 0.00 + N_2 \times 0.25 + N_3 \times 0.50 + N_4 \times 0.75 + N_5 \times 1}{N_1 + N_2 + N_3 + N_4 + N_5}$, where the sum
86 of the analyzed grains in each class was around 500. From a Student test the
87 maximum error on this measurement was estimated, to be lower than 2 units.
88 Grains harvested from two different locations (Cappelle ($50^\circ 29'N/3^\circ 10'E$) and
89 Maule ($48^\circ 54'N/1^\circ 51'E$)) were retained for this study as they were found to
90 display contrasting endosperm vitreousness [15].

91 2.1.1. *Light transmission through endosperm samples*

92 Wheat kernels were abraded longitudinally from the back and the ventral
93 sides with a 240 grit extra fine sandpaper (grit size $58.5 \mu\text{m}$). Abraded grains
94 were placed above a plate light (flat dome Light, LFX-100, CCS, Japan)
95 that diffuses uniform white LED lighting (Figure 1a). In order to avoid
96 straight light the plate light was covered with a black paper except at the
97 kernel position. Then light transmission through grain cut was collected in
98 the visible range using a $400 \mu\text{m}$ diameter optical fiber and measured using
99 a spectrometer (Ocean Optics USB 2000+XR extended range, FL, USA).

100 The number of photons (Intensity in counts) per unit of time (100 ms) was
101 obtained as a function of the wavelength (nm) between 400 and 800 nm and
102 analyzed with the SpectraSuite software (Ocean Optics, Dunedin, Florida,
103 USA). For each analyzed grain samples, thirty kernels were randomly selected
104 and 3 grain locations were probed into the endosperm cheeks (Figure 1b):
105 distal part (close to the brush), central, and proximal part (close to the germ).
106 Therefore 180 measurement values were obtained for each wheat sample.

Figure 1: (a) Schematic representation of the experimental assembly used for the spectroscopic analysis of the grain microstructure. h is for the sample thickness (b) Example of the analyzed grains differing by vitreousness. Circles represent the six areas where the transmission spectra were collected into the starchy endosperm.

107 2.2. Statistical analysis

108 The statistical analysis, such as statistical tests and box-plots, were per-
109 formed with R software (R Core Team (2016), R: A language and environ-
110 nment for statistical computing. R Foundation for Statistical Computing,
111 Vienna, Austria. URL <https://www.R-project.org/>).

112 3. Results

113 Wheat samples were selected from a previous study [15]. This study drove
114 us to select grains with a defined puroindoline genome leading to either soft
115 (native puroindoline a and b alleles) or hard (mutated puroindoline b) phe-
116 notype and grown in two contrasted environments resulting in different levels
117 of vitreousness for the starchy endosperm. Wheat grains were characterized
118 with a Pohl grain cutter as already described [23]. Considering the previous

119 study [15] which described the vitreousness range within different wheat grain
 120 samples grown in two years and in seven different sites, samples displaying
 121 vitreousness below 40%, were classified as mealy. Conversely, grains having a
 122 vitreousness score above 40% were considered as vitreous. This classification
 123 led us to identify four different grain samples defined by their genetic origin
 124 and vitreousness score (Soft Mealy, SM; Soft Vitreous, SV; Hard mealy, HM;
 125 Hard vitreous, HV) as summarized in Table 1.

Grain samples	Genomic <i>Pin</i> profile	Vitreousness (%)
Soft Mealy (SM)	<i>Pina-D1a/Pinb-D1a</i>	21.4 ± 1.2
Soft Vitreous (SV)	<i>Pina-D1a/Pinb-D1a</i>	48.2 ± 1.6
Hard Mealy (HM)	<i>Pina-D1a/Pinb-D1b</i>	23.1 ± 1.1
Hard Vitreous (HV)	<i>Pina-D1a/Pinb-D1b</i>	68.8 ± 1.7

Table 1: Genomic profile and vitreousness of the wheat near-isogenic lines differing by hardness and displaying contrasted vitreousness (as determined with a Pohl grain cutter)

126 3.1. Wheat grain endosperm vitreousness fits with a Beer Lambert law

127 Wheat starchy endosperm is a cohesive granular material [24] composed
 128 of various phases among which starch, protein matrix and voids occupy most
 129 of the volume. In such a material the pores induce differences in light trans-
 130 mission resulting in various vitreousness. For this type of porous materials
 131 the strong variation in optical indexes, which constitute massive scattering
 132 events, should follow the Beer-Lambert law:

$$I = I_0 \exp(-\kappa h) \quad (1)$$

133 where I_0 and I are the incident and transmitted light beam respectively,
134 κ is an absorption coefficient which depends on the material, and h is the
135 thickness of the sample.

136 Therefore, this law directly relates the attenuation of light to the optical
137 properties of the bulk microstructure. It is worth noting that κ depends only
138 on physical parameters without introducing any arbitrary variable.

139 To check the validity of this law, we performed light transmission measure-
140 ment at the center of HV kernel cheek cross-sections with thicknesses vary-
141 ing from 0.9 to 1.8 mm. Figure 2 shows the evolution of light transmission
142 $T = I/I_0$ as a function of h for three different samples. The results are well
143 fitted by the Beer-Lambert law (in dashed line) with $\kappa = (1.13 \pm 0.50) \text{ mm}^{-1}$.
144 The validity of the Beer-Lambert law to describe the transmission of light
145 through the wheat endosperm gives way to characterize wheat propensity to
146 transmit light with a sound physical parameter: the absorption coefficient.
147 In the following, we chose instead to use the transmission coefficient $\tau = 1/\kappa$
148 which increases with vitreousness.

Figure 2: Evolution of the light transmission as a function of the grain thickness for three different HV samples (fit dashed line $T(h) = e^{-1.13h}$). h is for the sample thickness.

149 The four wheat samples were then analyzed to determine τ (and thus
150 the vitreousness) with a specific focus on both inter-grain samples or intra-
151 grain sample variability. We performed measurements on 30 randomly se-
152 lected grains for each wheat samples (s) working at a carefully abraded fixed
153 thickness $h_s = (1.58 \pm 0.14) \text{ mm}$. This thickness was chosen to get easily
154 reproducible cross-sections with a good compromise between stiffness and
155 sensitivity to transmission measurement. τ values were directly derived from

156 the measurements of light transmission at this thickness, $T(h_s)$, using equa-
157 tion (2):

$$\tau = \frac{-h_s}{\ln T(h_s)} \quad (2)$$

158 3.1.1. Inter wheat grain endosperm texture comparison

159 Inter-grain comparison was performed by comparing the transmitted light
160 between the same specific grain location in the different wheat samples (cen-
161 tral areas of HV vs central areas of HM as an example). As far as the τ ob-
162 servations did not fulfill a Gaussian distribution, non-parametric tests were
163 performed to compare samples. Wilcoxon-Mann-Whitney tests were imple-
164 mented to compare the inter-grain observations (comparison of the median
165 of the transmission coefficient τ from an identical location between different
166 grain samples). Example of the inter-grain comparison corresponding to both
167 of the endosperm central areas is illustrated in Figure 3. The represented
168 boxplots highlighted the non-Gaussian and discontinuous distribution of the
169 transmission coefficient within the different samples with a few number of
170 grains displaying higher transmittance than the major part of the popula-
171 tion in each case, clearly reflecting the natural sample heterogeneity.

172 The statistical results showed that mealy grains displayed the lowest light
173 transmission coefficient, with no significant difference between soft and hard
174 lines. On the opposite, vitreous grains exhibit higher transmission coeffi-
175 cients, which were influenced by the genetic origin of the grains. Indeed, HV
176 grains present higher light transmission coefficients than SV grains (Figure
177 3).

178 For the different grain samples, the same comparison between transmis-

Figure 3: Boxplot for intergrain comparison of the transmission coefficient τ for HV, HM, SV and SM in both of the central cheek endosperm. The graph summarizes the distribution of data as the following : boxes enclose 50 % of the data (n=60) with the median value for variables displayed as a line, boxes span from the first quartile to the third quartile and vertical lines display 1.5 times the interquartile distance from the box (with extreme values of whiskers $[\max(\tau_{min}, Q3 - 1.5(Q3 - Q1)), \min(\tau_{max}, Q3 + 1.5(Q3 - Q1))]$ where $Q1$, $Q3$, τ_{max} and τ_{min} are the first and third quartiles, and max and min are the measured values for each texture respectively). Outliers are positioned outside the box and the vertical lines. *, ** and *** indicate significance of the differences with 90 %, 95 % or 99 % confidence level respectively, with n.s.= non significant effect. Each measurement was labeled with a dot.

179 sion coefficient τ either in the proximal or distal regions reveal similar trends:
 180 1) no statistical differences between mealy grains (low τ values), 2) light
 181 transmission decreases significantly in the order HV, SV and mealy (HM or
 182 SM).

183 3.2. Intra wheat grain endosperm texture comparison

184 Comparison between measurements in the endosperm central areas with
 185 those in the proximal and distal extremities were also conducted to exam-
 186 ine the intra-grain microstructure variability within each four wheat grain
 187 sample sets. Unilateral Wilcoxon signed rank tests were computed for these
 188 comparisons to determine if the median of the transmission coefficient τ
 189 measured at a specific endosperm location (central versus proximal and dis-
 190 tal and distal versus proximal) within grains of a specific wheat sample was
 191 significantly higher or lower than the median value observed for both other
 192 locations. Within each wheat sample, the central areas transmitted signifi-
 193 cantly higher amount of light than distal endosperm positions (brush side),

Figure 4: Boxplot for intragrain comparison of the transmission coefficient τ for HV, HM, SV and SM in the different endosperm regions : distal near the brush, central into the grain cheeks or proximal close to the germ. *, **, *** and n.s. as in Figure 3.

194 which transmitted more light than positions in the proximal regions (close
195 to the germ), except for the HV grains where proximal and distal regions
196 cannot be distinguished (Figure 4). However, within this HV sample, light
197 transmission measurements through the different grains appeared the most
198 variable as reflected by the boxplot height. Indeed vitreous samples clearly
199 showed more data spreading corresponding to different endosperm organi-
200 zation within each grain whereas light transmission through each grain in
201 mealy samples appeared more homogeneous.

202 4. Discussion

203 In this study, the endosperm microstructure was analyzed based on light
204 transmission measurements. As grain endosperm organization was found to
205 differ depending on the puroindoline allelic state which was suggested to play
206 a role in starch-protein network adhesion [6], near-isogenic grains differing
207 only by the puroindoline b allelic form (*PinbD1a* or *PinbD1b*) were used in
208 order to well define the genetic background. These wheat samples were grown
209 in two contrasted environments found to lead to distinct vitreousness level
210 [15] and thus to different level of wheat endosperm porosity. Here, we demon-
211 strated for the first time that vitreousness can be non-ambiguously quantified
212 and compared from light transmission measurements through wheat samples
213 displaying standardized thickness. As light transmission was found to follow
214 a Beer-Lambert law, it is clearly the results of changes in the endosperm

215 porosity as pores are known to scatter light. The statistical analysis showed
216 that grains grown in an environment leading to mealy kernels but presenting
217 distinct genetic background (as observed between mealy grains of distinct
218 hardness, HM and SM) cannot be distinguished by light transmission be-
219 cause they display a high porosity level. Importance of the environment was
220 also highlighted for grains within the same genetic background but grown in
221 different locations that were found to display distinct vitreousness and thus
222 light transmission (SM vs SV, HM vs HV). Consistency with these differ-
223 ences between wheat samples, presence of a pore network was shown to lead
224 to weaker mechanical strength whatever the genetic background [3]. These
225 results clearly highlight the importance of porosity or vitreousness characteri-
226 zation and of the identification of genetic background. Such a knowledge help
227 us to get a more accurate understanding of the grain mechanical properties
228 and thus the milling behavior as also previously noticed [15, 4]. Further-
229 more, SV and HV wheat grains displayed distinct vitreousness scores and
230 transmission coefficients even if they were grown in the same environment.
231 Therefore for vitreous grains, presence of both wild-type puroindolines results
232 in a significant decrease of τ revealing a more porous microstructure. This
233 observation possibly suggests that light transmission, and thus vitreousness,
234 is affected by the different genetic background of these wheat samples. In-
235 deed, the soft line containing the wild-type puroindolines would have a lower
236 starch-protein adhesion due to the higher puroindoline content at the starch
237 granule surface in the endosperm [25, 26]. The weak starch-protein adhe-
238 sion could act as a network of internal microcracks. They may constitute
239 another factor responsible for endosperm porosity that scatter the light and

240 result in lower light transmission. Recently, Oury et al.[15] demonstrated,
241 using similar genetic material that the soft near-isogenic lines never exceed
242 a vitreousness level of 60% in comparison with the hard near-isogenic lines
243 which were found to display a broader range of vitreousness levels (up to
244 80%). Additionally a larger data spreading was found within the hard vitre-
245 ous grain in accordance with the larger standard deviation observed in similar
246 samples for mechanical properties [3]. Our results also match with modeling
247 of the endosperm that also revealed a more important effect of changes in
248 the adhesion between starch and the protein network (related to the pres-
249 ence of wild or mutated puroindoline alleles) in the case of low porosity [27].
250 Moreover, light transmission analysis also highlighted the heterogeneity in
251 the endosperm microstructure depending on the location within the grain.
252 The highest light transmission obtained in the central endosperm suggests a
253 more compact organization in comparison with the distal or proximal regions
254 respectively close to the brush or the germ extremities . These differences in
255 compactness can be potentially related to the observed distinct behavior of
256 the grain extremities against pathogen attack [28] or water, gibberelic acid
257 and α -amylase diffusion along imbibition/germination [29, 30].

258 5. Conclusions

259 In this study, we first demonstrated a Beer-Lambert relationship for light
260 transmission through the endosperm allowing to quantify and compare en-
261 dosperm microstructure depending on both genetic background and environ-
262 mental conditions. We confirmed the role of the environment in endosperm
263 porosity and revealed a more pronounced effect of the genetic background for

264 grains with low vitreousness. Furthermore, local heterogeneity within the
265 endosperm was observed showing a more compact structure of the endosperm
266 central region in accordance with our knowledge on grain physiology.

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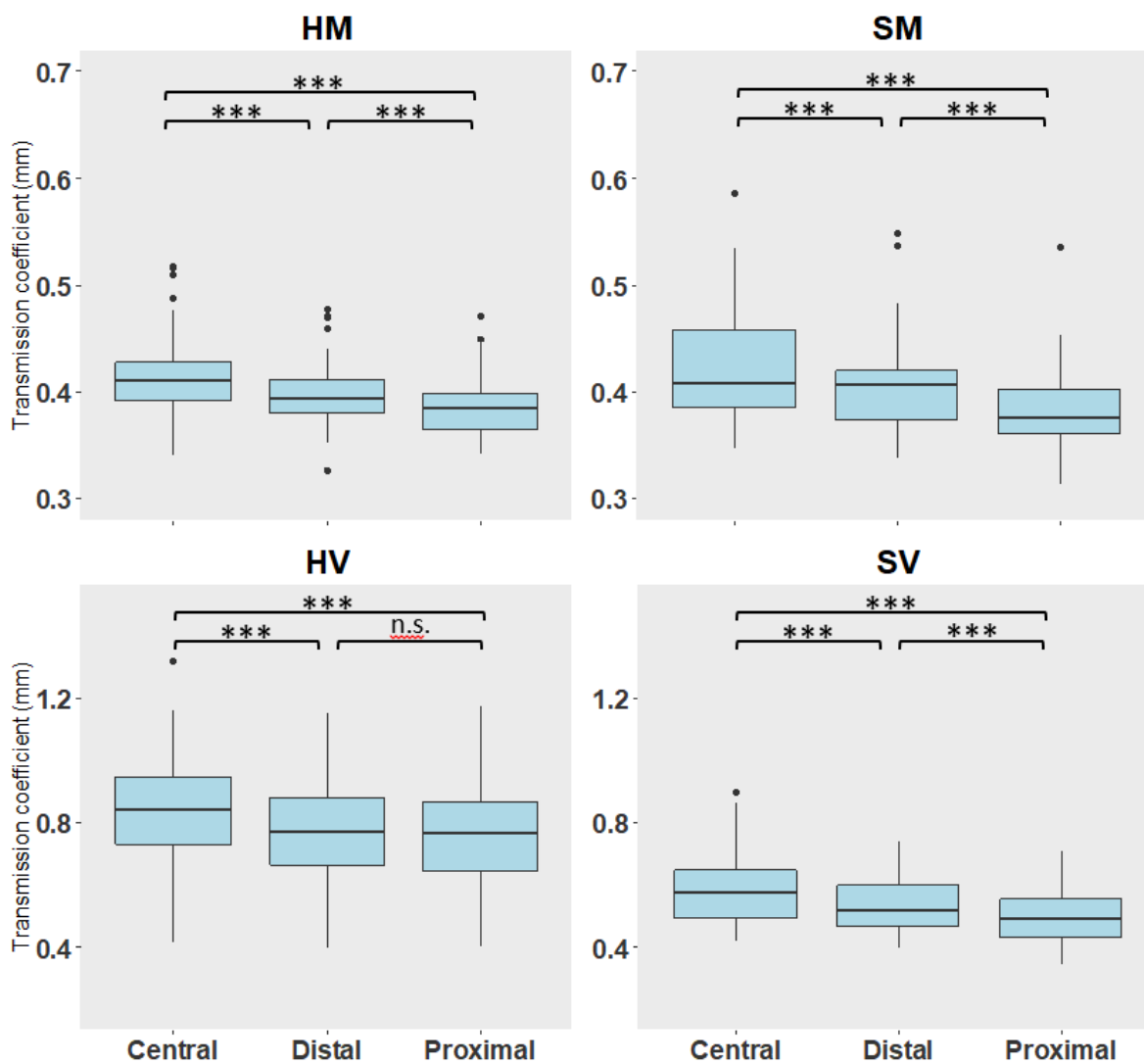
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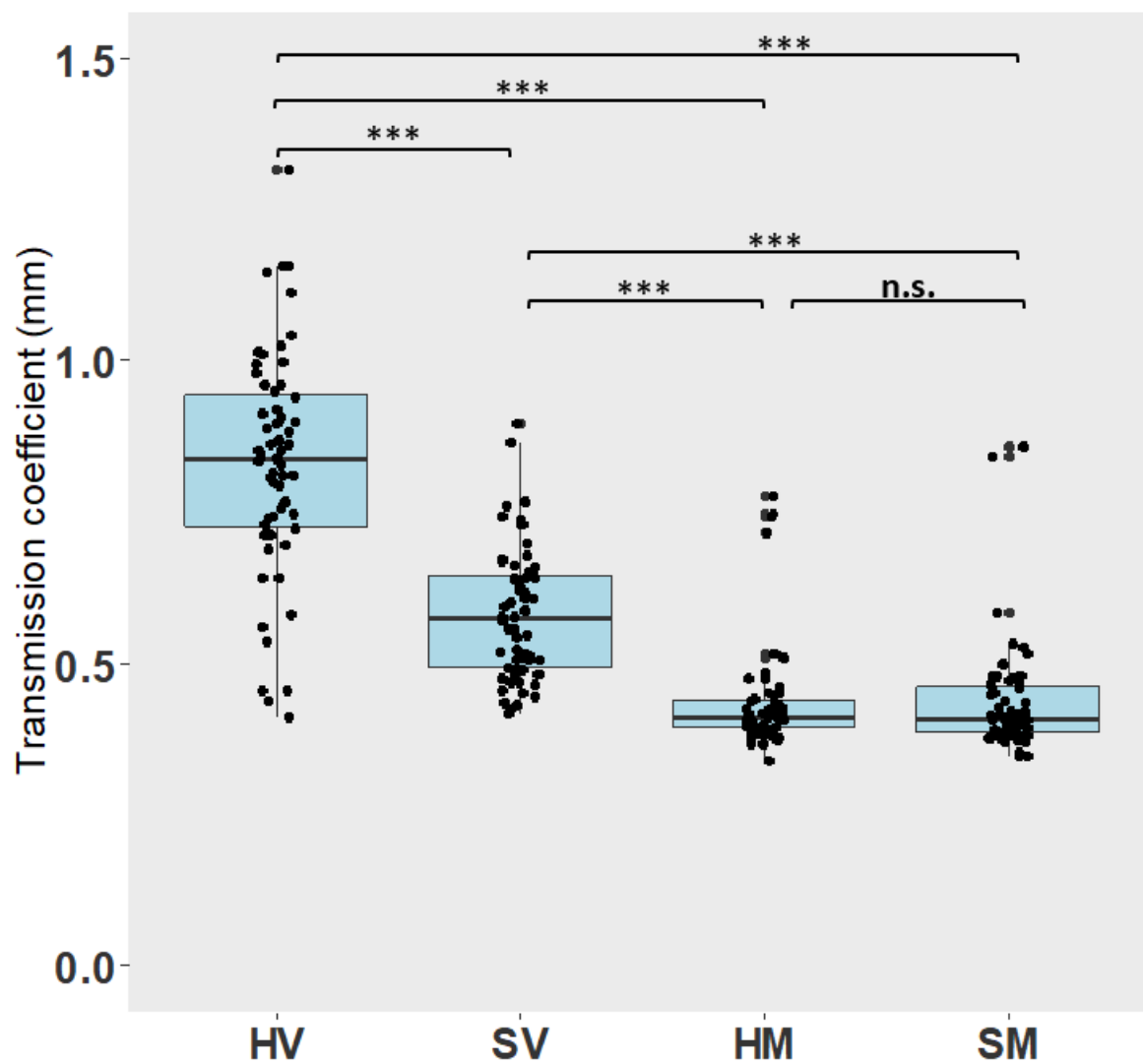
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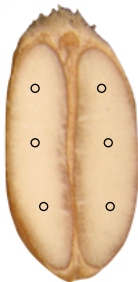
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vitreous

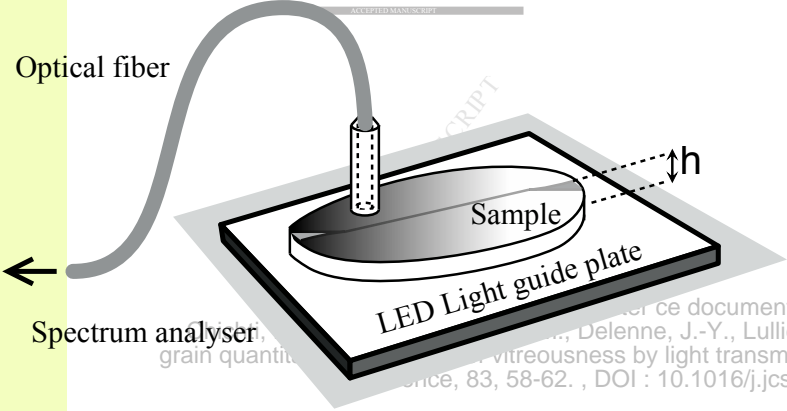


mealy

distal

central

proximal



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Highlights:

- Light transmission through the wheat endosperm follows a Beer-Lambert law
- Similar endosperm light transmission was found in grains displaying low porosity
- The highest transmittance was found with vitreous grains from hard genotype
- The proximal or distal part of the endosperm was more porous than the center

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