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Comparison of UV and visible autofluorescence of wheat grain tissues in macroscopic images of cross-sections and particles

Mathias Corcel [a], Marie-Françoise Devaux [a], Fabienne Guillon [a], Cécile Barron [b,*]

[a] INRA – Centre de recherche Angers-Nantes, UR BIA "Biopolymères Interactions Assemblages" 1268, Nantes, France
[b] UMR IATE, CIRAD, INRA, Montpellier SupAgro, Université de Montpellier, F-34060 Montpellier, France

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ABSTRACT

Many plant tissues can be observed thanks to the autofluorescence of their cell wall components. Multispectral autofluorescence imaging at the macroscopic scale is a rapid efficient way of observing samples with a large field of view (>1 cm^2) and a nice resolution (<3 µm per pixel). The objective of this work was to evaluate autofluorescence multispectral imaging at the macroscopic scale to identify the tissular origin of particles through their autofluorescence profiles. The pericarp and aleurone layer of wheat grain were used to compare contrasted autofluorescence profiles measured in cross sections and particles. Air and water mounting media were tested. Principal component analysis and variance analysis showed that the autofluorescence properties were retained from cross sections to particles. The mounting media modified the autofluorescence profiles and water was found to be better to observe both particles and sections. These results show that multispectral autofluorescence imaging at the macroscopic scale can be used to identify the histological origin of particles.

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1. Introduction

Plants are heterogeneous materials made up of organs (stem, grains, leaves, etc.) which are themselves made up of various tissues including different types of cells (parenchyma, pericarp, vascular bundles, etc.) with dedicated functions. The composition, structure and relative proportions of the different tissues and of the different types of cells in the organ are under biological control and determine the nutritional quality and use of the plant by humans. Studies on plant tissues are therefore of major importance for evaluating the quality of plant material for nutritional and industrial applications, as well as its ability to be processed (Ding et al., 2012). Plant fragmentation is often the first step in plant processing and use. Fragmentation often includes a grinding step that transforms the plant into powder and may also include separation by sieving for example. In the case of wheat straw, Silva et al. (2012) showed that the grinding step had a direct influence on the results of enzymatic degradation. In the cereal milling industry, wheat grains are ground at a histological scale to recover the starchy endosperm and process it into flour or semolina, and to collect the peripheral tissues (aleurone layer, testa and pericarp) as bran. The proportions of each tissue in the resulting fractions influence their nutritional or health quality, as well as their end-use properties (Brouns et al., 2012). In such cases, the tissue dissociation needs to be evaluated to understand, control and optimize the transformation of the plant.

Identifying tissues in cross sections of organs is easy, but not in powder form. Methods of identifying tissue in powder form are mainly based on their specific biochemical composition, which is theoretically the same after fragmentation. In wheat grain applications, different methods have been proposed to track tissues: measuring the amount of tissue-specific molecules used as biomarkers (Antoine et al., 2004; Hemery et al., 2009), using the spectroscopic response in the mid-infrared range (Barron, 2011), or monitoring their autofluorescence properties (Jensen et al., 1982; Symons and Dexter, 1993, 1996). In the latter, the specific autofluorescence properties of the aleurone layer and pericarp under UV and blue light have been exploited to quantify the contamination of the flour by bran particles (Brown, 1999; Lisio, 2001; Evers and Erdentug, 2003). These methods mainly focused on the detection of the most peripheral tissues of wheat grain and were developed to give quantitative assessments of a bulk tissue composition in flour or bran fractions. None of these methods allowed the estimation of tissue dissociation at the particle scale even when an imaging system was used, as it was the case of fluorescence measurements carried out using Maztech MicroVision equipment (Dipix I440, which is no longer available (Lisio, 2001)) or the Branscan Ltd. system (Fluoroscan, offline and online system (Brown, 1999)). Except in wheat, few studies have been conducted to monitor the constituents of

* Corresponding author.
E-mail address: cecile.barron@supagro.inra.fr (C. Barron).

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other plant material (organs, tissues) during fragmentation (Papatheofanous et al., 1998; Jin and Chen, 2007; Collins et al., 2014). More generally, no methods have been described to reveal the origin of single particles in an organ before fragmentation.

To observe tissue dissociation within particles, whose size can range from 100 μm to 2 mm, identification must be carried out at the micrometric scale. Fluorescence instruments make it possible to acquire images at the macroscopic scale. A large number of particles of variable size can be observed using mosaic images that combine a large field of view, one cm^2, and a fine resolution, <3 μm per pixel. In plant tissues, autofluorescence can be used to observe the structure of the cell wall. Plant cell wall autofluorescence is mainly linked to the presence of phenolic compounds such as lignin and hydroxycinnamic acids (ferulic acid, para-coumaric acid, etc.), as demonstrated in wheat peripheral tissues (Rost, 1995). Macroscopic imaging has great potential to reveal the composition of phenolic compounds (Yoshioka et al., 2013). However, considering the complexity of cell wall structure and composition, not only one compound is present or absent in a given cell type. Combining multiple autofluorescence excitation and emission is required to identify tissues with overlapping autofluorescence responses or in cases of the co-localization of constituents. The set of excitation-emission images form what is called a multispectral image. In wheat grain, identification of the testa, in addition to the aleurone layer and the pericarp was achieved by analyzing multispectral images using chemometric methods (Baldwin et al., 1997). All the autofluorescence intensities observed on each individual autofluorescence images are analyzed together and not only the presence and absence of autofluorescence in specific excitation conditions. Multispectral images can be used to build prediction models (Geladi and Grahn, 1996; de Juan et al., 2009). We propose to build models with cross-sections images used as calibration, where the different tissues can be clearly identified, and then apply the models to images of particles after fragmentation. The model applied to particles should be able to recover the tissular origin of the particles in the organ. To apply this approach in micro or macroscopy, several points first need checking: (1) the intensity of the autofluorescence profiles has to be maintained from structured organs to particles, (2) the way the samples are prepared must be suitable for both plant sections and particles. For the same fluorophore, fluorescence emission is known to be dependent, on physical-chemical characteristics such as polarity of the solvent or pH (Lakowicz, 2013). Potential distortion of autofluorescence profile can occur in the same tissue depending on whether it is a section or a particle and on the mounting media chosen for sample imaging.

The objective of the present study was to compare the autofluorescence properties of tissues in sections and particles in two mounting media, air and water, using a multispectral fluorescence macroscope. Wheat grain was chosen as the model organ on account of its well described histology and the possibility to separate and clearly distinguish autofluorescent external layers from the starchy endosperm. Two specific tissues, the aleurone layer and the pericarp, were used as their autofluorescence properties allows them to be identified on cross sections of wheat grain (Baldwin et al., 1997), and particles of pure tissue can be obtained after hand isolation or fractionation (Barron et al., 2007; Hemery et al., 2007).

2. Materials and methods

2.1. Cross section and powder samples

Cross sections and powders of the wheat cultivar *Tiger* (*Triticum aestivum* L.) were used in this study. The cross sections were prepared from six grains cut in half length. De-germinated grains were first hydrated and 80 μm thick sections were cut under water using a vibrating blade microtome (MICROM HM 650 V, Microm Microtech France).

The aleurone layer in powder form was supplied by Bühler A.G. (Uzwill, Switzerland). The sample corresponded to the "aleurone 2 < 180 μm" described by Hemery et al. (2009) and was produced using the physical dry processing (LEURON® bran fractionation process (Bohm et al., 2011)) with a particle size <180 μm. Pure pericarp powder was obtained from outer pericarp tissue isolated manually as described in Barron et al., 2007 and further ground in a mortar after drying at room temperature under phosphorus pentoxide. Particles size was mainly about 100–150 μm length with few bigger particles up to 500 μm.

2.2. Slide preparation: mounting media for image acquisition

Two mounting media, water and air, were tested for observation. As the cross-sections were cut under water, they were directly placed on a slide and observed in water. They were then dried in an oven (30 °C for 24 h) and observed. Aleurone and pericarp powders were observed mounted in air after dispersion on a slide using a dry powder disperser (SPD 1300, Malvern, England). Dispersion conditions applied were total cycle time <1 min, injection time 0.4 s, air pressure 4 bars. A small quantity of particles was suspended in water. The quantities were adjusted to ensure good dispersion of the particles. One drop of the suspension was put on a hybridization frame (Thermo Scientific, Gene Frame® in-situ hybridization microarray) for observation in a water medium.

2.3. Autofluorescence multispectral image acquisition

Between six and ten multispectral autofluorescence images were acquired for each modality: cross sections, aleurone powder, and pericarp powder, mounted in air and in water. Images were collected using the fluorescence macroscope Multizoom AZ100M (Nikon, Japan) equipped with a RGB DS-Ri1 camera (Nikon, Japan). The total magnification was set to 4X by combining the lens AZ-Plan Fluor 2X (NA: 0.2/WD: 45 mm) and setting the optical zoom to ×2. The system makes it possible to acquire RGB autofluorescence images with a resolution of 1280 × 1024 pixels per image with an intensity coded using 4,096 levels expanded between 0 and 65,536. The image resolution was 2.74 μm/pixel, and the field of view 3.5 × 2.8 mm^2. The macroscope was equipped with four different filter cubes corresponding to two UV and two visible - blue and green - excitations:

- Filter U1: Excitation 325–375 nm, Dichroic Mirror > 400 nm, Emission > 420 nm.
- Filter U2: Excitation 360–370 nm, Dichroic Mirror > 380 nm, Emission > 400 nm.
- Filter BL: Excitation 450–490 nm, Dichroic Mirror > 505 nm, Emission > 515 nm.
- Filter GR: Excitation 510–560 nm, Dichroic Mirror > 565 nm, Emission > 593 nm.

The order of acquisition was GR, BL, U2, and U1. Samples were illuminated with a mercury lamp (Intensilight C-HGFIE, Nikon, Japan). Exposure times (milliseconds) were fixed at 230 ms for U1, 350 ms for U2, 450 ms for BL, and 1,500 ms for GR, after looking at a few demonstration samples. The exposure time-ratios were green filter: 1, blue filter: 0.30, UV filter U2: 0.23, UV filter U1 = 0.15. Using these exposure times, saturation could occur in some images. When saturation was observed, exposure times were adjusted in order to respect the respective ratios and the

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