Laboratory Standard Operating Procedure

Characterization of RTB Starch Grain Size and Shape Through Imaging

High-Throughput Phenotyping Protocols (HTPP), WP3

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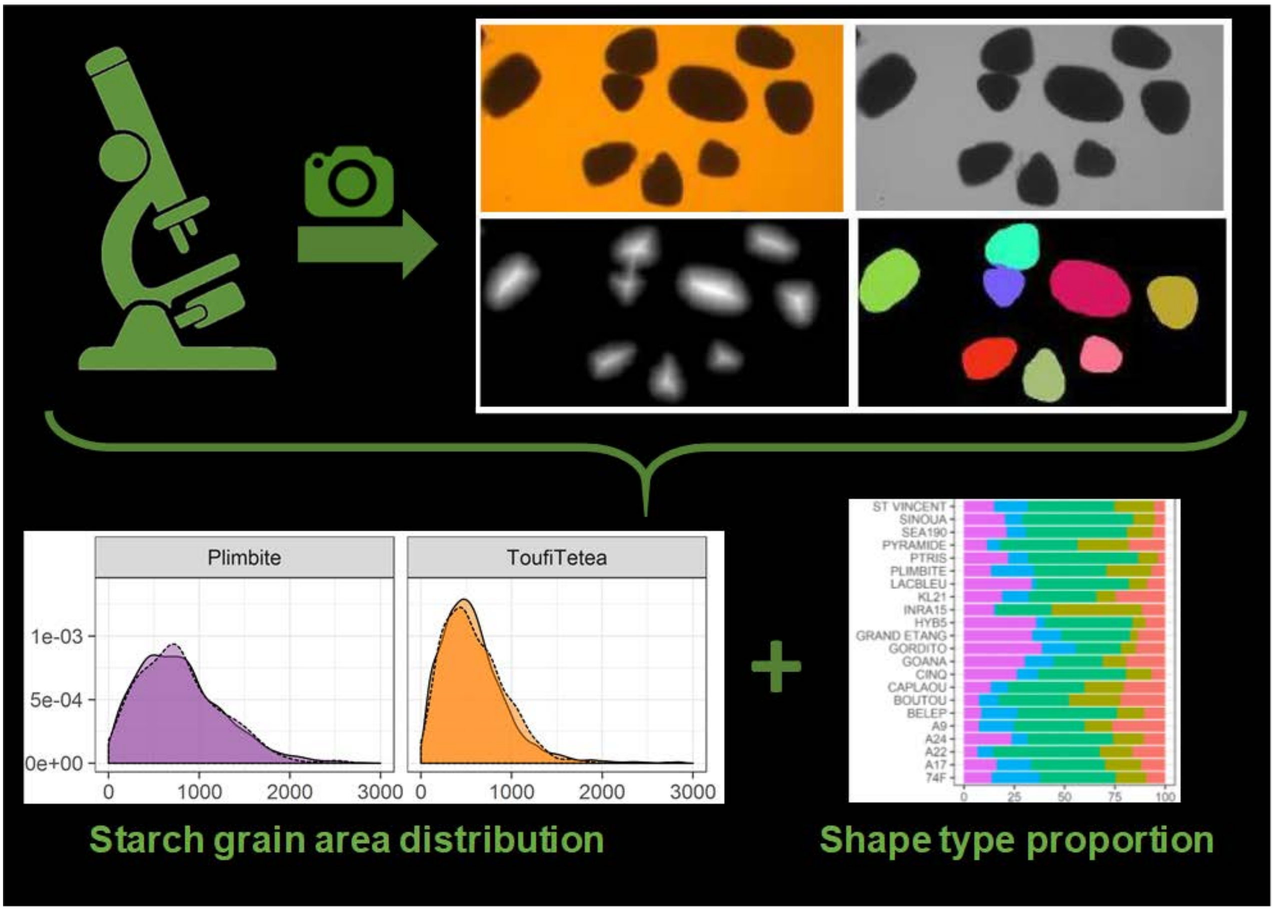

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RTBfoods

WP3: High-Throughput Phenotyping Protocols (HTPP)

SOP: Characterization of RTB Starch Grain Size and Shape Through Imaging

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ABSTRACT

Granules from different starch sources vary in composition, size and shape. The size distribution of starch grains and their shape contribute to some functional properties (e.g. viscosity, water retention capacity, starch gelatinization) but also certain mechanical properties. Conventional physicochemical measurements of starch properties are often costly and difficult to access. The work presented here aims to contribute to the evaluation of the quality of yam tubers through the development of an automated method for measuring the size and shape of starch grains in RTB products.

This protocol is based on the detection of starch grains by iodine staining of starch from RTB flour. Once stained, a drop of the solution is observed under the microscope and an image is taken. A morphological segmentation based on the watershed algorithm is then applied to images in order to detect every starch grain. Finally, the grain starch size and shape are calculated based on appropriate scale and indicators.

Key Words: Food quality; Starch granule; Computer vision; Granule size determination; Morphology

1 SCOPE AND APPLICATION

Granules from different starch sources vary in composition, size and shape. The size distribution of starch grains and their shape does not, on its own, appear to have a strong effect on starch performance. However, it contributes to certain functional properties, such as viscosity, water retention capacity, starch gelatinization, and its behavior during acid and enzymatic hydrolysis (Lindeboom et al 2004) but also certain mechanical properties. For example, Kang et al (2015) showed that the shape of the grains affects their hardness and consequently their transformation into flour (power required by the mill, fineness of the flours and rate of damaged starch grains). The size and shape of the grains will contribute to the speed of starch gelatinization and its gelatinization temperature (Eliasson et Karlsson 1983, Karlsson et al. 1983, Svihus et al., 2005, Puncha-arnon et al 2008). Acid and enzyme hydrolysis will be influenced by the size of the grains, in the sense that when we are in the presence of small grains, hydrolysis is carried out more rapidly with the acid or enzyme, than with large grains. This could be due to the larger surface area per unit weight of small grain starch (Lindeboom et al 2004). Large starch granules tend to build higher viscosity, but the viscosity is delicate because the physical size of the granule makes it more sensitive to shear. In spite of such differences, the more compact structure of a smaller molecule doesn't always mean a significant difference in gelatinization. Furthermore, starches having larger average granule size showed more amylose and phosphorus contents than those with smaller average granule size (Zhang and al., 2019).

Starch is mainly produced in the amyloplast and chloroplast of the plant. The membrane structures and physical characteristics of the plastids can give a particular shape or morphology to the starch grains and can affect the arrangement and association of amylose and amylopectin molecules in the grain (Lindeboom et al., 2004). The characteristics of starch grains are therefore partly under genetic control. Indeed, genetic mutations affect the content of amylose and amylopectin inducing effects on grain size distribution (Lindeboom et al., 2004) which varies from 3 to more than 100 microns (Hegenbart 1996). In yam, there are variations in the shape and size of starch grains, including from one variety to another, within the same species (Fauziah et al., 2016). Thus, several studies have highlighted the existence of different types of starch grains between yam species and varieties but also variability related to environmental conditions (Fauziah et al., 2016; Dabonne et al., 2010).

Given the importance of starch characteristics in terms of food safety, product acceptability or agro-processing potential, and because these characteristics vary according to genotype x environment interactions, it is necessary to be able to measure them quickly and at a lower cost. Conventional physicochemical measurements of starch properties are often costly and difficult to access for research in tropical environments. The work presented here aims to contribute to the evaluation of the quality of yam tubers through the development of an automated method for measuring the size and shape of starch grains in RTB products.

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2.2 Image analysis

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3 PRINCIPLE

This protocol is based on the detection of starch grains by colouring starch from RTB flour. The starch grains are highlighted by iodine staining. Indeed, in the presence of iodine solution in the medium, the starch grains are coloured. This iodine coloration of the starch is the result of the fixation of diiode **(**I_2**)** molecules inside the amylose helixes, i.e. one diiode molecule for every two turns of the helix. Amylose has a high binding capacity to iodine due to the linear structure of the latter, whereas amylopectin has a low capacity due to the presence of α-(1,6) type linkages on the chains and the preponderance of chains with a low degree of polymerization (Sahore , 2010). The colour of the starch-iodine complex thus formed, can range from brown to red, red-violet, blue-violet and finally blue, depending on the length of the starch polymer (Fauziah et al., 2016). The intensity of coloration will depend on the concentration of starch in the medium. Thanks to this coloration of the starch grains it is therefore possible to observe and determine the sizes and shapes of the starch grains with the help of image acquisition devices and image analysis software.

Once stained, a drop of the solution is observed under the microscope and an image is taken. A morphological segmentation based on the watershed algorithm is then applied to images in order to detect every starch grain. Finally, the grain starch size and shape are calculated based on appropriate scale and indicators.

4 APPARATUS

Oven (60°C) 	Grinders (0.2-0.5 mm) 	Laboratory precision balance 
Iodine solution 5% 	Micropipette 	Microtubes 
Lab vortex mixer 	Optical Microscope 	Camera 

5 PROCEDURE

5.1 Sample preparation

By genotype, at least three organ (i.e. tuber, fruit or root) are processed and characterized. RTB products are peeled, washed and cut into small cubes of 2 cm. Then the cubes are placed in the oven at 60°C for 72 hours before being grinded and sieved between 0.5 and 0.2mm.

A commercial betadine solution (5% diiodine) was used to prepare a working solution and to allow the colorimetric revelation of the starch. 30 mg of flour is poured in microtubes (Eppendorf, 2mL) before adding the iodine solution. Depending on the product, it is possible to determine the ideal concentration of flour in the iodine solution. For yam, an experiment has shown that a volume of 1.5mL of betadine added in each microtube gives an ideal solution of concentrated starch at 20g/L. This dilution allows a sufficient staining of the grains while avoiding a too strong or too weak concentration of grains in the solution; i.e. respectively causing numerous clusters of grains or making difficult to locate the grains under the microscope (Figure 1).

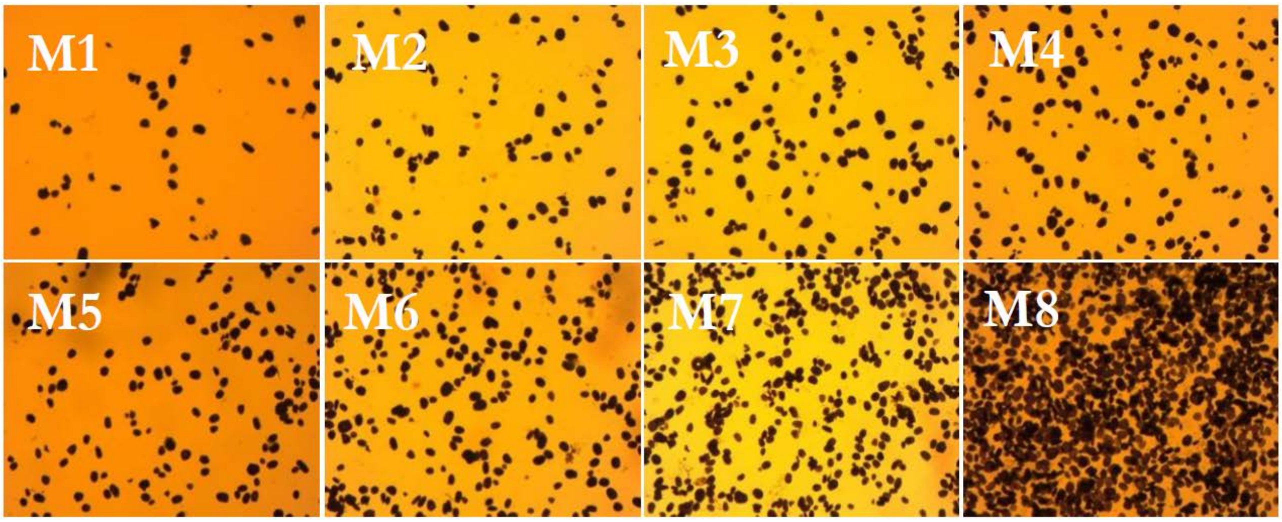

Figure 1: Microscopic images of starch grains with increasing flour concentrations from M1 to M8 (respectively 6.67, 13.33, 16.67, 20.00, 23.33, 33.33, 66.67, 133.33 mg mL^{-1}).

5.2 Image acquisition

After one hour of tinting at room temperature, the slides are mounted and analyzed under an optical microscope (e.g. Carl Zeiss Microscopy, Axio Scope A1). The slides are observed with an optical microscope according to two observation modalities: 100x and 400x magnification. The microscope is equipped with a camera (e.g. Axiocam ERc5s) to allow image acquisition. For each sample (e.g. a tuber), 3 slides are mounted and 5 images are saved for each slides for a total of 45 images per genotypes. Images are then labelled and saved in .jpg or .png for further analysis.

5.3 Starch grain segmentation

Even if shape and area of starch grain could be measure manually with open source software using freehand selection tool (e.g. ImageJ or Mesurim), this method is tedious and time consuming. Here we propose an automation of the method using image analysis algorithms. All the image processing and analysis steps were performed using the R programming language (R version 3.4.1) in order to guarantee the repeatability and accessibility. Image processing required two main steps: (i) image segmentation on the one hand, and (ii) object detection on the other hand.

5.3.1 Image segmentation

First, the images were converted to shades of grey to ensure the first segmentation (Figure 2A to B). This transformation is based on the Lightness values of each pixels (i.e. L* in the CIE L*a*b* color space). Then two successive morphological operations called a closing (i.e. an erosion followed by dilation) have been applied to remove cell particles and image noise (i.e. the random variation of brightness or color information, Figure 2B to C). This operation apply a disk shaped mask (i.e. kernel) by positioning its center over every of the image x, the output value of the pixel is the max/min value of pixels covered by the mask. Finally, a binarization of the images allowed the background to be separated from the regions corresponding to the starch grains (Figure 2C to D). The binarization is based on Otsu's method. Otsu's thresholding method is useful to automatically perform clustering-based image thresholding. The algorithm assumes that the distribution of image pixel intensities follows a bi-modal histogram, and separates those pixels into two classes (e.g. foreground and background). The optimal threshold value is determined by minimizing the combined intra-class variance.

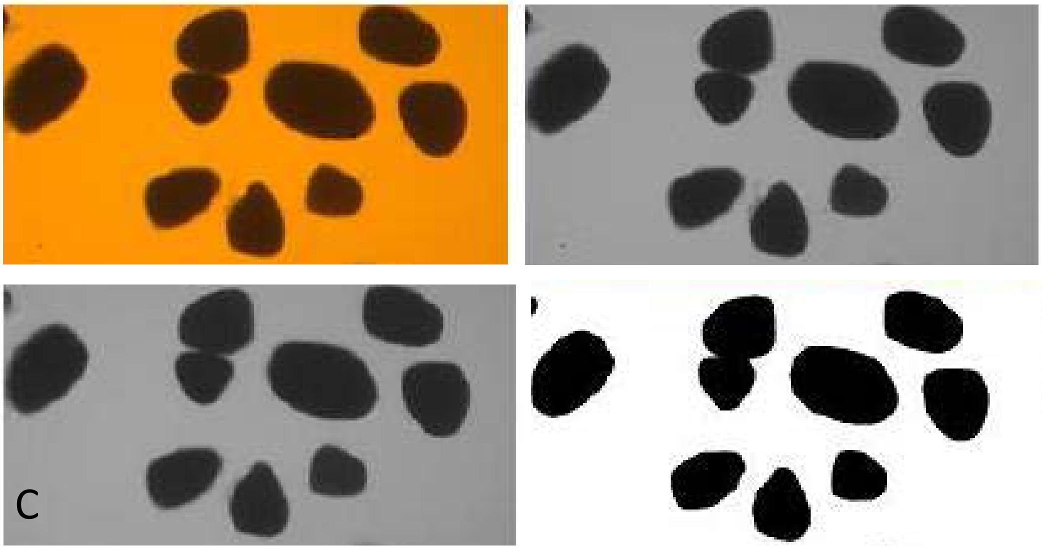

Figure 2: Morphological segmentation of starch grain.

5.3.2 Object detection

Nevertheless, the detection of objects on an image is sometimes problematic when the distance separating them is not sufficient for the break in homogeneity to be established during segmentation (Figure 3A). Binarization then leads to the merging of regions that actually correspond to distinct objects. To separate the merged starch grains, the watershed algorithm was applied (Figure 3B). The algorithm identifies and separates objects that stand out of the background (zero). It inverts the image and uses water to fill the resulting valleys (pixels with high intensity in the source image) until another object or background is met. The deepest valleys become indexed first, starting from 1 (Figure 3A to C). At the end of this step, the starch grains, initially agglomerated on the image, are separated and then labelled by different colours (Figure 3C). Finally, the truncated objects, positioned at the edge of the image, are removed so as not to compromise the measurements of size and shape of the starch grains (Figure 3C to D). To do so, we use the distance between the border and the centre of each object as opposed to the half of object maximum diameter.

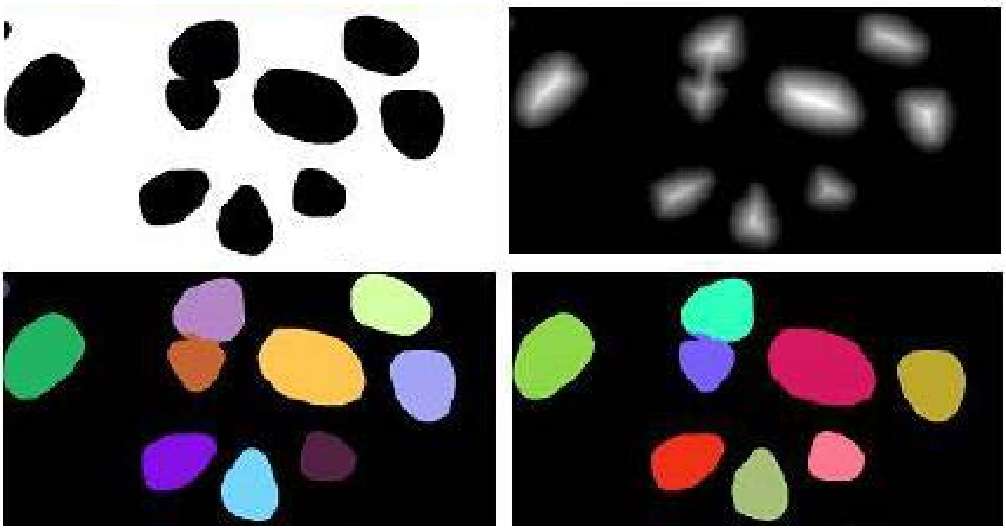

Figure 3: Individual starch grain detection.

6 Expression of Results

6.1 Starch grain size distribution

Once starch grains have been detected, it is easy to extract morphological information (e.g. area, maximum length, diameter). These metrics are first expressed in pixel number and need to be transformed in µm using the number of pixels of a known unit of length. Average dimension value can then be calculated by genotype. But even within a genotype there is a huge starch grain variability. So it is recommended to work with metric distribution rather than average (Figure 4).

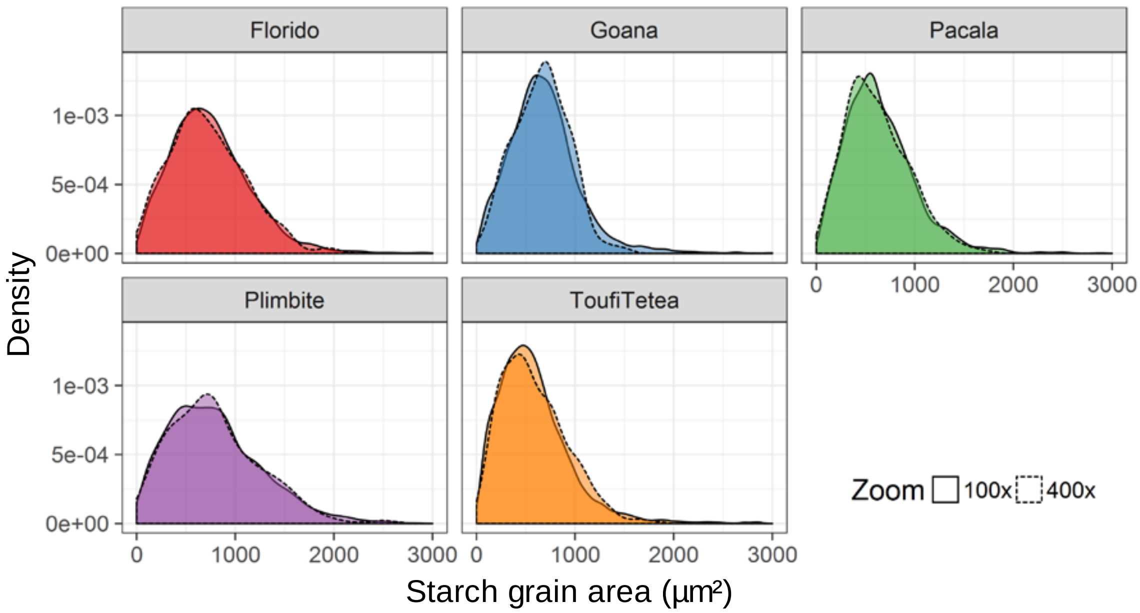

Figure 4: Example of distribution of starch grain area (µm²) calculated from images at 2 different magnifications for five yam genotypes.

6.2 Starch grain shape

The shape of a round-like object can be characterized using the flattening coefficient (i.e. the ratio of the major axis to the minor axis of its elliptic fit). This ratio allow to discriminate between genotype's starch grain shape (Figure 6).

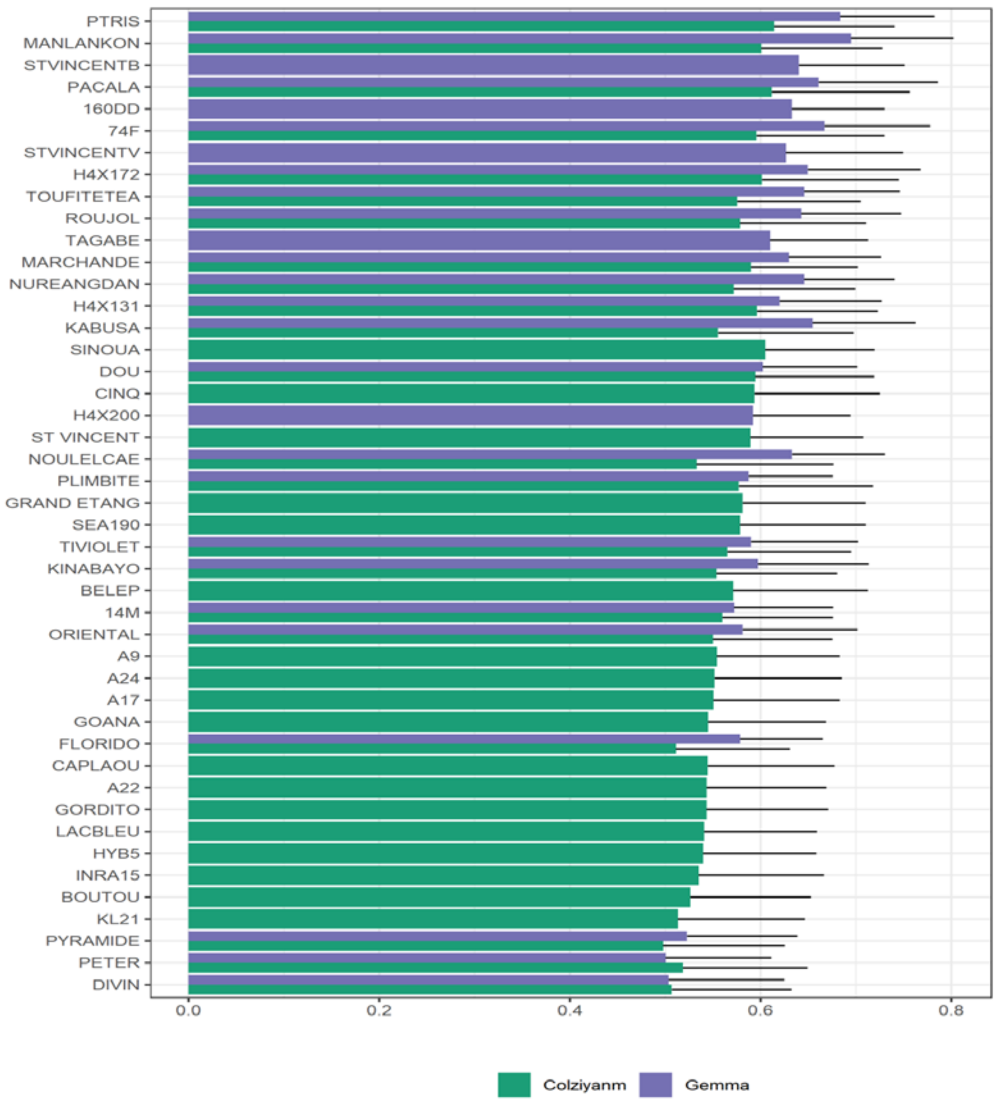

Figure 5: Example of genotypic diversity of starch grain flattening coefficient.

But this coefficient may not be sufficient to characterize the shape type (e.g. oval, round, triangular) of RTB starch grain (Figure 5). Many other shape index (e.g. Angel et al. 2010) could help to discriminate more precisely depending on the desired objective.

Figure 6: Yam starch grain shape diversity in Guadeloupe.

6.3 Repeatability

The use of ImageJ software allowed the manual measurement of the starch grain area as the reference method. A preliminary experiment allow measuring the starch grain area of 200 starch grains at 100x and 400x magnification with both the reference and the automatized image analysis method. Comparison of starch grain area at magnifications 100x and 400x, does not show any significant differences between both methods (Figure 4). Indeed, regardless of the magnification, the small RMSE (Root Mean Square Error) values show that automatized measurement is strongly linked with the reference method (ImageJ). Moreover, the random distribution of the errors on both sides of the 1:1 diagonal line, highlight the absence of bias. Even if the coefficient of determination is better at higher magnification, the lower magnification allows capturing a larger number of grains and thus reducing the uncertainty of the measurements by increasing the number of repetitions.

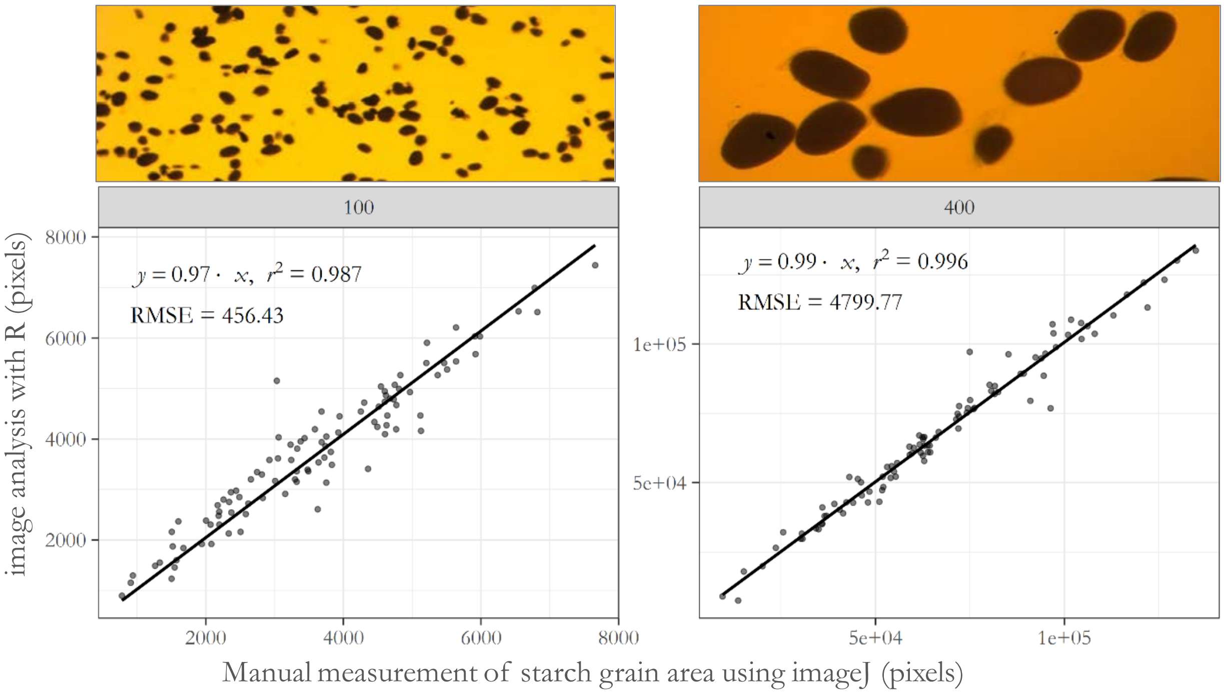

Figure 7: Comparison of manual and automatized measurements of starch grain area at magnifications 100x (left) and 400x (right) (n=100).

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