Rounding up plant cells

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Abstract

Compared to animal cells, plant cells are typically non-spherical, which may bias morphometric and fluorimetric analyses, including flow cytometry and other approaches used in the study of cellular biodiversity. The morphometric study of cotyledonary cells may serve to distinguish between genotypes, as cell shape is clearly an important issue when assessing flour quality and seed digestibility by animals, being affected by the surface and volume of particles. We devised a shape coefficient that resolves these difficulties with pea (*Pisum sativum L.*), and may find general applicability in cytological studies and for the characterization of biodiversity patterns.

Introduction

Using plant protein as animal feed, fodder or human food represents a strategic choice. Grain legumes are a source of high-quality protein for human consumption (e.g. beans, lentils, peas, chickpeas, faba beans)[1] and animal feed as seeds (e.g. peas, horse beans, soybeans, lupins)[2] or fodder (e.g. alfalfa, clover). Legumes have historically been utilized mainly as whole seeds but in recent years, interest has grown in their use in other forms (e.g. flour, concentrate, isolate). Pea (*Pisum sativum L.*) seed proteins are particularly rich in lysine and have a starch content close to cereals and a nitrogen content intermediate between soybean and cereals. Therefore, adding pea to industrial feed formulations is of relevance for pigs, ruminants and poultry. Given the differences between the digestion of feed by ruminants and monogastrics,[3] it is important to distinguish flours and grains that are adapted for either group of animals. This is particularly important in terms of particle size,[4] primary cell wall content[5] and biomechanical properties[6] which influence feed digestibility. However, in pea, published information on the final size (weight) of seeds[7,8] and on the size and characteristics of cotyledonary cells[9] is scant. In a previous study with 35 pea genotypes, Roche *et al.* determined the morphometry (number, surface, volume) and the wall thickness of cotyledonary cells isolated enzymatically (with Pectinase, see below) from mature seeds without affecting their shape, and were able to group the genotypes into different classes according to these parameters.[10] Interestingly, we verified several differences in the ranking of genotypes analyzed either in terms of their belonging to a particular class or within a same class of size. These differences, which concerned surface versus volume of cells, might be ascribed to the cell shape, and prompted the studies described here, where the main goal was to devise a reliable and robust shape coefficient (SC).

Materials and Methods

Pea (*Pisum sativum L.*) genotypes D 265, RIL156, Ballet, Princess and V380 were studied. Ground seeds were weighed and disinfected (ethanol 70% for 1 min, then NaOCl [2% active Cl$_2$] for 15 min, with three sterile milliQ water rinses) and, in order to disaggregate the ground particles, three different treatments without any observable effect on cell shape were applied: i) T, no treatment at all; ii) H, an acid hydrolysis (HCl 1 M, at 60°C, for 45 min); iii) H+P, the same acid hydrolysis followed by an enzymatic treatment as used to digest cotyledons (Pectinase 1% [w/v] dissolved in 0.2 M sodium acetate buffer plus 0.2M D-sorbitol, pH 5.5, at 37°C, with gentle shaking, for 2 h).

Given the very large number of measurements taken for each genotype, only one grinding grid was used (3 mm size) and particles recovered from 125, 160 and 200 μm sieving were analyzed.

The above preparations were used to study cell surface characteristics using Calcofluor White-stained cells using UV microscopically.[11] Microscopic observation of slides (2-D) was facilitated by this stain that labels cellulose by covalent binding at the β1-4 bonds of the cellulose molecule. The fluorescence can then be measured and cell surface dimensions determined using image acquisition programmes (ArchimedPlus and Histolab, Microvision, France).[4]

Cell volume was determined in 3-D by using a Multisizer II Coulter (EPICs) granulometric densitometer. This apparatus transforms the objects counted in a sphere, and determines their volume as the volume displaced following Archimedes principle. Hence, microscopically, the true shape of cells is assessed but only in 2-D, while with the microdensitometer above their 3-D image is considered.

A shape coefficient was devised and used in the characterization of the five tested pea genotypes, which were also analyzed for the same morphometric traits after seed grinding. This shape coefficient (SC) was calculated as:

$$SC = \frac{\sqrt{a^2 - b^2}}{a}$$

where, 'a' represents half the length of the cell along its longest axis and 'b' half the length of the cell at its shortest axis (Figure 1).

For each genotype, at least 250 cells were analyzed, experiments were repeated three times, and data were analyzed by one-way ANOVA (P=0.05),[12] with the significance of results among genotypes confirmed by LSD, and comparison of cell surfaces carried out by Student's t-test.

Results

Differences in cell shape between genotypes were apparent, and it was possible to distinguish between round and elongated cells. These, however, remain difficult to quantify. Thus, particularly for genotype Princess where the largest discrepancy was apparent between cell surface (Figure 2A) and cell volume (Figure 2B), it appeared that this could easily be explained by taking into account the elongated shape of its cells.

However, when measuring cell volume, a Coulter densitometer will assimilate all objects to a sphere and there is therefore the risk that two small cells stuck to each other might be counted as a single larger one. Conversely, when the cell surface is measured microscopically, the operator cannot assess cell depth and
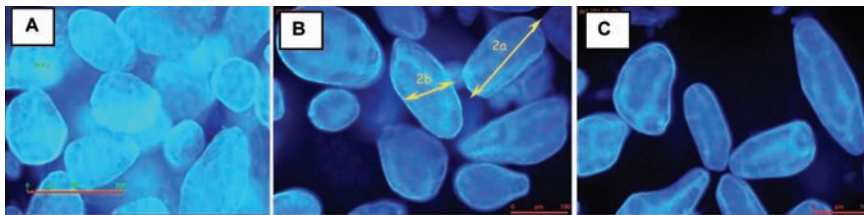
Figure 1. Typical cell shapes of genotypes D265 (A), RIL156 (B) and Princess (C). Arrows in (B) indicate the position of 'a' and 'b' used for calculation of the Shape Coefficient. Scale bars are 200 µm in (A) and 100 µm in (B and C).
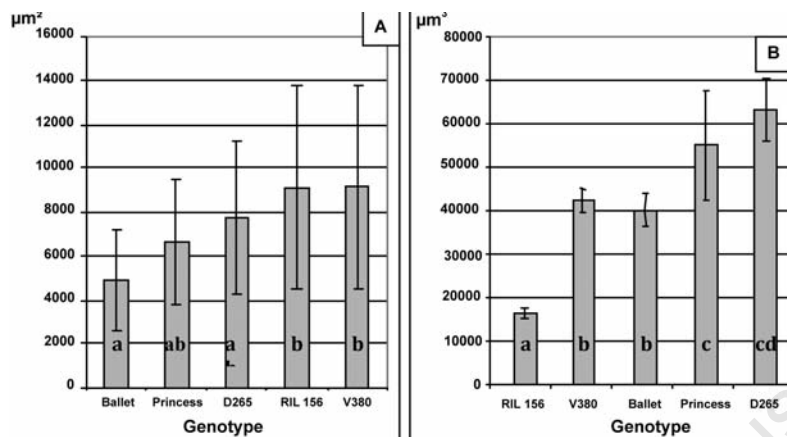

Figure 2. Surface (A) and volume (B) of cotyledonary cells of five pea genotypes. Data are expressed as the mean ± SE. Different letters within bars indicate significant differences (P=0.05).
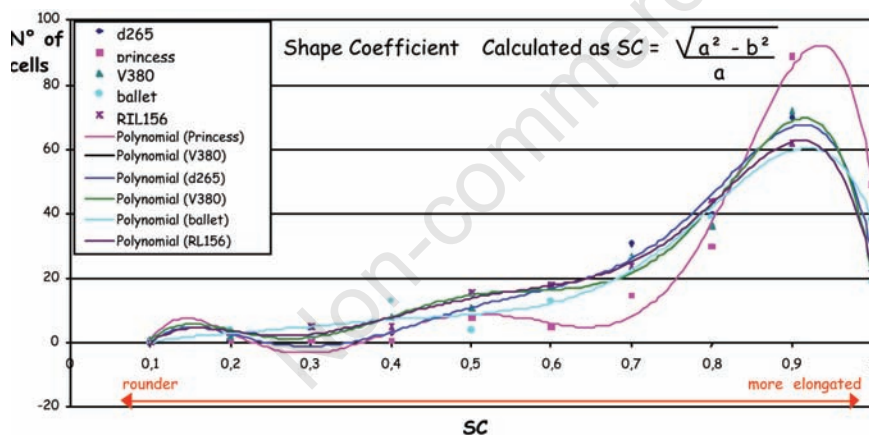

Figure 3. Shape coefficient (SC) at 0.1 intervals and polynomial regression curves for all genotypes.

this could cause bias in the data. This can explain the variability in figures for cell surface in a given genotype (Figure 1A). In addition, it is not uncommon that different cell shapes co-exist within a genotype, as shown in Figure 2.

The shape coefficient, SC, developed was able to distinguish the cell shape in all five genotypes (i.e. round versus elongated, as the SC value is close to 1.0 for elongated cells and lower than 0.5 for rounder ones; Figure 3).

For calculation of the polynomial curves in Figure 3, individual sections for each point in the curve were established at 0.1 intervals of the SC, which resulted in the generic equation $y = -x^6 + x^5 - x^4 + x^3 - x^2 + x - c$.

Against this background, the same five genotypes were analyzed for the morphometric parameters (cell surface, cell volume), using ground seeds. The results of mean cell volume are illustrated in Figure 4. The cell volume values obtained from intact mature seeds of the

same genotypes in a previous study[10] are also included as for reference (H+P coty). It is important to state that only particles of a size coherent with that of pea cotyledonary cells (i.e. between 39.66 and 179.7 µm) were considered, in order to avoid any bias from any cell debris that might have been present in the preparations.

In general, the genotype Ballet retained its ranking irrespective of the method used for particle disaggregation in the samples. However, the genotype Princess yielded smaller cell volumes than V380, contrary to results from intact mature seed cotyledons. The most strikingly heterogeneous data were observed for the genotype D265, which had the smallest volume of cotyledonary cells in former studies, but exhibited the largest volume for particles recovered after grinding through grids of 125 and 160 µm (T125 and T160), reflecting heterogeneity in particle shape. Secondly, the most reliable results are obtained when measurements are performed on samples that have been disaggregated both by hydrolysis and enzymes. It is with this treatment that results are most consistent, whatever particle size grid used to recover the ground samples. Data for genotype RIL156 are not coherent between ground and intact seeds, but when applying the SC calculated this can be explained by the heterogeneity inherent to this genotype in terms of cell shape, as it contains similar proportions of round, oval and elongated cells (cf. Figures 2B and 3).

Discussion

Morphological diversity has the potential to provide useful biodiversity by emphasizing essential aspects of diversity that are not necessarily picked up by taxonomic or phylogenetic indices, and the comparison between different aspects of diversity is frequently essential to understand the processes underlying the patterns of biodiversity observed,[13,14] for instance when describing genetic novelties such as those obtained via chemical, physical or insertional mutagenesis.

Among the most frequent problems for the microscopic and volumetric analysis of intact plant cells, shape is probably the most important and the most widely used.[5,14] Since plant cells (as opposed to animal ones) are rarely spherical they are difficult to measure. This is problematic when fluorochrome signals have to be used, as is the case in flow cytometry,[15] image analysis[14] and cell surface[4] or volume measurements.[10] One solution is to produce protoplasts, i.e. cells deprived of their walls which, being spherical, will not disturb the light trajectory as irregularly-shaped objects do.[15] This, however, requires a preparation that
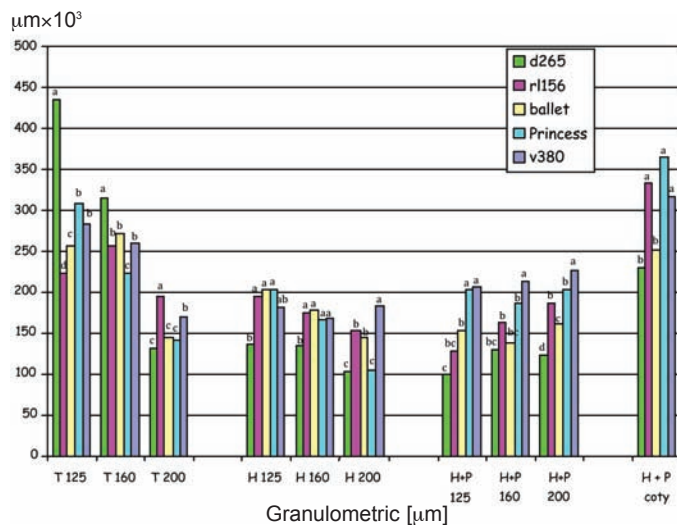
μm×10³

Figure 4. Mean volume of ground particles (diam. between 39.66 and 179.7 μm) for five genotypes, three granule size classes (125, 160 and 200 μm) and three treatments (T [none = control], H [hydrolysis], H+P [hydrolysis + pectinase]) per granulometry (i.e. T125, T160, T200, H125, H160, H200, H+P125, H+P160, H+P200), compared to the mean volume of cotyledonary cells (H+P coty) as reported in Roche *et al.*[10] Bars of genotypes with different letters within each treatment were significantly different at P=0.05 (n ≥ 50 and three independent experiments).

is far more difficult, long and costly than that of cells and with a composition of enzyme solutions for tissue digestion that is strongly dependent on genotype and tissue source, on developmental stage and, more importantly, that requires the use of osmotica so that isolated protoplasts will not burst. Unfortunately, such osmotic agents will also alter the original shape of cells by rendering them turgescent and concomitantly bias the results of such analyses.

Taxonomic patterns may mask other spatial patterns of morphological, functional or phylogenetic biodiversity.[13] Many published articles on the nature of biodiversity set on with the recognition that the richness of a species is only one way of measuring biodiversity and, yet, proceed to treat it as if it were its whole measure.[13,14] This is probably due to the relative success of different methods to measure and describe the shape of biological objects, plant cells in particular,[8,14] and the lack of reliability in the classing of different species or genotypes within a species when different methods are used to assess their morphometry.

The present results show the importance of shape as an additional parameter for cell characterization, particularly for the identification of pea ideotypes better adapted as feed for either monogastrics or ruminants. More gen-erally, the shape coefficient we devised should also find use in other studies (e.g. taxonomy) and with other species, where the non-sphericity typical of plant cells might bias analyses, such as in flow cytometry[15] or quantitative microfluorometric studies,[4] and also for the validation of gene function of genotypes modified genetically (by insertional or chemical mutagenesis) for genes involved in embryo development and seed filling, where a reliable characterization of the cotyledonary cells in their seeds will be required.

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