Flow cytometry a useful tool for measuring ploidy in *Passiflora* breeding programs.

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Flow cytometry is used to analyse the physical and chemical characteristics of particles in a fluid as it passes through a laser. It is a fast and simple method of analysis which involves measuring the fluorescence intensity of the cellular components after they have been labeled with a fluorescent dye. On exposure to the laser they emit light of various wavelengths. This article explores the methodology and the use of flow cytometry in passionflower breeding.

Flow cytometry applied to the study of plants

Flow cytometry is a technique useful both for research with plants from natural populations that have had no human intervention and for commercial crops and plants that are part of a breeding program. The most widespread studies using flow cytometry in plants have been to analyse the genetic material that is contained in the cell nucleus.

In natural populations, this method is used to estimate the amount of DNA and its composition to evaluate genetic variability, sometimes revealing plants to be either polyploids or aneuploids. Plants are often diploid, having two pairs of each chromosome, the structures containing DNA in the nucleus of cells. In comparison polyploids may have gained one complete set of chromosomes (triploid), two sets (tetraploid) or even more. Aneuploids on the other hand have lost or gained individual chromosomes from the diploid state.

Flow cytometry was used as part of our breeding programme to confirm the induction of polyploidy by detecting the multiplication of the number of chromosomes artificially and their stability.

Sample preparation

Flow cytometry with plants requires the cell nuclei to be freely suspended in solution. Thus sample preparation involves steps to extract the nuclei of living cells.

The tissue commonly used in this type of analysis is the leaf but other tissues can also be used such as roots, pollen, stems and seeds.

The tissue is chopped (**Fig. 1a** and **1b**) and passed through special filters leaving the cells nuclei in suspension and catching the debris that could clog the fine conducting tubes of the flow cytometer (**Fig. 1.c 1.d**). With the nuclei suspension ready, samples are chosen for the various fluorescent stains (**Fig. 1.e**) to be applied. (**Fig. 1.e**) There are stains for four DNA base pairs (adenine, thymine, cytosine and guanine) which are used to determine the total amount of DNA. For example, propidium iodide is a stain used to label the nuclei in red (**Fig. 2.a** and **2.b**). Other stains include DAPI (4',6-diamidino-2-phenylindole) dye which stains adenine and thymine bases to blue (**Fig. 2.c** and **2.d.**). The stained sample of nuclei is then ready to be analyzed in the flow cytometer (**Fig. 1.f**).

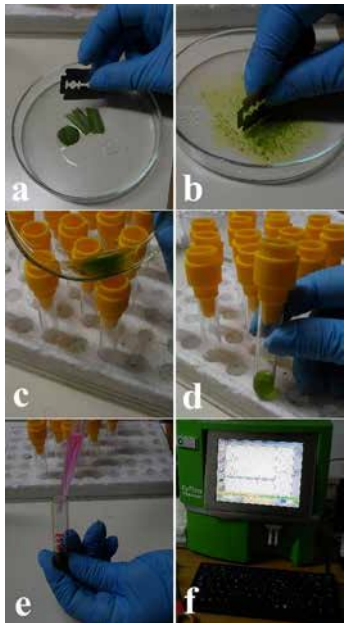

Fig. 1: Plant sample preparation for analysis by flow cytometry.
a: leaf portions of the control and plant to be analyzed; **b:** chopping up of the sample and control plants in the extraction liquid; **c** and **d:** filtration of the nuclei suspension; **e:** incorporation of propidium iodide stain (total DNA); **f:** the sample analysis in the flow cytometer.

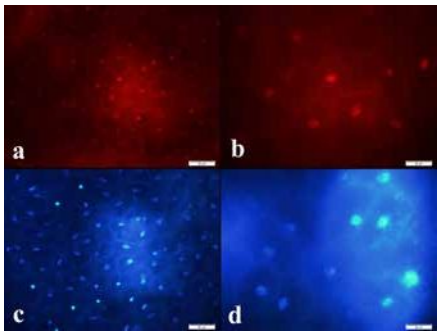

Fig. 2: Cell nuclei of the leaf of *Passiflora alata* stained with fluorescent dyes.
a and **b:** nuclei stained with propidium iodide (staining all four DNA bases); **c** and **d:** DAPI stained nuclei (stained bases adenine and thymine).

Operation of flow cytometer
Once prepared, the sample of stained nuclei is injected into the flow cytometer. This device has a series of tubes that that lead single nuclei one by one past the 'interrogation point' where each nucleus is scanned with a laser to excite the fluorescent dye in the DNA, emitting photons that are collected by an optical system. The optical signals are converted into electronic signals, converting the data for graphical analysis on the cytometer screen.

Results and interpretation
Each sample analyzed produces a histogram displayed by the flow cytometer, showing the number of nuclei that passed through the interrogation point and their fluorescence intensity. The fluorescence intensity produces a peak in the histogram with a value (**Fig. 3**). If a nucleus is twice the size of another, its fluorescence is doubled, since this value is directly proportional to the amount of stained DNA. Depending on the purpose of analysis, the fluorescent dye selected can be total, dyeing all of the four bases of DNA, or partial dyeing only two bases.

Estimation of total nuclear DNA amount
To determine the total DNA amount of a passionflower all the bases of the DNA are stained. For this purpose, as stated above, propidium iodide can be used to stain all four DNA bases. In addition, it is necessary to incorporate a control plant with a known DNA amount. Portions of the two plants are chopped at the same time (**Fig. 1a** and **1b**) and the amount of DNA in the control plant is used to estimate the amount in the other plant. For example, to estimate the amount of DNA of *Passiflora amethystina*, *Hordeum vulgare* cv. "New Golden", a barley cultivar with a known amount of DNA (10.4 picograms) was used. The resulting histogram is shown in **Figure 3.** In the sample, 4226 cores of *P. amethystina* were counted with an average fluorescence of 4875, while for *Hordeum vulgare* cv. "New Golden" 665 cores were counted with an average fluorescence of 13347. Using the fluorescence data the obtained DNA amount can be calculated, in this case *P. amethystina* has 3.79 picograms of DNA.

Confirmation of hybrid plants
In plant breeding, hybridization between closely related species is used to try to combine the best features of both parents in their offspring, if possible in the first (**F1**) generation. Flow cytometry with a partial staining can confirm the success of the cross between two plants if they have different amounts of DNA. As every male pollen donor gives the offspring plant half the genetic material from the nucleus, then the hybrid will have an intermediate amount between both parents.

For example, *P. alata* (**Fig. 4a**) and *P. caerulea* (**Fig. 4c**) were crossed to evaluate the possibility of obtaining the flower color of the first combined with low temperature tolerance of the second. Success in hybridizing can be noted by color of the hybrid flower (**Fig. 4-b**), flow cytometry can confirm that there has been a successful cross long before the blooms (**Fig. 4d**).

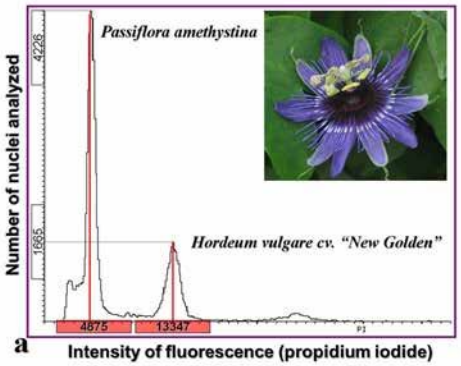

Fig. 3: Histogram for the estimation of the amount of DNA of *Passiflora amethystina* by flow cytometry (total fluorescent staining, propidium iodide).
Formula: (DNA of *P. amethystina* = *Hordeum vulgare* cv. "New Golden" DNA x *P. amethystina* fluorescence/b *Hordeum vulgare* cv. "New Golden" fluorescence)
Data: DNA *Hordeum vulgare* cv. "New Golden" = 10.4 picograms;
P. amethystina fluorescence = 4875
Hordeum vulgare cv. "New Golden" fluorescence = 13347
Result: DNA *P. amethystina* = 10.4 × 4875/13347 = 3.79 picograms

Detection of induced polyploidy
The induction of polyploidy by chemical means, is used in breeding programs as doubling the amount of DNA of a plant will increase the size of its cells. This may lead to stronger rapid growing thicker root systems, vigorous plants, striking sturdier foliage, bigger flowers with wider petals and sepals, flowers that stay open longer and superior hardiness. Treatment is not always successful and it is desirable to have a method to confirm whether a plant is polyploid or the attempt failed.

Flow cytometry can detect polyploidy because a plant with twice the amount of DNA per nuclei will have twice fluorescence. For example, in the hybrid (*P.* 'Amethyst´ x *P. caerulea*) x *P. amethystina* a treatment was applied to obtain larger flowers (**Fig. 5.a**). The results were evaluated by flow cytometry. Histograms of untreated plants and treated unsuccessfully showed fluorescence value 50 (**Fig. 5.b** and **5.c**) while this value for induced polyploids was 100, twice the previous (**Fig. 5 d**).

Future of flow cytometry in plants
While flow cytometry has been a useful tool in the determining of the ploidy of plants in the breeding process, there are still unstudied applications. Future research, in both genetics and plant physiology, will surprise us with new uses for this technique.

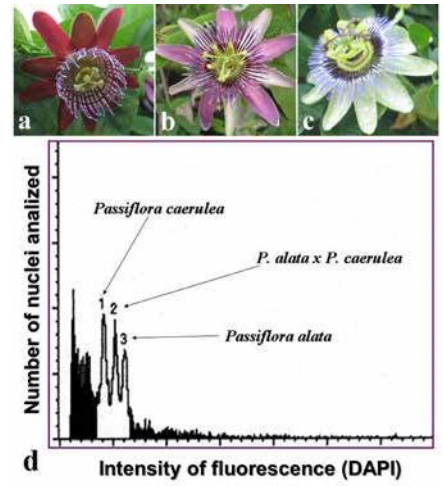

Fig. 4: Detection by flow cytometry of a hybrid of *Passiflora alata* and *Passiflora caerulea* (DAPI fluorescent staining partial).
a: *P. alata*; **b:** interspecific hybrid *P. alata* x *P. caerulea*; **c:** *P. caerulea*; **d:** histogram of the number of nuclei analyzed according to their DAPI intensity of fluorescence for a combined sample of *P. alata* (♀) + *P. caerulea* (♂) + *P. alata* x *P. caerulea* (hybrid).

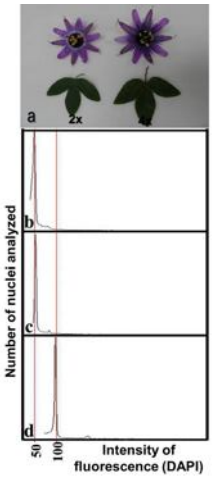

Fig. 5: Detection of induced polyploids by flow cytometry.
a: Flower and leaf of the diploid hybrid (*P.* 'Amethyst´ x *P. caerulea*) x *P. amethystina* (2x) and its induced polyploid (4x); **b, c** and **d**: flow cytometry histograms for untreated plants (2x); unsuccessfully treated plant (2x) and obtained polyploid plant (4x), respectively.

Literature on the subject

Bugallo, V; Pannunzio, M J; Cardone, S; Facciuto, G. 2011. Breeding Advances in *Passiflora* Native to Argentina. Floriculture and Ornamental Biotechnology, Global Science Books, UK. 5 (1): 23-34.

Dolezel, J; Greilhuber, J; Suda, J. 2007. Flow cytometry with plant cells: analysis of genes, chromosomes and genomes. Wiley 1-454.

King, LA; Irvine, MS. 2010. Investigation of *Passiflora* hybrids using flow cytometry. *Passiflora* 20(1): p*P.* 5-11.

Orchatt, S J. 2008. Flow cytometry in plant breeding. Cytometry (73 A) 7: 581-598.

Souza MM, Palomino G, Pereira TN, Pereira MG, Viana AJ? 2004. Flow cytometric analysis of genome size variation in some *Passiflora* species. Hereditas 141(1):31-8.