Improved Procedures to Assess Plant Protoplast Viability: Evidencing Cytological and Genomic Damage

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Summary Plant protoplasts are valuable in biotechnology, enabling from plantlet regeneration to the determination of gene function. Viability tests are required in protoplast applications to determine the rate of viable cells, allowing to decide on the most adequate isolation and purification procedures and to verify whether sufficient cells are available for subsequent steps. Fluorescence microscopy is usually employed for this purpose. However, obstacles have pointed out: the long time required to count a relatively small number of protoplasts, cell clumps preventing their observation, and the observer’s subjective visual perception of the fluorescence. Therefore, this study aimed to establish procedures for the viability test adapted for flow cytometry (FCM), Muse cell analyzer (Muse) and Comet Assay (CA). Capsicum annuum L. was chosen as a study species based on the recalcitrant morphogenic nature of its protoplasts. After isolation and purification, the FCM and Muse allowed assessing large numbers of protoplasts, and the CA the protoplast nuclei within a short period. Using the adjusted procedures, various cytological characters could be evidenced by FCM and Muse, and different levels of genomic damage evaluated by CA, allowing the discrimination and measurement of the viable protoplasts. Considering these results, the present study introduces improved quantitative procedures for viability tests. Moreover, aiming at plantlet regeneration, distinct applications should employ to measure the protoplast viability and thus define the most adequate isolation and purification procedures. Contributing with this purpose, guidelines present here to adequate and standardize the laboratory conditions for protoplast viability test using FCM, Muse and CA.

Key words Flow cytometer, Muse cell analyzer, Comet assay, Tissue culture, Viability test, Protoplast culture.

Protoplasts are isolated, "naked", cells whose cell walls are removed by mechanical or, as in most cases, enzymatic pool treatment (Klercker 1892, Cocking 1960). If viable, the cells can dedifferentiate under in vitro culture conditions, becoming totipotent (Cocking 1972, Eeckhaut et al. 2013). Besides the synthesis of new walls, these totipotent cells also resume the cell cycle, giving rise to proembryogenic masses so-called calli (Nolan and Rose 2010, Neelakandan and Wang 2012). Some cells of a callus acquire competence, following a different morphogenetic route, organogenesis or embryogenesis, according to the influence of genetic, epigenetic and physiological features, as well as the environment in vitro conditions (Fehér et al. 2003). Thus, totipotent protoplasts are considered valuable for somatic hybridization, cybrid generation (Pelletier et al. 1983) and genetic transformation (Rhodes et al. 1988). Furthermore, protoplasts are flexible for studies in polysaccharide biosynthesis during cell wall regeneration (Klein et al. 1981), signal transduction (Sheen 2001), intracellular traffic vesicles (Faraco et al. 2011), membrane physiology (Ma et al. 2015), temporal gene transcript profiling (Chupeau et al. 2013), and determination of gene function from transient expression analysis (He et al. 2016). In spite of its many applications, protoplast recalcitrance due to lack of totipotency renders plantlet regeneration impossible in some species (Davey et al. 2005). Protoplast viability, the living cells without the cell wall, is influenced by genetic and epigenetic factors, besides the chronologic, physiologic and ontogenetic age of the explant. The viability also affected by cell isolation stress generated in the enzymatic pool treatment usually applied for cell wall removal (Papadakis et al. 2001, Neelakandan and Wang 2012). During the isolation process, oxidative compounds such as hydrogen peroxide and superoxide can be accumulated (Papadakis et al. 2001), affecting membrane integrity through the attack to ester bonds of the phospholipids (Niehaus 1978). Another limiting aspect in obtaining viable protoplasts is the osmolality during pre-plasmolysis, cell isolation and in the propagation media. Besides, controlled physical conditions (as temperature, photoperiod and light qual-
ity) are required to maintain the cell viability in protoplast cultures (Neelakandan and Wang 2012).

In viability tests, fluorochromes are frequently used to discriminate between living and dead cells (Kepp et al. 2011). Fluorescein diacetate (FDA) is widely employed for this purpose (Larkin 1976). This fluorochrome penetrates into living and dead cells, allowing their differentiation after fluorescein cleavage by cellular esterases, which are only present in viable cells (Schoor et al. 2015). Esterases cleave an acetate residue of fluorescein, promoting the emission of green fluorescence (Widholm 1972). However, Zilkah and Gressel (1978) considered the observation of FDA fluorescence using microscopy to be subjective, varying among analysts due to the leakage of esterases and fluorescein from defective cells into the external medium. Furthermore, a long time is necessary for the evaluation of protoplast viability using microscopy (Aoyagi 2011).

Differently, from fluorescence microscopy, FCM is a rapid, objective and multiparametric application. FCM histograms and dot plots show subpopulations, which are denominated as subclusters, defined by intrinsic optical parameters of cellular constituents, such as cell wall and chloroplast, and/or optical properties of the fluorochromes (Shapiro 2007). In FCM, other fluorochromes have been used besides FDA, employing their optical parameters of excitation and emission λ, and principles to discriminate between living and dead cells. Propidium iodide (PI), 4′,6-diamidino-2-phenylindole (DAPI) (Kepp et al. 2011), and 7-amino-actinomycin D (7-AAD) (Zimmermann and Meyer 2011) do not cross the intact plasma membrane of living cells. Hence, populations of dead cells showing PI, DAPI or 7-AAD fluorescence are separated in FCM, due to membrane permeability and the binding of these fluorochromes to the DNA (Zimmermann and Meyer 2011, Adan et al. 2016). Apart from these fluorophores, also the red endogenous autofluorescence of chlorophyll has been explored for viability tests using FCM (Coury et al. 1995, Guzzo et al. 2002).

Whilst most use the fluorescence microscopy, FCM has been applied for viability test of protoplasts from macroalgae (Coury et al. 1995), bacteria (Amor et al. 2002), phytoplankton (MacIntyre and Cullen 2016), and human lymphocytes (Vermes et al. 1995, Włodkowic et al. 2011, Sauvat et al. 2015). However, protoplast viability assessments using FCM are scarce in angiosperms. This application has been fundamental for screening and sorting of Zea mays L. protoplasts transgene for the green fluorescent protein (Galbraith et al. 1995). In protoplast suspensions of Nicotiana plumbaginifolia, FCM was used to relate chromatin condensation and DNA fragmentation to apoptosis (O’Brien et al. 1998). FCM was applied to discriminate and sort protoplast subpopulations of Daucus carota L., showing subpopulations with distinct morphogenic potential (Guzzo et al. 2002). Thus, FCM is an important application to screen and select viable protoplasts exhibiting cellular, morphological and/or physiological features with potential for other areas.

Besides FCM, the Muse, a compact flow cytometer (Merck Millipore Corporation 2013), has also been used for viability test of human (Ueda et al. 2013, Marusik et al. 2014) and fish cell suspensions (Nynca et al. 2016). The data obtained with Muse show the high correlation to conventional FCM, with the advantage of rapid detection of cellular samples even from a minimal cell suspension volume of only 200 μL (Merck Millipore Corporation 2011).

Another method that can be adapted for protoplast viability test is the Comet Assay (CA), which evidences damage in nuclear DNA (Kuzminska et al. 2016). CA, or single cell gel electrophoresis assay, is a relatively sensitive, rapid quantitative tool that assesses the DNA damage in eukaryotic cells (Collins 2004, Zhang et al. 2011). CA was first described by Ostling and Johanson (1987) to assess DNA damage of murine lymphocytes subjected to gamma radiation by neutral electrophoresis. The methodology using neutral electrophoresis identifies single DNA strand breaks. Subsequently, neutral electrophoresis was replaced by alkaline electrophoresis, being applied to human lymphocytes exposed to X-rays (Singh et al. 1988). The main advantage of using alkaline CA is the detection of single and double DNA strand breaks (Afanasieva et al. 2010).

Considering the relevance of protoplasts in the biotechnology era and the subjectivity that may exist during viability tests based on fluorescence microscopy, this study introduces rapid, reliable and reproducible quantitative procedures to evaluate protoplast viability using FCM, Muse and CA. For this, C. apsicum annuum was chosen as model owing to its severely recalcitrant morphogenetic nature, with the inability to respond under in vitro culture conditions, and constant efforts made to explore its morphogenetic potential and regeneration (Kothari et al. 2010).

Materials and methods

Plant material and in vitro plantlet growth

C. annuum L. ‘Itapuã 501’ (Solanaceae) seeds were disinfested in laminar flow chamber using 70% ethanol for 1 min, followed by a volume of 50% of 2.0–2.5% NaOCl with one drop of Tween 20 (Merck KGaA) for 20 min. Next, the seeds were rinsed with sterilized distilled water (dH2O) for three times of 5 min, then dried on sterile filter paper. The seeds were germinated in glass jars containing 100 mL of half-strength MS basal salts (Sigma-Aldrich) supplemented with 10 mL L−1 MS vitamins (Murashige and Skoog 1962), 30.0 g L−1 sucrose, 0.1 g L−1 myo-inositol and 6.5 g L−1 agar (Sigma-Aldrich). Prior to autoclaving, the pH was adjusted to 5.6.
and the medium was sterilized for 20 min at 120°C and 1.5 atm. The cultures were kept in a growth room under 25±2°C and photoperiod cycle of 16/8 h of the light/dark.

**Protoplast isolation and purification**

After three weeks, the leaves were used for protoplast isolation and purification. In laminar flow chamber, 0.5 g of leaves were transversely cut into strips with a sterile scalpel in a Petri dish of 60×15 mm, and maintained in 15 mL of autoclaved 13M cell protoplast washing solution (CPW): 0.272 g L⁻¹ K₂HPO₄, 1.01 g L⁻¹ KNO₃, 14.08 g L⁻¹ CaCl₂·2H₂O, 2.46 g L⁻¹ MgSO₄·7H₂O, 0.0016 g L⁻¹ KI, 0.0003 g L⁻¹ CuSO₄·5H₂O, and 130 g L⁻¹ mannitol (Sigma-Aldrich), pH 5.6. All tissues were pre-plasmolyzed in CPW solution for 1 h at 25±2°C and 40 rpm, in the dark. The 13M CPW solution was discarded using a Pasteur pipette, and 15 mL of 13M CPW supplemented with 1.5% (w/v) cellulase Onozuka R-10 (Yakult), 0.5% (w/v) Macerozyme R-10 (Yakult) and 0.25% (w/v) Driselase (Sigma-Aldrich) were added (pH 5.6). Prior to use, the pool was centrifuged at 3000 rpm for 5 min, the pellet was discarded, and the supernatant filter-sterilized through 0.22 µm millipore filter (Milllex). The leaves were incubated in the same medium at 25±2°C and 40 rpm for 10 h, in the dark. Subsequently, the protoplasts were harvested by filtering through a 70 µm nylon cell strainer (BD Falcon); the filtrate was collected using a Pasteur pipette and transferred to 15 mL conical tube. The filtrate was diluted by addition of 15 mL of 13M CPW, and centrifuged (Excelsa II, model 206 MP, Fanem) at 700 rpm for 5 min. The debris and the solution were removed using a Pasteur pipette, and the pellet was resuspended in 15 mL of 13M CPW, with two repetitions for purification using the modified procedure of Anthony et al. (1999). After isolation, images of freshly isolated protoplasts were captured using an inverted phase-contrast microscope IX70 (Olympus). The protoplast isolation and data analysis below were accomplished in six randomized days.

**Protoplast viability analyses in FCM and Muse**

Two treatments were performed for viability test in FCM: 1.5 mL of unstained protoplast suspensions (control) and 1.5 mL of protoplast suspensions stained with 0.2 µM FDA for 5 min in the dark (Guzzo et al. 2002). For each treatment, eight repetitions, each presenting over 10000 protoplasts, were evaluated in a Flow Cytometer III Partec equipped with UV lamp (388 nm) and laser source (488 nm). The autofluorescence emitted by chlorophyll a (max. ca. 670 nm; Cerovic et al. 2002) was collected through RG 610 nm filter, while FDA fluorescence (max. ca. 497 nm; Thermo Fisher Scientific) was collected through an EM 520-nm band-pass filter. Dot plots were generated from FL-1 green and FL-3 red parameters using the FlowMax software (Partec). Dot plots of log 90° light-scatter versus log green fluorescence, and log red fluorescence of chlorophyll versus log green fluorescence were created for a total of ca. 10000 protoplasts. The verification of proper FCM alignment and instrument performance was done by checking the data obtained from running calibration beads of 10 µm (F13838, Life Technologies).

Protoplast viability was also evaluated by the Muse Cell Analyzer (Merck Millipore) equipped with a laser source (532 nm) through a red filter (680 nm) for fluorescence emission of protoplasts stained with PI (max. ca. 620 nm; Thermo Fisher Scientific). For this analysis, 0.2 mL of unstained protoplast suspension (control) and protoplasts stained with 7.48 µM PI for 5 min in the dark (Watanabe et al. 2002) were used. Eight repetitions were performed for each treatment, with about 5000 protoplasts collected per microtube. Fluoresbrite microspheres (10 µm diameter) were also used for calibration and setup. The protoplasts evaluated in Muse were analyzed by the Flowing software version 2.5 (Perutto Terho, Centre for Biotechnology, Turku, Finland).

**Protoplast viability by alkaline CA**

All steps described below were carried out in the dark to prevent additional DNA damage. CA was accomplished using protoplasts (sample), nuclei (negative control) and nuclei treated with hydrogen peroxide (positive control). Immediately after isolation and purification, the protoplast suspensions were centrifuged (Excelsa II, model 206 MP, Fanem) at 700 rpm for 5 min, the CPW was discarded and 3 mL of phosphate buffer saline (PBS) was added. For positive and negative controls in CA, nuclei suspension was obtained from C. annuum leaves gently sliced and, after, maintained in 100 µL of PBS for 15 min (modified of Gichner et al. 2009).

Clean slides were covered with 1% normal melting point agarose (Sigma-Aldrich) at 50°C, which was solidified for 12 h (Zhang et al. 2011). In the second layer, 22 µL of protoplast suspension or nuclei suspension (controls) were embedded in 88 µL of 0.75% low melting point agarose (Sigma-Aldrich) at 37°C on the slide. Next, each slide was covered with a coverslip in order to ensure a homogeneous distribution. The coverslip was removed after gel solidification at 4°C for 20 min. Only for the positive control, the slides were incubated in 150 µM hydrogen peroxide for 5 min at 4°C in the dark (modified of Collins et al. 1997).

The slides were immersed into lysis solution (2.5 M NaCl, 100 mM ethylenediamine tetraacetic acid (EDTA), 10 mM tris(hydroxymethyl)aminomethane (Tris), 1% Triton X-100 and 10% dimethyl sulfoxide (Sigma-Aldrich), pH 8) at 4°C for 1 h. For DNA denaturation before electrophoresis, slides were placed in a horizontal electrophoresis system containing an alkaline buffer (250 mM Tris, 75 mM NaOH, 10 mM EDTA, pH 13) for 5 min. Electrophoresis was carried out for 18 min at 18 V cm⁻¹ with amperage of approximately 26 mA. Af-
ter electrophoresis, the slides were washed three times in neutralization buffer (400 mM Tris–HCl, pH 7.5; Ojima et al. 2009) at 4°C for 5 min. The slides were stained with 100 µL of 50 mM acridine orange (Sigma-Aldrich) for 15 min and rinsed three times with dH₂O.

CA analysis was performed considering 100 comets per slide, with overlapping comets and comets in areas near the slide edges not being counted. Six slides (replicates) of the protoplasts, positive and negative controls were analyzed using a DP-71 video camera (Olympus), mounted on a BX-60 fluorescence microscope (Olympus) equipped with a stabilized light source, an MPlanApo 20x objective (Olympus). The images were evaluated by a visual method, classifying the comets in five categories, from 0 (no apparent tail) to 4 (largest quantity of DNA in the tail) (Collins 2004, Araldi et al. 2015). A guideline on the protoplast viability test with alkaline CA is shown in Fig. 1.

Results

Physical and chemical in vitro conditions were suitable for C. annuum seed germination, enabling the development of seedlings showing vigorous leaves after three weeks. Protoplast isolation and purification were carefully accomplished under low light to avoid DNA damage. The pre-plasmolysis and isolation procedures provided protoplasts with roundish shape and heterogeneous size ranging from 12 to 38 µm of diameter (Fig. 2). The centrifugation step removed the cellular debris, maintaining a population of intact protoplasts.

The protoplast viability rate was determined for FCM by comparing the results generated from unstained protoplasts, taking advantage of the chlorophyll autofluorescence. A single cluster emitting a red autofluorescence (max. emission ca. 670 nm) signal derived from the chlorophyll a (max. excitation ca. 370 nm) (Fig. 3A). Based on this control FCM dot plot, two quadrants were previously defined: the lower exhibiting all of the cells, and the upper without cells (Fig. 3A). Considering the same position of the quadrants previously delimited (Fig. 3A), the FCM dot plots obtained from FDA-stained protoplasts were analyzed. The FDA-stained protoplast suspensions (max. excitation ca. 497 nm) also exhibited a higher level of green fluorescence (max. emission ca. 517 nm), being segregated as a second cluster of protoplasts (Fig. 3B). This way, two subpopulations were found in all FCM dot plots provided by FDA-stained protoplasts, being that the viable protoplasts were bound to the upper quadrant, while unviable protoplasts remained in the lower quadrant (Fig. 3B). This enhanced green fluorescence is consistent with the supposition that the upper cluster contains viable protoplasts. The viability counts were performed from eight repetitions with each over 10000 protoplasts in three distinct days. The percentage of the total number of cells for the quadrants (upper and lower) was determined, showing a mean of 65.22% of viable and 34.78% of unviable cells.

Similarly, to FCM, the viability test in Muse also allowed screening viable and unviable protoplasts. Unstained protoplast suspensions generated one single cluster based on red chlorophyll a autofluorescence (Fig. 4A). From this control Muse dot plot, initially, two quadrants were determined: the left quadrant composed by all protoplasts and the right quadrant with 0%. In contrast, the PI-stained (max. excitation ca. 533 nm) protoplast suspensions provided dot plots evidencing other populations. So, the number of quadrants was revisited and adjusted by four quadrants (Fig. 4). The upper left quadrant refers to the population of unstained viable protoplasts, which emitted through autofluorescence of...
chlorophyll \( \alpha \), while the PI-stained unviable protoplasts remained in upper right quadrant (Fig. 4B). Cellular debris was limited to the lower left quadrant. The viability rate was also calculated from eight repetitions with each over 5000 protoplasts in three distinct days. The percentage of the total number of protoplasts for each quadrant was obtained, whereby a mean of 66.20% of viable protoplasts and 33.80% of unviable protoplasts and cellular debris were measured.

Based on all analyses (eight repetitions in three distinct days) from FCM and Muse, the procedures for protoplast isolation yielded a mean percentage of 65.71% viable protoplasts. Considering the debris evidenced in Muse, unviable protoplasts corresponded to 34.29%.

For CA, the proportion \( 88:22 \mu \text{L} \) between the volume of low melting point agarose and that of sample protoplasts or control nuclei suspensions was considered ideal to evaluate the comets, as it enabled the visualization of more than 100 comets without overlapping per slide. The lysis solution allowed clear visualization of the comets by removing autofluorescent chloroplasts and nuclear membrane fragments. The combination of EDTA, NaOH, and Tris in the alkaline buffer favored the preservation of comets. The electrophoretic conditions of time and voltage allowed the comet tail (DNA strand break) to remain attached to the comet head.

Comets were found in all slides, being considered over 100 comets in six slides for each of the three independent protoplast isolation procedures. The comets were classified into five types (0–4) upon visualization of the tail length (Araldi 2015) as 0 (without DNA damage), 1 (low damage), 2 (medium damage), 3 (large damage) or 4 (maximum detected damage). Bar=5 \( \mu \text{m} \).
Discussion

The viability test in plants allowed evaluating large populations of protoplasts, with more than 10,000 for FCM, 5,000 for Muse and 100 for CA in each repetition. For FCM and Muse, the tests were performed within a relatively short time, using stained and unstained protoplast suspensions in the eight independent replicates over each random six-day evaluation periods. The results corroborated that large numbers of cells in suspension can be quickly analyzed in FCM (Adan et al. 2016) and Muse (Merck Millipore Corporation 2011). In comparison, the test conducted using fluorescence microscopy required a long period for observations of a relatively small number of protoplasts. In Capsicum, the viability test was accomplished using FDA after counting 200 cells for each of three replicates (Lindsey and Yeoman 1984).

Besides rapidity and large protoplast sample size, the viability tests by FCM and Muse were reproducible and accurate, as reflected in the standard deviation values and the identical dot plot profiles (Figs. 3, 4) obtained for all replicates and days. Additionally, the objectivity of the analyses was ensured by automatic measurements through the software. The mean percentages of viable and unviable protoplasts were calculated numerically, with graphs generated by the FlowMax software (Partec) for FCM, and the Flowing Software version 2.5 software for Muse. In contrast, the viability test via fluorescence microscopy can be influenced by the individual experience of each observer, resulting in inaccurate data (Zilkah and Gressel 1978, Aoyagi 2011). In addition, some data may be compromised by the presence of cell clumps, in that some cells are not seen, interfering with the rate of viable and unviable cells (Ishikawa et al. 1995).

Evaluation of the autofluorescence emitted by the chloroplasts was essential to provide the control FCM and Muse dot plots (Figs. 3A, 4A). The analysis of unstained protoplasts, exploiting the red autofluorescence emitted by chlorophyll a (COURY et al. 1995), generated a single indiscriminate population containing viable and unviable protoplasts. Similarly, a single cluster emitting a high level of red fluorescence has also been evidenced in the FCM dot plots from unstained protoplasts of Zea mays (Galbraith et al. 1995) and D. carota (GUZZO et al. 2002). Therefore, the control from unstained plant protoplasts should be established beforehand in order to identify viable and unviable protoplasts in the FCM and Muse dot plots generated from the stained population, regardless of the type and action mechanism of the used fluorochromes.

FDA in FCM and PI in Muse were also fundamental for discrimination of viable and unviable protoplast populations (Figs. 3B, 4B). In FCM, non-FDA-stained subpopulations were considered unviable, while in Muse the non-PI-stained subpopulations were interpreted as viable. This difference is related to the fluorescence principle: FDA fluorescence is emitted in viable cells (Widholm 1972), whereas PI stains the nuclei acids of unviable cells (Adan et al. 2016). An alternative to FDA in viability tests is carboxyfluorescein diacetate (eFDA), which also indicates membrane integrity and activity of cytoplasmic enzymes (Amor et al. 2002). PI can also be replaced by DAPI or 7-AAD, which indirectly also show the loss of membrane integrity (Kepp et al. 2011, Zim- 

mermann und Meyer 2011). In keeping with the possibility of using distinct fluorochromes, it is further necessary that the configuration of FCM or Muse is calibrated to verify the excitation source with UV light or laser for the filters of the wavelength emitted by the respective fluorochrome.

Dot plots with high resolution, clustering distinct subpopulations of the C. annuum protoplasts, were shown for FCM and Muse. For all FCM dot plots, two subpopulations were clearly identified, evidencing viable and unviable protoplasts (Fig. 3B). Viable and unviable subpopulations have also been discriminated by exploiting chloroplast autofluorescence together with FDA in macroalgae (COURY et al. 1995) and D. carota (GUZZO et al. 2002) protoplasts. Just as for FCM, the procedure involving Muse proved to be powerful in clustering viable and unviable subpopulations and, additionally, cell debris (Fig. 4B). Based on the results from Muse, the low mean rate (3.95%) of cell debris demonstrated that the isolation and purification of the protoplasts, besides removal of the enzymatic solution, effectively eliminated the cell wall residues, free and fragmented organelles, and nuclei. In protoplast culture, if the percentage of cell debris is high, phenolic oxidation may occur (DAVEY et al. 2010), impairing or even hindering the regeneration of in vitro plantlets from isolated protoplasts.

The mean rates of viable C. annuum protoplasts were 65.22±9.03% for FCM and 66.20±5.79% for Muse. Using fluorescence microscopy and FDA, only PRakash et al. (1997) reported the percentage of viable protoplasts for C. annuum to be 70–75%. Yet, in the latter plantlets were regenerated from mesophyll protoplasts, differently from cotyledon ones. Due to recalciitrant nature, so far protoplasts of the genus Capsicum do not respond to in vitro culture (Kothari et al. 2010, do Rêgo et al. 2016).

Differently, from FCM and Muse, alkaline CA allowed the identification and classification of DNA strand breaks by visualization of comets, which were classified in 1 to 4 (Fig. 5). Based on the controls, negative with 10.00% and positive with 35.00% of comets, the protoplast isolation, and purification procedures caused damage to the DNA structure of C. annuum, a mean of 20.88% of comets. Although the literature does not present an ideal parameter of the percentage of comets in the negative control, it was established as an acceptable
value up to 10%. Therefore, CA revealed DNA structure changes that may be related to the viability of the protoplasts and their recalcitrance in tissue culture. Hence, besides affecting the membrane integrity (Niehaus 1978, Papadakis et al. 2001), the oxidative compounds generated during protoplast isolation and purification can promote DNA damage (Costa et al. 2012). In the initial studies involving CA, the presence of DNA strand break was associated with the early apoptosis process (Olive et al. 1993). Indeed, in the early stages of apoptosis, the chromatin is condensed and the DNA begins to be degraded, while the protoplast’s plasma membrane remains intact (O’Brien et al. 1998). However, Collins (2004) reported that CA does not identify apoptotic cells because the DNA fragments have the size similar to nucleosome oligomers, and may disappear during lysis or electrophoresis. However, this author does not reject the possibility of cells with highly damaged DNA entering the apoptotic process.

Based on the mean 20.88% of comets measured from over 100 comets in six slides for each of the three independent protoplast isolation procedures, 79.12% of the protoplasts showed nucleoids without DNA damage, which were classified as type 0. According to CA, this 79.12% of protoplasts were considered viable cells. This mean value differs from the 65.22% and 66.20% obtained with FCM and Muse, respectively. Therefore, an accurate protoplast viability test should perform using distinct methods, evaluating the integrity of the plasma membrane as well as of the DNA.

In conclusion, the viability test performed using FCM and Muse was objective, accurate, reproducible, and very clear to separate viable and nonviable protoplasts using chlorophyll autofluorescence, combined with FDA to evaluate enzymatic cell activity or PI to assess membrane integrity. Besides FCM and Muse, the use of CA for viability test detected different levels of DNA strand breaks. Considering these results, the protoplast isolation and purification procedures promote cytological disruption shown by FCM and Muse, and genomic damage evidenced by CA that can prevent the in vitro responses associated with plantlet regeneration. As presented here, distinct approaches should adopt to assess protoplast viability in order to define the most adequate procedure. Additionally, guidelines produce regarding CA application (Fig. 1) and comparing the main aspects of the viability tests using the FCM and Muse methodologies (Table 1). From these guidelines, new procedures can be standardized according to specific laboratory conditions.

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References


| Table 1. Guide comparing the main aspects of the viability test using the Muse™ cell analyzer and flow cytometer. |
|------------------------------------------------------|---------------------------------------------------------------------------------------------------------------|
| **Viability test in protoplasts** | **Muse cell analyzer** | **Flow cytometer** |
| Excitation source | Laser 532 nm | UV lamp 388 nm |
| Filter | Red 680 nm | |
| Parametric evaluation | FSC×PL fluorescence | Chlorophyll autofluorescence×FDA fluorescence |
| Fluorochromes | PI–excitation ca. 533 nm emission ca. 620 nm | FDA–excitation ca. 497 nm emission ca. 517 nm |
| Histogram quality | High | High |
| Clustering of the protoplast subpopulations | Yes | Yes |
| Cell debris identification | Yes | No |
| Volume of the protoplast suspension | 0.2 mL | 1.5 mL |
| Protoplasts collected for each sample | ca. 5000 | ca. 10000 |
| Time for analysis of the sample | ca. 1–2 min | ca. 1–2 min |
| Histogram analysis | Flowing software 2.5.1 | FlowMax software |
| Accuracy and reproducibility of the analysis | High | High |
| Cost | Relatively low | Relatively high |


