NATÁLIA LAYANE BADARÓ COSTA

IMPROVED PROCEDURES TO ASSESS PLANT PROTOPLAST VIABILITY: EVIDENCING CYTOLOGICAL AND GENOMIC DAMAGES

Dissertation presented to the Universidade Federal de Viçosa as part of the requirements of the Cell and Structural Biology Graduate Program, to attainment of the title *Magister Scientiae.*

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APPROVED: July 21th, 2017.

Marcia Flores da Silva Ferreira

Wellington Ronildo Clarindo

Carlos Roberto de Carvalho
(Advisor)

Ao meu orientador Prof. Carlos Roberto de Carvalho, ao Prof. Wellington Clarindo, aos meus pais Manoel e Gildelice.

DEDICO

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“Looking down the microscope at disrupted DNA molecules can seem as inspirational as stargazing”.

(Azqueta and Collins 2013)

ABSTRACT

COSTA, Natália Layane Badaró, M.Sc., Universidade Federal de Viçosa, July, 2017. **Improved procedures to assess plant protoplast viability: evidencing cytological and genomic damages.** Advisor: Carlos Roberto de Carvalho.

Plant protoplasts are valuable in biotechnology, enabling the plantlet regeneration until the gene function determination. In all applications, viability test is required to measure the rate of viable protoplasts, allowing to decide on the most adequate isolation and purification procedures and to verify whether there are sufficient cells for subsequent steps. Fluorescence microscopy is usually employed for viability test. However, some problems have been pointed out: long time required to count a relatively small number of protoplasts, cell clumps preventing their observation, and the subjective visual perception of the fluorescence by observer. This study aimed to establish procedures for viability test adapted for flow cytometry (FCM), Muse™ cell analyzer (Muse) and Comet Assay (CA). For this, *Capsicum annuum* was chosen due to recalcitrant morphogenic nature of its protoplasts. After isolation and purification, the applications allowed assessing large numbers of protoplasts (FCM and MUSE) and protoplasts nuclei (CA) in a short time period. From the adjusted procedures, different types and levels of cytological (FCM and Muse) and genomic damages (Muse and CA) were evidenced, allowing to discriminate and measure the viable protoplasts. Considering the results, this study introduces improved quantitative procedures for viability test. Besides of these and aiming the plantlet regeneration, different methods can be applied to assess the protoplast viability, defining the more adequate isolation and purification procedures. Contributing with this purpose, guides were showed for FCM, Muse and CA to standardization of viability tests in plant protoplasts.

RESUMO

COSTA, Natália Layane Badaró, M.Sc., Universidade Federal de Viçosa, julho de 2017. **Procedimentos aprimorados para avaliar a viabilidade de protoplastos de planta: evidenciando danos citológicos e genômicos.** Orientador: Carlos Roberto de Carvalho.

Os protoplastos de plantas são valiosos na biotecnologia, possibilitando desde a regeneração das plântulas até a determinação da função de genes. Em todas as aplicações, o teste de viabilidade é necessário para medir a taxa de protoplastos viáveis, possibiltando decidir os procedimentos de isolamento e purificação mais adequados e verificar se há células suficientes para as etapas subsequentes. A microscopia de fluorescência geralmente é empregada para o teste de viabilidade. No entanto, alguns problemas têm sido apontados: longo tempo necessário para contar um número relativamente pequeno de protoplastos, aglomerados de células que impedem a observação dos protoplastos e percepção visual da fluorescência subjetiva ao observador. Este estudo teve como objetivo estabelecer procedimentos para teste de viabilidade adaptado para citometria de fluxo (FCM), Muse™ cell analyzer (Muse) e Ensaio cometa (CA). Para isso, *Capsicum annuum* foi escolhido devido à natureza morfogênica recalcitrante dos protoplastos. Após o isolamento e a purificação, as aplicações permitiram avaliar um grande número de protoplastos (FCM e MUSE) e núcleos dos protoplastos (CA) em um curto período de tempo. A partir das adaptações nos procedimentos, foram evidenciados diferentes tipos e níveis de danos citológicos (FCM e Muse) e genômicos (Muse e CA), possibilitando discriminar e mensurar os protoplastos viáveis. Considerando os resultados, este estudo introduz procedimentos quantitativos melhorados para o teste de viabilidade. Além disso, visando a regeneração de plântulas, diferentes métodos podem ser aplicados para avaliar a viabilidade de protoplastos, definindo os procedimentos de isolamento e purificação mais adequados. Corraborando para este propósito, foram mostrados guias para FCM, Muse e CA visando a padronizaçao dos testes de viabilidade em protoplastos de plantas.

SUMMARY

1 INTRODUCTION

Protoplasts are isolated cells (“naked” cells) whose cell walls have been removed by mechanical or, as in most cases, enzymatic pool treatment (Klercker 1892; Cocking 1960). If viable, these cells can be dedifferentiated under in vitro culture conditions, becoming totipotent (Cocking 1972; Eeckhaut et al. 2013). Besides the synthesis of new walls, these totipotent cells resume the cell cycle, giving rise to proembryogenic masses – so-called calli (Nolan e Rose 2010; Neelakandan and Wang 2012). Some cells of a callus acquire competence, following a different morphogenic route (organogenesis or embryogenesis), according to the influence of genetic, epigenetic and physiologic features and the in vitro environmental 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 the applications, the protoplast recalcitrance in some species still prevents their totipotency (Davey et al. 2005).

Protoplast viability is influenced by genetic and epigenetic factors, besides the chronologic, physiologic and ontogenetic age of the explant. The viability is 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 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 (temperature, photoperiod and light quality) 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). Esterase enzymes 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, flow cytometer (FCM) is pointed out as a rapid, objective and multiparametric application. FCM histograms and dot plots show subpopulations (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 (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 segregated 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; Wlodkowic 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 (GFP) (Galbraith et al. 1995). In protoplast suspensions of *Nicotiana plumbaginifolia*, FCM has been used to relate chromatin condensation and DNA fragmentation to apoptosis (O'Brien et al. 1998). FCM has also been 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™ Cell Analyzer (Muse), a compact flow cytometer (Merck 2013), has also been used for viability test of human (Ueda et al. 2013; Marusiak et al. 2014) and fish cell suspensions (Nynca et al. 2016). The data obtained with Muse show 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 2011).

Other alternative method that can be adapted for protoplast viability test is the Comet Assay (CA), which shows damage in nuclear DNA (Kuzminsky et al. 2016). CA, or single cell electrophoresis gel, is a relatively sensitive, rapid, quantitative method that assesses the DNA damage in eukaryotic cells (Collins 2004; Zhang et al. 2011). CA was first described by Östling and Johason (1984) to assess DNA damage of murine lymphocytes subjected to gamma radiation by neutral electrophoresis. The methodology using neutral electrophoresis identifies double DNA strand breaks. Subsequently to neutral electrophoresis, the alkaline electrophoresis was developed, applying it to human lymphocytes exposed to X-ray (Singh et al. 1988). Usually, the alkaline CA detect 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, *Capsicum annuum* L. (Solanaceae) was chosen as model owing to its severely

recalcitrant morphogenic nature, with inability to respond under in vitro culture conditions, and constant efforts made to explore its morphogenic potential and regeneration (Kothari et al. 2010).

2 OBJECTIVE

2.1 GENERAL OBJECTIVE

The present study had as main objective to evaluate the viability of plant protoplasts by adapting protocols of Flow cytometry, Muse™ cell analyzer and Comet assay.

2.2 SPECIFIC OBJECTIVES

i. Adapt methodology to evaluate the viability of plant protoplasts by means of Flow cytometry using the FDA fluorochrome.

ii. Adapt methodology to evaluate the viability of plant protoplasts by means of Muse™ cell analyzer using the PI fluorochrome.

iii. Adapt the Alkaline comet assay procedure to identify possible nuclear DNA damage of protoplasts.

iv. Compare the methodologies: Flow cytometry, Muse ™ cell analyzer and Comet assay.

3 MATERIALS AND METHODS

3.1 PLANT MATERIAL AND IN VITRO PLANTLET GROWTH

Capsicum annuum L. (Solanaceae) 'Itapuã 501' seeds were disinfested in laminar flow chamber using 70% ethanol for 1 min, followed by a volume of 50% of 2.0–2.5% Na_2OCl with one drop of Tween 20 (Merck® KGaA, Darmstadt, Germany) for 20 min. Next, the seeds were rinsed with sterilized distilled water (dH_2O) 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® Chemical Co., USA) 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® Chemical Co., USA). Prior to autoclaving, the pH was adjusted to 5.6 and the medium was sterilized for 20 min at 120ºC and 1.5 atm. The cultures were kept in a growth room under a temperature regime of 25 ± 2°C and photoperiod cycle of 16/8 h of light/dark.

3.2 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 (CPW) solution: 0.272 g L^{-1}

KH_2PO_4, 1.01 g L^{-1} KNO_3, 14.08 g L^{-1} $CaCl_2.2H_2O$, 2.46 g L^{-1} $MgSO_4.7H_2O$, 0.0016 g L^{-1} KI, 0.0003 g L^{-1} $CUSO_4.5H_2O$, and 130 g L^{-1} mannitol (Sigma® Chemical Co., USA), 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 Pharmaceutical Ind. Co. Ltd), 0.5% (w/v) macerozyme R-10 (Yakult Pharmaceutical Ind. Co. Ltd) and 0.25% (w/v) driselase (Sigma Chemical Co.) were added (pH = 5.6). Prior to use, the pool was centrifuged at 3,000 rpm for 5 min, the pellet was discarded, and the supernatant filter-sterilized through 0.22 µm Millipore (Millex®) filter. 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 70-µm nylon cell strainer (BD FalconTM); the filtrate was collected using a Pasteur pipette and transferred to 15-mL Falcon 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 (modified from Anthony et al. 1999). After isolation, images of freshly isolated protoplasts were captured using an inverted phase-contrast microscope IX70 (Olympus TM), with objective LUCPlanFLN – UIS 2 40×/0.60 Ph2. The protoplast isolation and data analysis below were accomplished in six randomized days.

3.3 PROTOPLAST VIABILITY ANALYSES IN FCM AND MUSE

Two treatments were performed for viability test in FCM: (i) 1.5 mL of unstained protoplast suspensions (control) and (ii) 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 10,000 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. ~670 nm; Cerovic et al. 2002) was collected through RG 610 nm filter, while FDA fluorescence (max. ~497 nm; Thermo Fisher Scientific™ 2017a) 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 (chlorophyll) versus log green fluorescence were created for a total of ~10,000 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, Germany) equipped with laser source (532 nm) through red filter (680 nm) for fluorescence emission of protoplasts stained with PI (max. ~620 nm; Thermo Fisher Scientific™ 2017b). 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 ~5,000 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 (Perttu Terho, Centre for Biotechnology, Turku, Finland, www.flowingsoftware.com).

3.4 PROTOPLAST VIABILITY BY ALKALINE CA

All steps described below were carried out under darkness to prevent additional DNA damage. The CA was accomplished from protoplasts (sample), nuclei (negative control) and nuclei treated with hydrogen peroxide (positive control) of *C. annuum*. Immediately after isolation and purification, the protoplast suspensions were centrifuged (Excelsa II, model 206 MP – Fanem®) of at 700 rpm for 5 min, the CPW 13M was discarded and added 3 mL of phosphate buffer saline (PBS). For positive and negative controls of the CA, nuclei suspensions were 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 Chemical Co.) at 50ºC, which was solidified during 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 Chemical Co.) at 37ºC on the slide. After, each slide was covered with a coverslip in order to ensure a homogeneous distribution. The coverslip was removed after the gel solidification at 4°C during 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 in 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 Chemical Co.), pH = 8] at 4ºC for 1 h. For DNA denaturation before electrophoresis, slides were placed in a horizontal electrophoresis system with 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 with amperage of approximately 26 mA. After electrophoresis, the slides were washed three times in neutralization buffer (400 mM Tris-HCl, pH = 7.5; Ojima et al. 2009) at 4ºC for 5 min. The slides were stained with 100 μL of 50 mM acridine orange (Sigma Chemical Co.) for 15 min and rinsed three times with dH_2O.

CA analysis was performed from 100 nucleoids per slide, being that overlapping nucleoids and it in areas near the edges of slide were not accounted. Six slides (replicates) of the protoplasts, positive and negative controls were analyzed using a DP-71 video camera, mounted on a BX-60 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a stabilized light source, an MPlanApo 20×/0,50 objective. The images were evaluated by visual method classifying the nucleoids in 5 categories, from 0 (no apparent tail) to 4 (largest quantity of DNA in tail) (Collins 2004; Araldi et al. 2015). A guideline of the protoplast viability test from alkaline CA has been showed in the Figure 1.

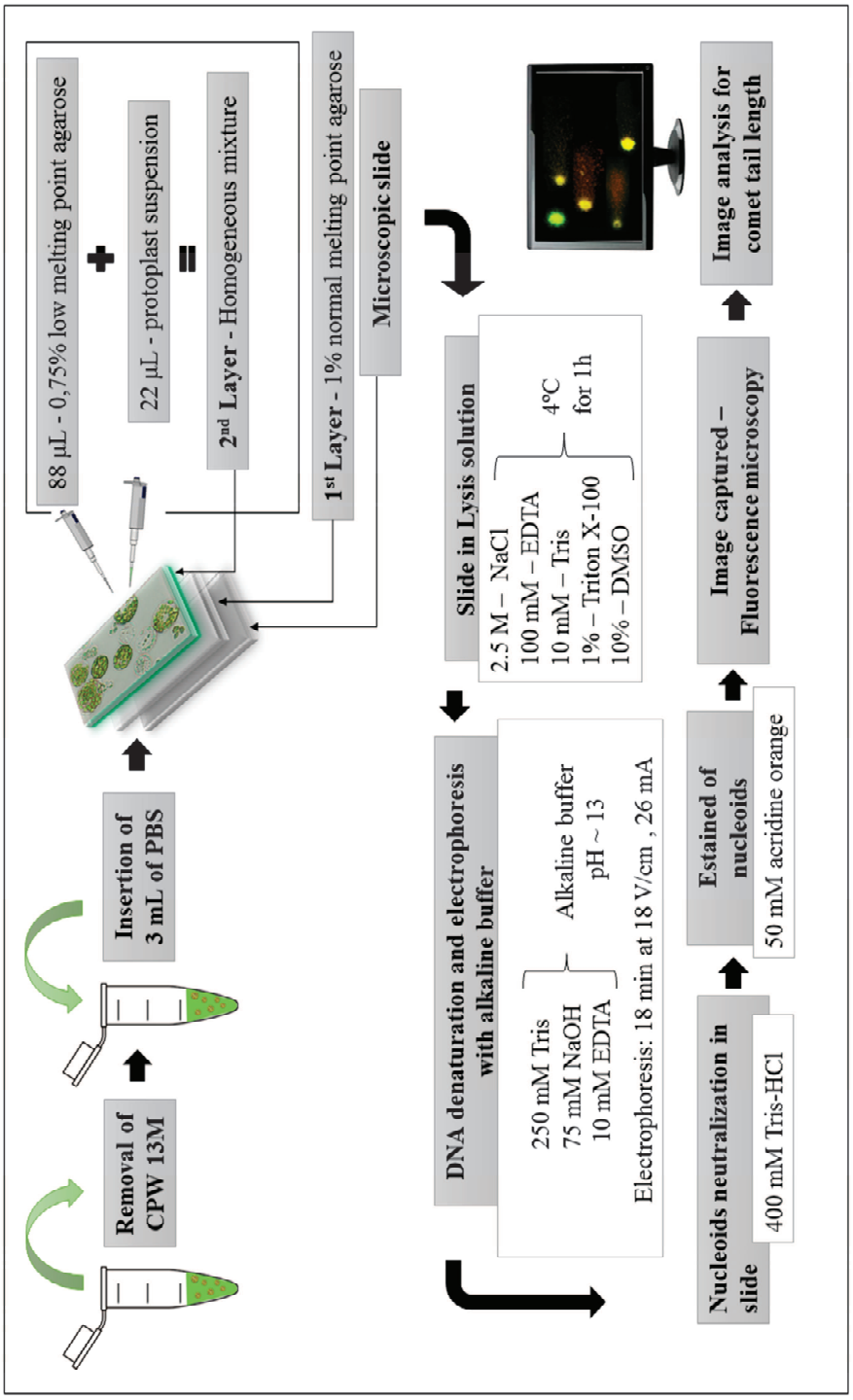

Fig. 1 – Guideline about the adaptation procedure performed in the CA alkaline for viability evaluation in C. annuum protoplast.

4 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 in low light to avoid DNA damage. The pre-plasmolysis and isolation (enzymatic pool and time) procedures provided protoplasts with roundish shape and heterogeneous size ranging approximately from 12 to 38 µm of diameter (Fig. 2). The centrifugation step removed the cellular debris, maintaining a population of intact protoplasts.

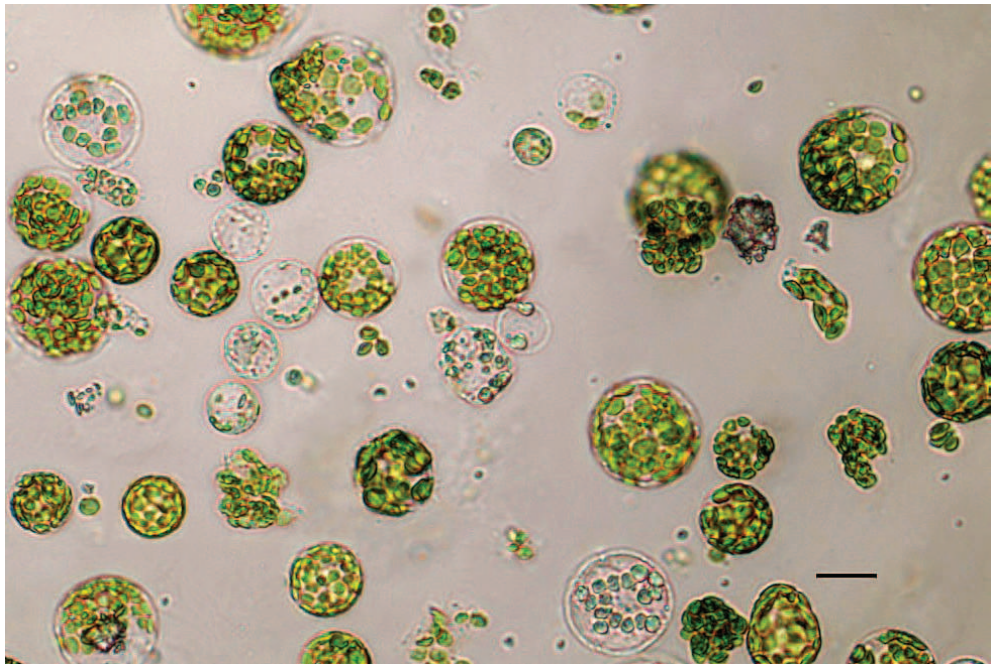

Fig. 2 – Freshly isolated *C. annuum* protoplasts showing roundish shape and heterogeneous size, ranging approximately from 12 to 38 µm of diameter. The image was captured by inverted phase contrast microscope. Bar = 10 µM.

The protoplast viability rate was determined for FCM by comparing the results generated from unstained protoplasts, taking advantage of the chlorophyll

autofluorescence, in relation to the FDA-stained protoplasts. When subjected to FCM with UV light (388 nm), unstained protoplast suspensions were characteristically located as a single cluster emitting a red autofluorescence (max. emission ~670 nm) signal derived from the chlorophyll *a* (max. excitation ~370 nm) (Fig. 3a). Based on this control FCM dot plot, two quadrants were previously defined: the lower exhibiting all (100%) 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 protoplast were analyzed. The FDA-stained protoplast suspensions (max. excitation ~497 nm) also exhibited a higher level of green fluorescence (max. emission ~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 (FDA-positive) were bound to the upper quadrant, while unviable protoplasts (FDA-negative) 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 established as a percentage of the total number of cells for the quadrants (upper and lower), showing 65.22% of viable and 34.78% (± 9.03%) of unviable cells.

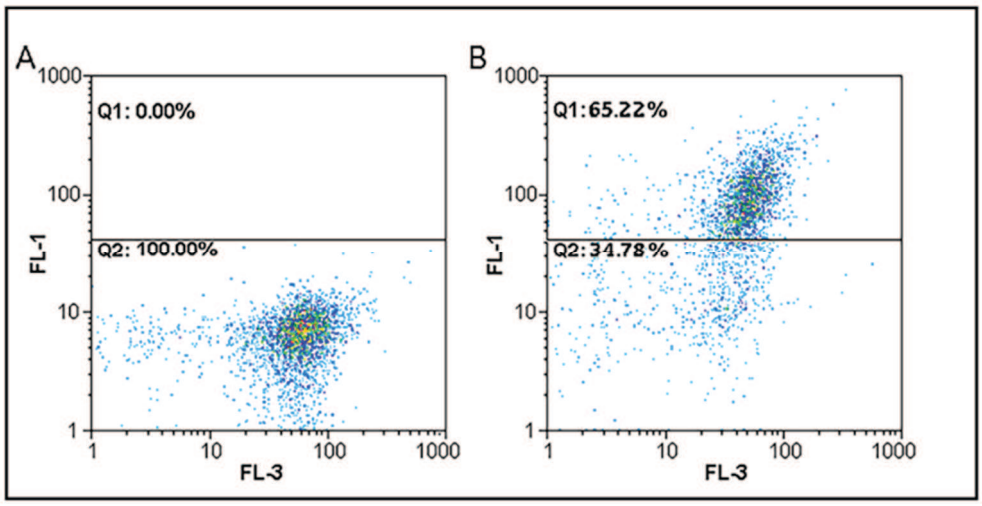

Fig. 3 – Dot plots generated from *C. annuum* protoplasts with flow cytometer using FL-3 (*x* axis, red) and FL-1 (*y* axis, green). (a) Control dot plot showing a single indiscriminate protoplast population formed based on chlorophyll *a* autofluorescence. (b) Viable (upper quadrant, FDA-stained) and unviable subpopulations (lower quadrant, non-FDA-stained protoplasts) clustered after emission of chlorophyll *a* autofluorescence *vs* FDA fluorescence.

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: left quadrant composed by all protoplasts (100%) and the right quadrant with 0%. In contrast, the PI-stained (max. excitation ~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 *a*, while the PI-stained (unviable) protoplasts remained in upper right quadrant (Fig. 4b). Cellular debris were limited to the lower left quadrant. The viability rate was also calculated as percentage of the total number of protoplasts for each quadrant, whereby 66.20% of viable

protoplasts and 33.80% (± 7.75%) of unviable protoplasts and cellular debris were measured.

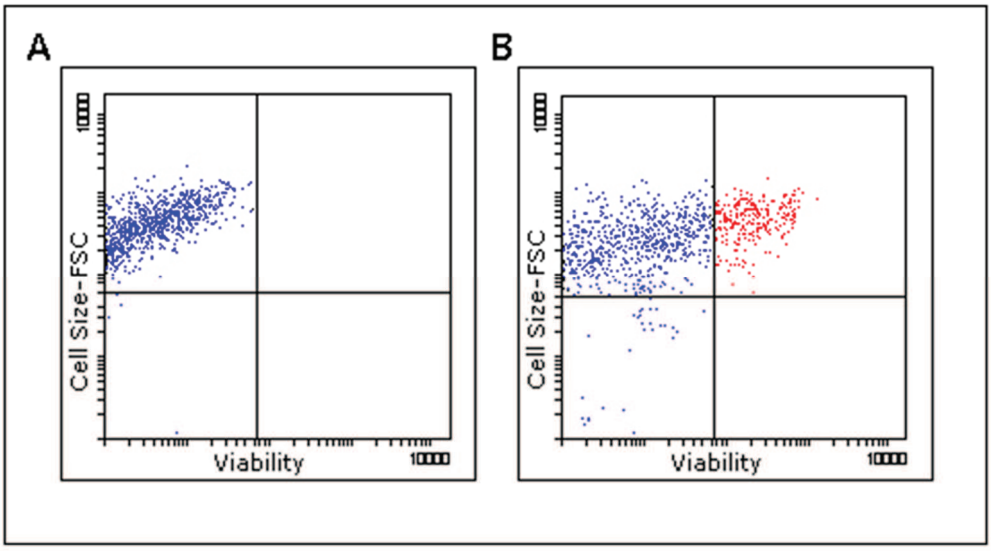

Fig. 4 – Dot plots generated from *C. annuum* protoplasts with Muse™ cell analyzer. (a) As in FCM, control dot plots exhibiting a single indiscriminate protoplast population from chlorophyll *a* autofluorescence. (b) Viable (upper left quadrant, non-PI-stained) and unviable subpopulations (upper right quadrant, PI-stained) and cell debris (lower left quadrant) were clustered based on emission of chlorophyll *a* autofluorescence *vs* PI fluorescence.

Based on the results obtained from FCM and Muse, the procedures for protoplast isolation yielded 65.71% of viable protoplasts. Considering the debris evidenced in Muse, unviable protoplasts corresponded to 34.29%.

For CA, the proportion (88:22 µL) between the volume of low melting point agarose volume and the volume of protoplast (sample) or nuclei (controls) suspension was considered ideal for evaluation of the comets, due to visualization of more than 100 nucleoids no overlapping per slide. Lysis solution allowed a clean visualization of the comets by removing of autofluorescent chloroplast and fragments of nuclear membrane. The combination of EDTA, NaOH and Tris (hydroxymethyl) aminomethane (Tris) in the alkaline buffer favored the preservation of nucleoids. The electrophorese conditions (time and

voltage) allowed the comet tail (DNA strand break) to remain attached to the comet head.

Comets were found in all slides, being classified in five types (0 – 4) by visualization of the tail length. Comets without apparent tail were classified in 0, and the 1 at 4 were classified according the increase of the tail length. The negative control presented the mean of 10.00% ± 1.44% of comets, with the most defined as the type 2. 35.00% ± 3.27% of comets was visualized in the slides of the positive control, being the majority categorized in 2 and 3. From the protoplast suspensions was observed a mean of 20.88% ± 2.60% comets, predominantly of the type 3 and 4 (Fig. 5).

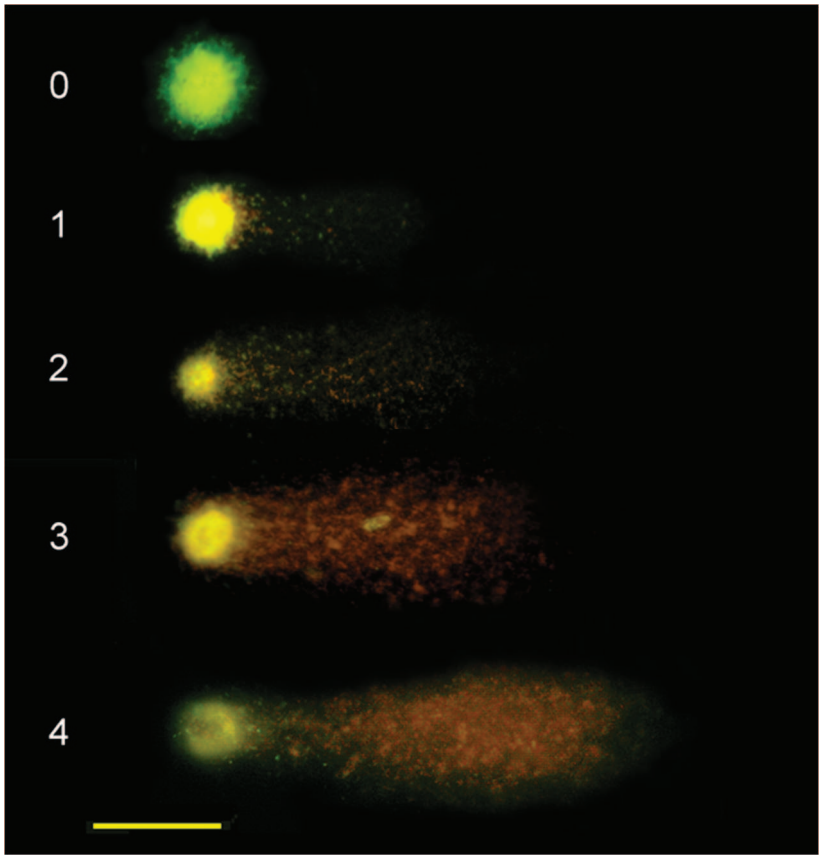

Fig. 5 – Comet types found in all slides produced from *C. annuum* nuclei (positive and negative controls) and protoplasts. The comets were classified according to tail length (Araldi et al. 2015) in 0 (without DNA damage), 1 (low damage), 2 (medium damage), 3 (large damage) or 4 (maximum of damage detected).

5 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 the 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 days of evaluation. The results corroborated that large numbers of cells in suspension can be quickly analyzed in FCM (Adan 2016) and Muse (Marck 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 identical dot plot profiles (Fig. 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 version 2.5 software for Muse. In contrast, 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 (Fig. 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 stained population, regardless of the type and action mechanism of the used fluorochromes.

FDA (FCM) and PI (Muse) were also fundamental for discrimination of viable and unviable protoplast populations (Fig. 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 nucleic acids of unviable cells (Adan et al. 2016). An alternative to FDA in viability tests is carboxyfluorescein diacetate (cFDA), 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; Zimmermann e Meyer 2011). In keeping with the possibility of using distinct fluorochromes, it is further necessary that the configuration of FCM or Muse be calibrated to verify the excitation source (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 of cell debris (3.95%) 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% (FCM) and 66.20% (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 recalcitrant nature, so far protoplasts of the genus *Capsicum* do not respond very well in vitro culture (Kothari et al. 2010; do Regô et al. 2016).

Differently of the FCM and Muse, the alkaline CA allowed the identification and classification of DNA strand break from visualization of nucleoids, which were classified from 0 to 4 (fig. 5). Based on the controls (positive with 10.00% of comets, and negative with 35.00%), the protoplast isolation and purification

procedures promoted damages in the DNA structure (20.88% of comets) of *C. annuum*. Although the literature does not present an ideal parameter of percentage of comets in the negative control, it was established as an acceptable value up to 10,00%. The CA showed DNA structure changes that can be related with the viability of the protoplasts and their recalcitrance in tissue culture. Moreover, the oxidative compounds generated during protoplast isolation besides of affecting the membrane integrity (Niehaus 1978; Papadakis et al. 2001), can also promote DNA damages (Costa et al. 2012). In the initial studies involving CA, the presence of DNA strand break, was associated to the early apoptosis process (Oliver et al. 1993; Charzyńska et al. 2000). Indeed, in stage early of the apoptosis, the chromatin is condensed and the DNA begins to be degraded, while the plasma membrane of the protoplast retains intact (O'brien et al. 1998). However, Collins (2004) reported that CA does not identify apoptotic cells because the DNA are fragments in oligonucleotides size and it can disappear during lysis or electrophoresis. However, this author does not reject the possibility of cell with highly damaged DNA entering in apoptotic process.

Based on 20.88% of comets measured, 79.12% of the protoplasts showed nucleoids without DNA damage, which were classified as type 0. From CA, these protoplasts (79.12%) were considered viable cells. This mean value is distinct in relation to the 65.22% and 66.20% obtained from FCM and Muse, respectively. Therefore, an accurate protoplast viability test should be performed using distinct methods, evaluating of the plasm membrane or the DNA integrity.

6 CONCLUSIONS

In conclusion, the viability test performed using FCM and Muse was objective, accurate, reproducible, and very clear to separate viable and unviable protoplasts using chlorophyll autofluorescence combined with FDA for evaluation of cell enzymatic activity or PI for identify of membrane integrity. Besides FCM and Muse, the use of CA for viability test detected different levels of DNA strand breaks. Considering the results, the protoplast isolation and purification procedures promote cytological (showed by FCM and Muse) and genomic damages (evidenced by Muse and CA) that can prevent the in vitro responses associated to plantlet regeneration. As presented here, distinct viability test can be realized in plant protoplast in order to define the more adequate isolation and purification procedure. Additionally, guides were produced about CA application (Fig. 1) and comparing the main aspects of the viability test using FCM and Muse methodologies (Table 1). From these guides, new procedures can be standardized according to specific laboratorial conditions.

Table 1 – Guide comparing the main aspects of the viability test using flow cytometer and the Muse™ cell analyzer

Viability test in plant protoplasts	Muse ™ Cell Analyzer	Flow cytometer
Excitation source	Laser 532 nm	UV lamp 388 nm
Filter	Red 680 nm	FL-1 520 nm; FL-3 610 nm
Parametric evaluation	Biparametric: FSC x PI fluorescence	Biparametric: chlorophyll autofluorescence x FDA fluorescence
Fluorochromes	PI – excitation ~533 nm; emission ~620 nm	FDA – excitation ~497 nm; emission ~517 nm
Dot plots 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	~5,000	~10,000
Time for analysis of the sample	~1–2 min	~1–2 min
Histogram analysis	Flowing software 2.5.1 (Perttu Terho, Centre for Biotec., Finland)	FlowMax® software (Partec®)
Accuracy and reproducibility of the analysis	High	High
Cost	Relatively low	Relatively high

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