Electronic Journal of Biotechnology ISSN: 0717-3458
Vol.9 No.5, Issue of October 15, 2006

Received June 6, 2005 / Accepted April 5, 2006
DOI: 10.2225/vol9-issue5-fulltext-4
RESEARCH ARTICLE

Viability in protoplasts and cell suspensions of *Coffea arabica* cv. Catimor

Rafael Fernandez-Da Silva
Laboratorio de Clonación y Genética Vegetal
Instituto de Biología Experimental
Facultad de Ciencias
Universidad Central de Venezuela
Calle Suapure, Colinas de ello Monte
Caracas, Venezuela
Tel: 58 212 7510111
Fax: 58 212 7535897
E-mail: rfernandez2@uc.edu.ve

Andrea Menéndez-Yuffá*
Laboratorio de Clonación y Genética Vegetal
Instituto de Biología Experimental
Facultad de Ciencias
Universidad Central de Venezuela
Calle Suapure, Colinas de ello Monte
Caracas, Venezuela
Tel: 58 212 7510111
Fax: 58 212 7535897
E-mail: amenendez@cantv.net

Financial support: Project S1-98003209 FONACIT (Ministerio de Ciencia y Tecnología de Venezuela).

Keywords: cell suspensions, cell viability, coffee, Evans blue, MTT, protoplasts.

Abbreviations:
2,4-D: 2,4-dichlorophexyacetic acid
BA: Benzyladenine
FDA: fluorescein diacetate
MES: (2-[morpholino]ethanesulfonic acid)
MTT: (3-[4,5-Dimethyltiazol-2-yl]-2,5-diphenyltetrazolium bromide)
TTC: triphenyl tetrazolium chloride

Two methods were evaluated in order to assess the viability of protoplasts and cell suspensions of *Coffea arabica* cv. Catimor used in a protocol of transformation by electroporation. One method consisted of staining with 1 % Evans blue and the other staining with 1% 3-[4,5-dimethyltiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). When Evans blue was applied to viable cells and protoplasts they either did not stain or acquired a faint blue colour. However, it was difficult to distinguish the non-viable cells in the wide spectrum of clear blue tonalities. In contrast, with the MTT assay only the viable cells and protoplasts reduced this salt to the red coloured formazan; viable and non-viable cells were distinguished more clearly with MTT than with Evans blue. The optimal temperature for the reaction with MTT was 37ºC. The time of incubation was shown to be important, since longer times improved the reaction; the highest viability value was obtained after incubation for 120 min.

For many years coffee has been the subject of improvement by conventional methods; the application of biotechnology in the improvement of this crop has recently progressed and procedures to estimate cell viability have become necessary. One of the requirements for the establishment of a cell culture or protoplast system is to count on a reliable and efficient method to estimate cell viability. Several methods allow the evaluation of cell viability under different conditions, among them, the application of selective agents (antibiotics, herbicides) added to the media. In the protoplast system the estimation of viability is necessary to evaluate the efficiency of the protoplast isolation procedure and to establish the culture conditions.

The methods used to evaluate viability can be classified in two groups: those that stain only the dead cells, and those in which only the living cells are coloured, because the colour is a product of cell metabolic activity (Widholm, 1972). The most used stains for dead cells are Evans blue, bromophenol blue, methylene blue and phenosafranin, whereas fluorescein diacetate (FDA) is used for living cells. Evans blue and methylene blue are reduced by the living

*Corresponding author

cells turning colourless while dead cells remain blue. When fluorescein diacetate is used, the coloration (fluorescence) is the product of esterase activity in the cells; this non-polar compound can enter the cell, where it releases fluorescein (Huang et al. 1986) but requires a costly fluorescence microscope to be detected.

Viability can also be quantified with activity assays, using for example the tetrazolium salts 3-[4,5-dimethyltiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and triphenyl tetrazolium chloride (TTC). Tetrazolium salts are reduced in viable cells to a red coloured water-insoluble formazan by the action of dehydrogenases (Tisserat and Manthey, 1996; Verleysen et al. 2004). Black coloured or non-coloured cells are considered dead (Khatun and Flowers, 1995).

Different compounds have been used to determine viability in cell suspensions, pollen and spores of phytopathogenic fungi. For example, cell suspensions of *Glycine max* infected by *Fusarium solani* were stained with 0.1% phenosafranin, the living cells acquiring a red colour (Li et al. 1999). Khatun and Flowers (1995) estimated the viability of pollen of *Oryza sativa* with fluorescein diacetate, aniline blue, TTC and MTT (0.1-1%); the best results were obtained with MTT, with viable cells staining light red to red and yellow, non-stained or black pollen were considered non-viable.

In cell cultures it is very important to have an effective and cheap method to determine clearly and efficiently the presence of viable and non-viable cells. Even though the procedures to determine viability with MTT are well established for animals, reports of the use of MTT in plants are scarce. The MTT assay was used by Nyange et al. (1997) to evaluate the effect of filtrates of the fungus *Colletotrichum kahawae*in cell suspensions and protoplasts of *Coffea arabica*. Yamamoto et al. (2002) used MTT to evaluate mitochondrial activity in tobacco (*Nicotiana tabacum*) and pea (*Pisum sativum*) subjected to toxicity by aluminium. Wang et al. (2004b) estimated the pollen viability in transgenic and non-transgenic tall fescue (*Festuca arundinacea*) plants by different staining methods, such as X-gal, aniline blue, TTC, MTT, lugol solution and fluorescein diacetate. None of the staining methods could effectively distinguish between viable and dead pollen. Gracia-Medrano and Miranda-Ham (2003) strongly recommended the use of an assay based on the reduction of tetrazolium salts (MTT or TTC) to evaluate the viability change due to hypersensitive response in *Lycopersicon esculentum* suspension cultures, because they provide an adequate indicator of changes in metabolic activity. Vižintin and Bohanec (2004) indicate that MTT and FDA are both adequate for viability testing of damaged and undamaged pollen grains or microspores of cucumber.

TTC has been useful in the evaluation of viability in cryopreservated cell suspensions of *Medicago sativa* (Shibli et al. 2001), cryopreserved tips of african violet

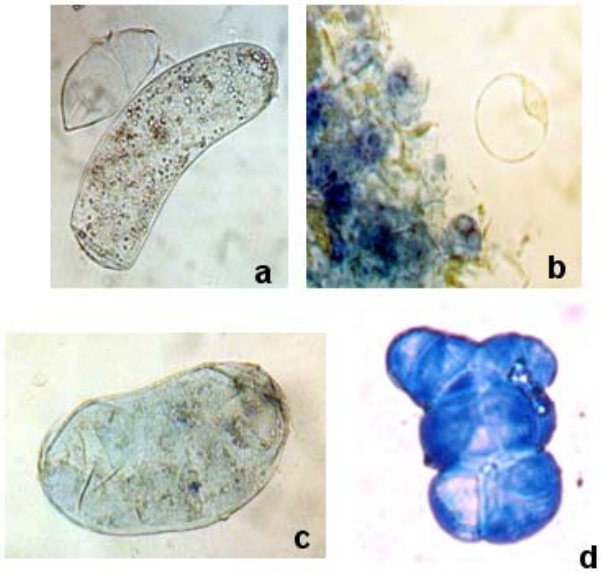

Figure 1. Cells and protoplasts stained with Evans blue.
(a) Non-stained cells (viable).
(b) Viable protoplast surrounded by blue cellular aggregates
(c) Cell with blue cytoplasm.
(d) Control of Non-viable cells dead with FAA.

(*Saintpaulia ionantha*) (Moges et al. 2004), embryogenic cells of *Vitis* spp. (Wang et al. 2004a), and thalli of *Ulva fasciata* under salinity stress (Chang et al. 1999). The TTC viability index was higher in high than low-vigour radicles of cucumber (Kang and Saltveit, 2002).

The main objective of this research was to establish a clear, efficient and cheap method to estimate the viability of protoplasts and cell suspensions of Catimor coffee, in order to apply this method to cell suspensions, protoplasts isolation, and genetically transformed material.

MATERIALS AND METHODS

Plant material

Plants of *Coffea arabica* cv. Catimor (Catimor is the product of the cross *C. arabica* cv. Caturra x Hybrid of Timor) were grown and maintained in bags with soil in the gardens of the Instituto de Biología Experimental, Caracas.

Tissue culture

Coffee cell suspensions were obtained following Hermoso-Gallardo and Menéndez-Yuffá (2000). In the first stage callus formation was induced from leaf sections, with the following modifications in culture media (M1): ½ Murashige and Skoog (1962) salts, 10 mg/l thiamine-HCl, 100 mg/l myo-inositol, 50 mg/l cysteine-HCl, 1 mg/l pyridoxine, 1 mg/l nicotinic acid, 2 mg/l kinetin, 0.5 mg/l 2,4-dichlorophexyacetic acid (2,4-D) and 30 g/l sucrose,

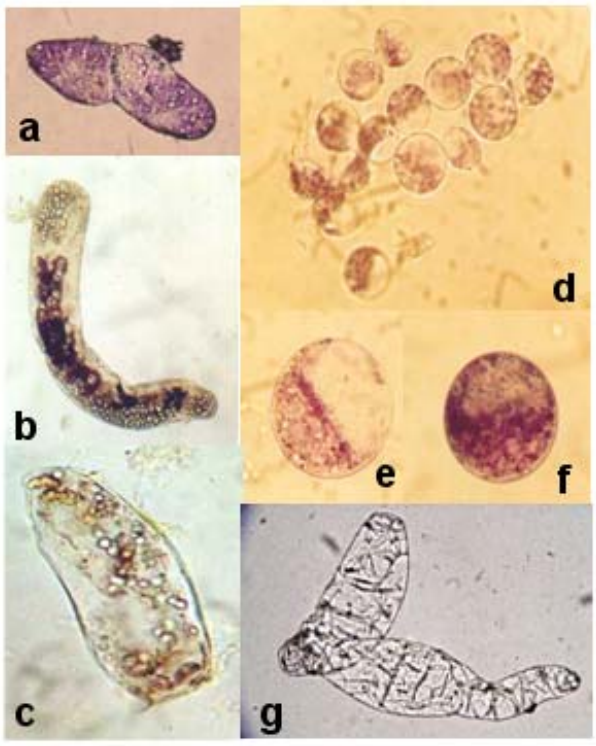

Figure 2. MTT assay in cells and protoplasts.
(a) Pink coloured cells (viables).
(b) Cell of purple colour (viable).
(c) Colourless cell (non-viable).
(d) Pink and red coloured protoplasts (viable).
(e) Protoplast with red cytoplasm (viable).
(f) Protoplast with purple cytoplasm (viable).
(g) Control of non-viable cells dead with FAA.

adjusting pH to 5.8 and solidifying with 3 g/l phytagel (SIGMA, St. Louis, USA) before sterilization.

The explants were incubated for 12 weeks in the dark at room temperature. The calli were then transferred to a second medium (M2) of similar composition as M1, with 5 mg/l benzyladenine (BA) as the only growth regulator, and maintained in the dark at room temperature. In the third phase the embryogenic calli were transferred to a liquid medium with 8 mg/l BA under continuous shaking at 160 rpm for 15 days in the dark and at room temperature. The suspension was filtered through a steel sieve (60 mesh), changing to fresh medium every eight days.

The isolation and culture of protoplasts were based on the procedures described by Tahara et al. (1994) with modifications, and proceeded as follows: 0.5 g of four-month old embryogenic calli were treated with a digesting solution composed of 1 % macerozyme R-10; 1-2% cellulase R10 "Onozuka" (both enzymes from Yakult Pharmaceutical), 0.5 M manitol, 25 mM $CaCl_2$ in buffer MES (2-[morpholino]ethanesulfonic acid) at pH 5.8. The preparation was placed under slow shaking (50 rpm) for 24 hrs, to obtain the enzymatic digestion of the cell walls. The digestion was sieved through a 60-mesh steel sieve. The enzymatic solution was eliminated by centrifugation and the pellet was resuspended in a medium composed of MS (1962) salts, 1 mg/l BA, 0.5 M manitol and 25 mM $CaCl_2$.

Viability tests

Viability was determined by two methods, Evans blue and the MTT assay. One ml samples of 4-week-old cell suspensions or protoplasts were placed in micro-centrifuge tubes, centrifuged at 1000 rpm, and 100 µl of either Evans blue or MTT were added to the sample. The procedures were based on Widholm (1972) for Evans blue and on Tisserat and Manthey (1996) for MTT. Cells were incubated with 1% Evans blue for 10 min at room temperature, centrifuged at 1000 rpm and resuspendedin distilled water. Ten random counts were done by means of a hemacytometer. A 1% MTT solution was used to test the following incubation conditions: 10, 30 or 120 min at room temperature or 37ºC. Viability was determined by counting the coloured cells in a hemacytometer.

Control tests of viability were done with suspension cultures fixed in 70% ethanol or in FAA (5% of 37% formaldehyde, 5% glacial acetic acid, 90% of 70% ethanol).

RESULTS AND DISCUSSION

According to previous reports Evans blue is an indicator of non-viable cells; in the present study the viable cells were non-stained or clear blue (Figure 1a), whereas the viable protoplasts did not stain (Figure 1b). In contrast, the non-viable cells and protoplasts, were distinguished by an intense blue colour (Figure 1c and Figure 1d).

Viable cells treated with MTT were distinguished by a pink, red or purple colour in the cell cytoplasm (Figure 2a and Figure 2b) or protoplasts (Figure 2d, Figure 2e, and Figure 2f), and the non-viable cells either showed a clear yellow colour or did not change their colour (Figure 2c and Figure 2g).

Evans blue stains only the dead cells because in the living cells the dye is reduced to a colourless form, but it is difficult to distinguish the dead cells in a blue background due to the low extinction coefficient of this dye (Huang et al. 1986). In agreement with these authors, we also observed that it was difficult to discriminate between living and dead cells. In contrast, the MTT assay based on the cell metabolic activity changes the colour of living cells by the reduction of the salt (MTT), forming an insoluble compound of red or purple colour (formazan) through the action of the mitochondrial dehydrogenase (Tisserat and Manthey, 1996), whereas the black colour or no change in colour is a sign of cell death (Khatun and Flowers, 1995).

The Evans blue method in protoplasts yielded a 93.8% of viability, whereas with the MTT assay applied to the same

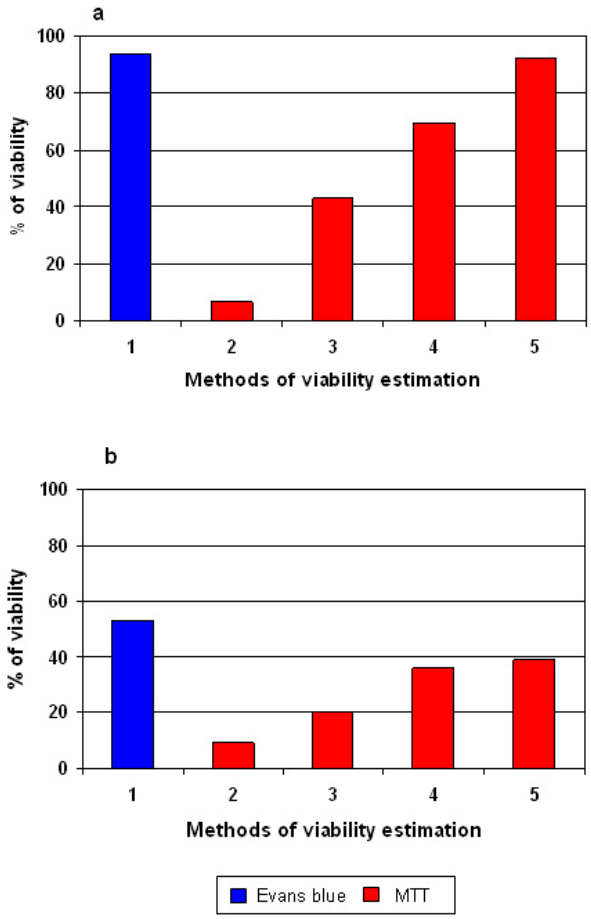

Figure 3. Comparison of methods to evaluate the viability.
(a) In protoplasts.
(b) Cell suspensions. Methods of viability estimation: 1. Evans Blue. 2. MTT (room temperature). 3. MTT 15 min at 37ºC. 4. MTT 30 min at 37ºC. 5. MTT 120 min at 37ºC.

preparation the percentage of viable protoplasts varied with the conditions. The viability was low at room temperature (6.6%) and increased at 37ºC. The time of incubation was also important, increasing viability from 43.1% to 92.2% when the interval varied from 15 min to 120 min (Figure 3).

In cell suspensions Evans blue detected 53% of viable cells and the MTT varied with the temperature and the incubation time, from 9.6% at room temperature to 20% at 37ºC (15 min) or 39% at 37ºC (120 min).

Previous research has also showed that the time required to observe the colours in the cells varies with the compound. For example, Evans blue stains the cells rapidly (10 min) similar to fluorescein diacetate (3 min) (Widholm, 1972). We observed that incubation time was important when detecting viability, the MTT assay having an optimum incubation time of 120 min. These results are in accordance with previous work: the time for the MTT reaction can vary from 15 min for pollen (Khatun and Flowers, 1995) to 24-48 hours for spores of phytopathogenic fungi (Sutherland and Cohen, 1983). These authors also consider that MTT concentration and incubation temperature and time are important factors in the assay. Gracia-Medrano and Miranda-Ham (2003) report that they used an incubation time of 8 hrs with MTT or TTC to evaluate changes in viability of *Lycopersicon esculentum* suspension cultures.

An incubation temperature of 37ºC was better than room temperature to detect viability with MTT, probably because this temperature was better for the activity of the mitochondrial dehydrogenase.

The Evans blue method and the MTT reaction can be seen in a light microscope, in contrast to the FDA, which requires a fluorescence microscope with a suitable barrier filter (Widholm, 1972), Vižintin and Bohanec (2004) also considered as advantage in the use of MTT over FDA the lack of need for fluorescence; in the latter method the cost of the equipment is a disadvantage.

CONCLUDING REMARKS

In this research we demonstrate the usefulness of the MTT assay to evaluate viability in protoplasts and cells suspensions of coffee; additionally, that of the two methods assayed, MTT is the best system to determine without ambiguity the viability of cells and protoplasts of coffee 'Catimor', since in appropriate conditions of temperature and time of incubation it allows the clear discrimination between viable and non-viable cells and avoids the need for expensive equipments of fluorescence required by the method of FDA.

ACKNOWLEDGMENTS

To FONACIT for the financial support of this research (Project S1-98003209). To Dr. Ana Herrera for the revision of the manuscript and to Lic. Luis Hermoso-Gallardo for his assistance in tissue culture.

REFERENCES

CHANG, Wan-Chun; CHEN, Ming-Hwa and LEE, Tse-Min. 2,3,5-Triphenyltetrazolium reductions in the viability assay of *Ulva fasciata* (Chlorophyta) in response to salinity stress. *Botanical Bulletin of Academia Sinica*, July 1999, vol. 40, no. 3, p. 207-212.

GRACIA-MEDRANO, Escobedo and MIRANDA-HAM, Lourdes. Analysis of elicitor-induced cell viability changes in *Lycopersicon esculentum* mill. suspension culture by different methods. *In Vitro Cellular and Developmental Biology-Plant*, March 2003, vol. 39, no. 2, p. 236-239.

HERMOSO-GALLARDO, Luis and MENÉNDEZ-YUFFÁ, Andrea. Multiplicación masiva del café (*Coffea arabica* L. cv. Catimor) mediante cultivo de suspensiones celulares embriogénicas. *Acta Científica Venezolana*, July 2000, vol. 51, no. 2, p. 90-95.

HUANG, C.N.; CORNEJO, M.J.; BUSH, D.S. and JONES, R.L. Estimating viability of plant protoplasts using double and single staining. *Protoplasma*, June 1986, vol. 135, no. 2-3, p. 80-87.

KANG, H.-M. and SALTVEIT, M.E. Effect of chilling on antioxidant enzymes and DPPH-radical scavenging activity of high- and low-vigour cucumber seedling radicles. *Plant, Cell and Environment*, October 2002, vol. 25, no. 10, p. 1233-1238.

KHATUN, S. and FLOWERS, T.J. The estimation of pollen viability in rice. *Journal of Experimental Botany*, January 1995, vol. 46, no. 1, p. 151-154.

LI, S.; HARTMAN, G.L. and WIDHOLM, J.M. Viability staining of soybean suspension-cultured cells and a seedling stem cutting assay to evaluate phytotoxicity of *Fusarium solani* f. Sp. *glycines* culture filtrates. *Plant Cell Reports*, January 1999, vol. 18, no. 5, p. 375-380.

MOGES, Asmara D.; SHIBLI, Rida A. and KARAM, Nabilas S. Cryopreservation of African violet (*Saintpaulia ionantha* Wendl.) shoot tips. *In Vitro Cellular and Developmental Biology-Plant*, July-August 2004, vol. 40, no. 4, p. 389-395.

MURASHIGE, Toshio and SKOOG, Folke. A revised medium for rapid growth on bioassays with tobacco tissue culture. *Physiologia Plantarum*, July 1962, vol. 15, no. 3, p. 473-497.

NYANGE, N.; WILLIAMSON, B.; LYON, G.D.; MCNICOL, R.J. and CONNOLLY, T. Responses of cells and protoplasts of *Coffea arabica* genotypes to partially purified culture filtrates produced by *Colletotrichum kahawae*. *Plant Cell Reports*, September 1997, vol. 16, no. 11, p. 763-769.

SHIBLI, R.; HAAGENSON, D.; CUNNINGHAM, S.; BERG, W. and VOLENEC, J. Cryopreservation of alfalfa (*Medicago sativa* L.) cells by encapsulation-dehydration. *Plant Cell Reports*, July 2001, vol. 20, no. 5, p. 445-450.

SUTHERLAND, E.D. and COHEN, S.D. Evaluation of tetrazolium bromide as a vital stain for fungal oospores. *Phytopathology*, May 1983, vol. 73, p. 1532-1535.

TAHARA, M.; YASUDA, T.; UCHIDA, N. and YAMAGUCHI, T. Formation of somatic embryos from protoplasts of *Coffea arabica* L. *HortScience*, March 1994, vol. 29, no. 3, p. 172-174.

TISSERAT, B. and MANTHEY, J.A. *In Vitro* sterile hydroponic culture to study iron chlorosis. *Journal of Plant Nutrition*, January 1996, vol. 19, no. 1, p. 129-143.

VERLEYSEN, H.; SAMYN, G.; VAN BOCKSTAELE, E. and DEBERGH, P. Evaluation of analytical techniques to predict viability after cryopreservation. *Plan Cell, Tissue and Organ Culture*, April 2004, vol. 77, no. 1, p. 11-21.

VIŽINTIN, L. and BOHANEC, B. *In vitro* manipulation of cucumber (*Cucumis sativus* L.) pollen and microspores: isolation procedures, viability tests, germination, maturation. *Acta Biologica Cracoviensia Series Botanica*, 2004, vol. 46, p. 177-183.

WANG, Qiaochun; MAWASSI, Munir; SAHAR, Nachman; LI, Ping; VIOLETA, Colova-Tsolova; GAFNY, Ron; SELA, Ilan; TANNE, Edna and PERL, Avihai. Cryopreservation of grapevine (*Vitis* spp.) embryogenic cell suspensions by encapsulation-vitrification. *Plant Cell, Tissue and Organ Culture*, June 2004a, vol. 77, no. 3, p. 267-275.

WANG, Zeng-Yu; GE, Yaxin; SCOTT, Megann and SPANGENBERG, German. Viability and longevity of pollen from transgenic and nontransgenic tall fescue (*Festuca arundinacea*) (Poaceae) plants. *American Journal of Botany*, April 2004b, vol. 91, no. 4, p. 523-530.

WIDHOLM, J.M. The use fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. *Stain Technology*, July 1972, vol. 47, no. 4, p. 89-94.

YAMAMOTO, Yoko; KOBAYASHI, Yukiko; DEVI, S. Rama; RIKIISHI, Sanae and MATSUMOTO, Hideaki. Aluminium toxicity is associated with mitochondrial dysfunction and the production of reactive oxygen species in plant cells. *Plant Physiology*, January 2002, vol. 128, no. 1, p. 63-72.