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Abstract

The vanilla plant is an orchid mainly commercialized for the production of vanillin. This compound is considered the second largest natural flavoring source in the world. In recent years, price of vanillin has increased considerably, which has generated the necessity of improving the quality of the plant materials. There are few works related to the isolation, fusion and regeneration of protoplasts in vanilla, not allowing to boost the benefits that this technique can generate for crop breeding. This work presents efficient protocols for protoplast isolation and fusion from leaf and protocorm like bodies (PLBs) of vanilla (*Vanilla planifolia* and *Vanilla pompona*) in order to contribute to the genetic improvement of the genus.

A three-week pre-treatment in the dark was standardized before placing the explants in an osmotic solution (0.06 M MES, 0.4 M mannitol, pH 5.7) for one hour at 50 r.p.m. This solution was then replaced with different enzymatic solutions for three hours at 25 ± 1 ºC and 50 r.p.m. The isolated protoplasts were filtered (320 mesh), centrifuged (100 xg for 5 min) and re-suspended in a 0.6 M sucrose solution. Subsequently, a washing solution (50% MS salts with 0.03 M MES and 0.2 M mannitol, pH 5.7) was added to separate protoplasts by flotation-centrifugation. Protoplasts` viability was evaluated with 0.01% Evans blue. Enzymatic solution containing 1% cellulase, 1% pectolyase and 0.5% hemicellulase (pH 5.7) yielded the highest amount of protoplasts from *V. planifolia* leaf explants ($2{,}9 \times 10^5 \pm 0{,}7 \times 10^5$ protoplasts/g fresh weight, with a viability of 81%) and from PLBs ($2{,}8 \times 10^5 \pm 0{,}7 \times 10^5$ protoplasts/g fresh weight, viability 80%). In *V. pompona*, yields of $2{,}8 \times 10^5 \pm 0{,}8 \times 10^5$ protoplasts/g fresh weight from leaf explants (viability 79%) and $2{,}5 \times 10^5 \pm 0{,}8 \times 10^5$ protoplasts/g fresh weight from PLBs (viability 79%) were obtained.

For electrofusion, a hypoosmolar solution (Eppendorf®, HA, AL) was used, and the alignment and fusion parameters were standardized. The fusion parameters U1 = 8 V, 60 s; A = 170 V, 30 µs, n 3; U2 = 8 V, 60 s generated the highest number of fusion events (8.9%). Highest number of microcalli (plating efficiency 9.4%) was observed on media containing 50% MS salts supplemented with MS vitamins, 1% $CaCl_2$, 1 mg/L benzyladenine, 1 mg/L 2,4-dichlorophenoxyacetic acid, 0.2 M mannitol, 0.03 M 2-(N-morpholino) ethanesulfonic acid, 1 g/L hydrolyzed casein, 20 g/L sucrose, and 6.2 g/L agar as a gelling agent (pH 5.7) in diffuse light (16 hours to 1000 lux).

Keywords: Protoplasts, isolation, electrofusion, *V. planifolia*, *V. pompona*.
Acknowledgments: Authors want to thank the Consejo Nacional de Rectores (CONARE) in Costa Rica by partially funding this investigation.

Introduction

In recent years, the international price of natural vanilla has been increasing considerably. For 2011, the Gross Production Value of vanilla was US$ 24 million. Production Value was highest in 2005, when it reached US$ 51 million (vanilla benefit) caused by the 2004 tsunami; in 2007 it dropped back to US$ 16 million and since 2008 it has stabilized. With these prices, the vanilla global area planted grew up 4.9% between 2001 and 2011 (FAOSTAT 2014), and the world demand increased to 12 000 tons per year (Dignum *et al.* 2001). However, the global production of natural vanilla has only increased by 473 tons per year (average), being the deficit covered by synthetic compounds (Havkin *et al.* 2004, Havkin and Frenckel 2006).

Traditionally, vanilla is propagated by cuttings (Besse *et al.* 2004); however, this process can be performed only on a small scale, due to the mechanical transmission of diseases. In addition, the collection of stem cuttings prevents that mother plants can be exploited for production (Parra 1987). Because of these reasons, it is difficult to meet the demand of plants through this technique. Therefore, micropropagation has been presented as an alternative to solve the shortage of vegetative propagules (George and Ravishankar 1997, Geetha and Shetty 2000, Giridhar *et al.* 2001).

Currently, due to the profitability of the vanilla production and the high susceptibility of commercial materials to the wilt caused by *Fusarium oxysporum* and *Phytophthora meadii* or *Sclerotium rolfsii* (Parthasarathy *et al.* 2005, Pinaria *et al.* 2010), there is an increasing interest interest for the production of new and better vanilla materials. Some *in vitro* non-conventional breeding techniques can facilitate the production of new genotypes that better fulfill the producers' needs. This may be the case of the protoplast fusion technique, which has proved its potential in the development of new materials in several plant species (Szabados 1991). The main objective of this work was to establish protocols for the isolation, culture and protoplasts fusion of two species of vanilla (*Vanilla planifolia* and *Vanilla pompona*).

Methodology

Study Location

Research was conducted at the Plant Biotechnology Laboratory of the Sustainable Agriculture in the Humid Tropics Research and Development Center (CIDASTH), the Molecular Biology Laboratory of the Science and Letters School at the Technological Institute of Costa Rica (San Carlos, Alajuela) and the Plant Biotechnology Laboratory of the Grain and Seed Research Center (CIGRAS) located at the University of Costa Rica (Ciudad Universitaria Rodrigo Facio, San Pedro de Montes de Oca).

Plant Material

Worked was conducted with *V. planifolia* (G Jackson, 2n=32) and *V. pompona* (Schiede, 2n=32) material established *in vitro* at the ITCR San

Carlos. Plants were multiplied in order to obtain leaf material and protocorm like bodies (PLBs) in both vanilla species for protoplast isolation, culture and fusion testing. These tissues were used because of their high regenerative capacity, which was confirmed in previous studies (Alatorre 2002; Palama *et al.* 2010).

For PLB induction, root-tip explants were used. They were placed inverted (negative geotropism) on a medium containing MS salts (Murashige and Skoog 1962) supplemented with 1 mg/L, 6-benzylaminopurine (BA), 1 g/L hydrolyzed casein (CH), 0,5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g/L sucrose and 8 g/L of agar as gelling agent. To obtain leaf tissue, *in vitro* plants were used. They were placed on medium containing MS salts, supplemented with 1 mg/L BA, 1 g/L CH, 30 g/L sucrose and 8 g/L agar as gelling agent. Explants were cultured at 27 ± 1 °C; with a 16 hours (8.6 μmol/m^2s) photoperiod, and subcultured regularly every 30 days (Montero 2009).

Protoplast Isolation

For the isolation of protoplasts, three procedures (optimization of the isolation protocol, purification of isolated protoplasts and enzymatic assays) needed to be defined. A pretreatment of three weeks in the darkness was standardized prior to placing the explants, leaf fragments (1 g, segmented in squares of 0.3 x 0.3 cm) or poorly differentiated PLBs (1 g), in the osmotic solution (0.06 M MES and 0.4 M mannitol, pH 5.7) for one hour. Subsequently, this solution was replaced with the enzyme solution described in table 1 for three and four hours at 28 °C. Both processes were performed in the dark at 50 rpm. The isolated protoplasts were filtered (320 mesh), centrifuged and washed three times by gentle centrifugation (800 rpm, 100 xg) for 5 min in washing solution (50% MS salts with 0.03 M MES and 0.2 M mannitol at pH 5.7). Protoplast were resuspended in 1 mL of washing solution and carefully overlaid on 5 mL of floating solution containing 0.6 M sucrose and 0.03 M MES (pH 5,7) before final centrifugation. Protoplasts were removed using a Pasteur pipette from the interface of the two solutions and were resuspended in washing solution.

Table 1. *Enzyme Concentrations Evaluated for Isolation of Protoplasts from Vanilla Leaf Explants and PLBs Protoplast Isolation.*

Enzyme Solution [1]	Cellulase [2]	Pectolyase [3]	Hemicellulase [3]	Macerozyme [2]
A	0.8%	0.5%	0.2%	---
B	1%	1%	0.5%	---
C	1%	---	---	0.5%
D	1%	---	---	1.0%
E	1%	---	---	1.5%
F	2%	---	---	0.5%

[1] Enzymatic treatments (0.01 M MES + 0.7 M mannitol, pH 5.7) were adapted from Szabados (1991), Price and Earle (1984) and Seeni and Abraham (1986, cited by Arditti and Ernst 1993).
[2] Karlan Biochemicals, AR, EEUU.
[3] MP Biomedicals, CA, EEUU

The process efficiency was evaluated by means of the yield of isolated protoplasts per g of tissue, using a Fuchs-Rosenthal chamber. The treatments were distributed in a completely randomized design, factorial arrangement of treatments 6 x 2 (enzyme solutions x hours) and nine replicates per treatment. Each experimental unit consisted of a Petri dish with 4 mL of solution. Analysis of variance and comparison of means (Tukey, $\alpha = 0.05$) were performed using the SAS System ® (Proc GLM) program.

Viability Tests

The number of viable protoplasts per g of tissue was determined with the Evans blue test (Gaff and Okong´o-Omgola 1971). For this purpose, 200 µL of the protoplast suspension were placed for 10 min on a hanging drop slide. One Evans blue 0.01% drop (approximately 100 µL) was subsequently added and allowed to stand for 20 min. The viability was assessed by the percentage ratio between the number of viable protoplasts and the number of isolated protoplasts per g of tissue, using a Fuchs-Rosenthal chamber. A descriptive analysis was performed using percentages obtained during tests. Each experimental unit consisted of 200 µL of protoplast suspension (at a concentration of 1 $x10^5$ protoplasts/mL) with six replicates per treatment.

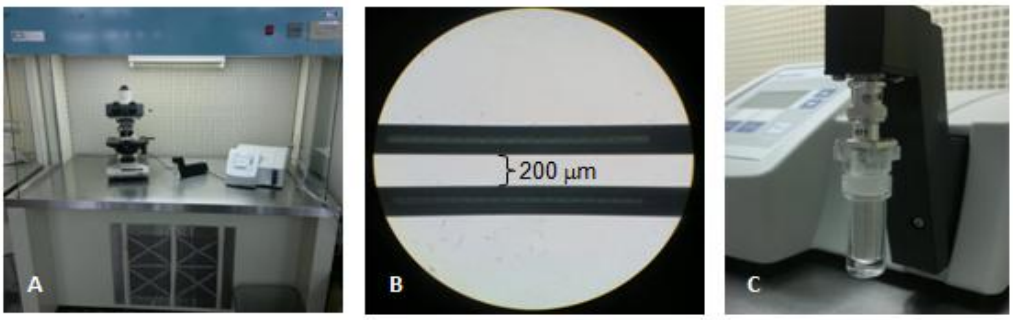
Protoplasts Electrofusion

Isolated and purified protoplasts were centrifuged at 800 rpm for 5 minutes, the supernatant was discarded and the pellet was suspended in hypoosmolar solution (Eppendorf ®, AH, GE) to a concentration of 1 $x10^6$ protoplasts/mL. Alignment and Testing electrofusion process (2 MHz, 100 V/cm) was performed on mesophyll protoplasts - etiolated PLBs mixtures from the two species tested (*V. planifolia* + *V. pompona*).

To determine the necessary alignment voltage (U1), 200 µL of each protoplast mixture were placed on the electrofusion chamber (see Figure 1). Different AC voltages (5 to 10 V increased by 1 V) at constant 60 seconds were evaluated. A descriptive analysis was performed using alignment rates observed in the visual field of the microscope during tests. Each experimental

unit consisted of 200 µL protoplast suspension with six replicates per treatment.

Figure 1. *Equipment Used for Electrofusion of Protoplasts Obtained from Vanilla Leaf Explants and PLBs (A. Workstation; B. Electrofusion Chamber, 40X; C. Helix Chamber)*



For the electrofusion process, different fusion parameters (Table 2) were evaluated. Process efficiency was evaluated by the number of fusion events counted (200 µL at 1 x10^6 protoplasts/mL) during the post-alignment (U2) in the electrofusion chamber. Fusion events were performed with protoplasts from different explants (*V. planifolia* leaf segments and PLBs *V. pompona* or vice versa) in order to facilitate the identification (by coloration or size) of fusion events obtained by microscopic observations. The treatments were evaluated by descriptive analysis of efficiency rates observed in the visual field of the microscope during tests. Each experimental unit consisted of 200 µL of fusion events in solution with six replicates per treatment.

Table 2. *Electrofusion Parameters Used for Vanilla Protoplasts Obtained from Leaf Explants and PBLs (Modified from Montero, 2009)*

Parameter	Fusion (Un)		
	Voltage (V)	Time (µs)	n [1]
A	170	25	2
B	170	25	3
C	170	30	2
D	170	30	3

[1] n = number of DC pulses which underwent fusion events.

Once the proper alignment voltage was established, fusion tests were performed with the parameters described above. For this, approximately 700 µL protoplast mixtures were placed in the Helix electrofusion chamber (Figure 1). Finally, 700 µL, containing fusion events were pipetted and plated on culture medium for subsequent regeneration.

Culture and Regeneration of Fusion Events

The fusion products were distributed by dripping on culture medium containing 50% MS salts supplemented with MS vitamins, 1% $CaCl_2$, 1 mg/L BA, 1 mg/L 2,4-D, 0.2 M mannitol, 0.03 M MES, 1 g/L CH, 20 g/L sucrose and 6.2 g/L of agar as gelling agent (pH 5.7). Osmotic potential was gradually decreased (every third day) by adding fresh medium with variations of 50% (0.1 M), 25% (0.05 M) and without mannitol added to the culture medium as an osmotic regulator. Microcalli were grown at 25 ± 2 °C in diffuse light conditions (16 h light photoperiod, 4.3 μmol/m^2s). The process was evaluated by the efficiency rates between the number of protoplasts presenting cell division and the amount of protoplasts placed (1 mL at 1 x10^6 protoplasts/mL). Each experimental unit consisted of 700 μL protoplast solution with six replicates per treatment.

Results and Discussion

Protoplasts Isolation

Once the isolation and purification protocols were optimized (see Montero 2009), the different enzyme solutions were tested, in order to maximize the protoplasts yield. Experiments were first conducted with leaf explants of the two vanilla species studied. Afterwards, the best enzyme solution was used for PBL protoplast isolation.

For *V. planifolia* protoplast isolation, the enzyme solution B (1% cellulase, 1% hemicellulase and 0.5% pectolyase) showed the highest yields for 3 to 4 hours of digestion (4.1 x10^5 ± 0.3 x10^5 and 3.2 x10^5 ± 0.2 x10^5 protoplasts/g tissue, respectively, Figure 2). The lowest number of protoplasts was obtained with the enzyme solution E (1% cellulase and 1.5% macerozyme), with 0.5 x10^5 ± 0.1 x10^5 protoplasts/g tissue at 4 hours digestion (see Figure 2). Similarly, the *V. pompona* protoplast isolation showed the highest yields with in the enzyme solution B for 3 and 4 hours of digestion (4.0 x10^5 ± 0.4 x10^5 and 3.2 x10^5 ± 0.3 x10^5 protoplasts/g tissue, respectively). The lowest number of isolated protoplasts was obtained with the enzyme solution D (1 % cellulase and 1% macerozyme) with 4 hours digestion, presenting yields of 0.4 x10^5 ± 0.2 x10^5 protoplasts/g tissue (see Figure 3).

Figure 2. *Protoplast Number Obtained from Six Enzyme Solutions Evaluated in V. planifolia (3 and 4 Hours Digestion). Similar Letters Indicate Absence of Significant Differences (Tukey, P = 0.0001). Bars Represent the Standard Deviation*

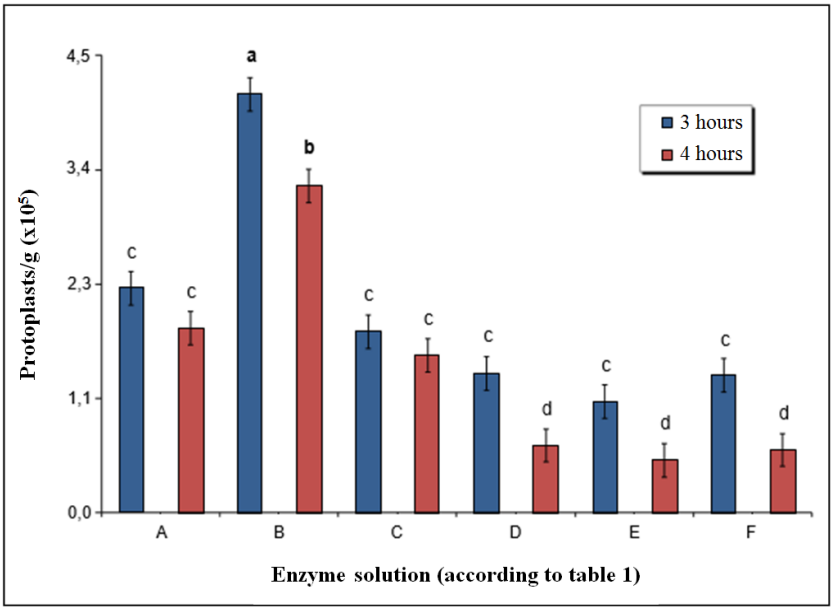
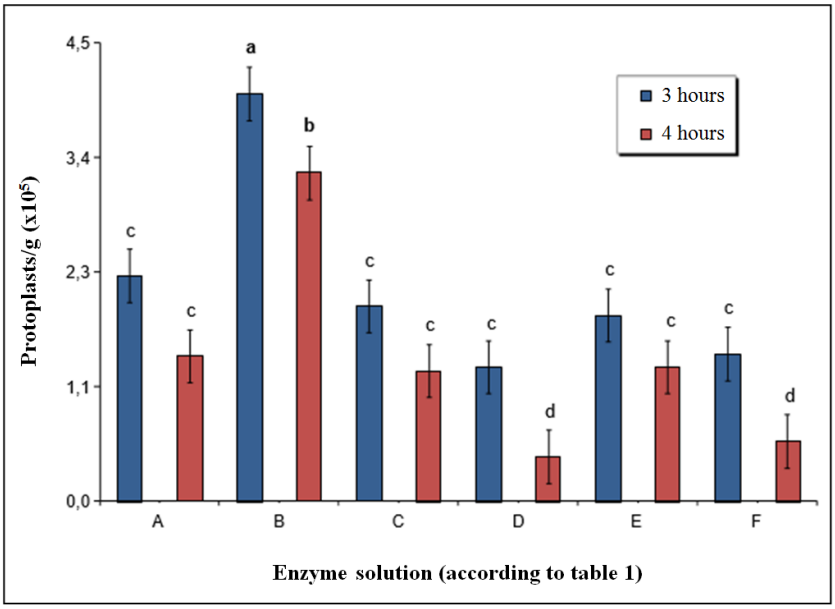


Figure 3. *Protoplast Number obtained from six enzyme solutions evaluated in V. pompona (3 and 4 hours digestion). Similar letters indicate absence of significant differences (Tukey, P = 0.0001). Bars represent the standard deviation*



Yields obtained during *V. planifolia* protoplast isolation from leaf mesophyll in this study were similar to those reported by Price and Earle (1984), with values up to 1.8 x10^5 protoplasts/g fresh leaf tissue using an

enzyme solution of 2% celulisine with digestion times of five hours. Divakaran *et al.* (2008) reported performance of up to 2.5 x10^5 protoplasts/g for *V. planifolia* leaf tissue, using purified enzyme solution of cellulase 2% and 0.5% macerozyme digestion with periods up to 8 hours. No data on *V. pompona* protoplast isolation has been reported in the literature up to now. However, the number of protoplasts isolated in this study was similar to that reported for *V. planifolia* (Price and Earle 1984) and *V. wightiana* (1.6 x10^5 protoplasts/g fresh tissue; Seeni and Abraham 1986, cited by Arditti and Ernst 1993).

In monocots, the cell wall has a large number and variety on hemicelluloses and a smaller range of pectins (Ishii, 1989). This composition possibly facilitated the digestion with pectolyase and hemicellulase, increasing the yield of isolated protoplasts in both species at the highest concentrations tested for these enzymes (1% and 0.5% respectively). According to Ishii (1989), although the presence of cellulase is sufficient for the degradation of the cell wall in monocots, addition of pectolyase improved performance on the isolated protoplast yield. In *V. andamica*, adding hemicellulase increased the number of protoplasts isolated from leaf explants (Divakaran *et al.* 2008).

Utilization of the enzyme solution B for the isolation of protoplasts from PLBs showed the following yields: For *V. planifolia*, 2.9 x10^5 ± 0.7 x10^5 protoplasts/g tissue, and for *V. pompona* 2.5 x10^5 ± 0.8 x10^5 protoplasts/g tissue (see Figure 4). No significant differences (Tukey, P = 0.7407) occurred between the two vanilla species evaluated when enzyme solution B was used, while highest yields were consistently obtained with leaf explants.

Protoplasts isolated from leaf explants, in both *V. planifolia* and *V. pompona*, showed characteristic green color of the chloroplasts presence in mesophyll cells. Protoplasts isolated from PBLs exhibited a creamy color due to the absence of chloroplasts and the presence of protoplastids (see Figure 5).

Figure 4. *Number of Protoplasts Obtained in the Isolation from Two Explants (Leaf and PLB) in the Two Vanilla Species Tested (3 Hours of Digestion) with Enzyme Solution B. Similar Letters Indicates Absence of Significant Differences (Tukey, P < 0.05). The Bars Represent the Standard Deviation*

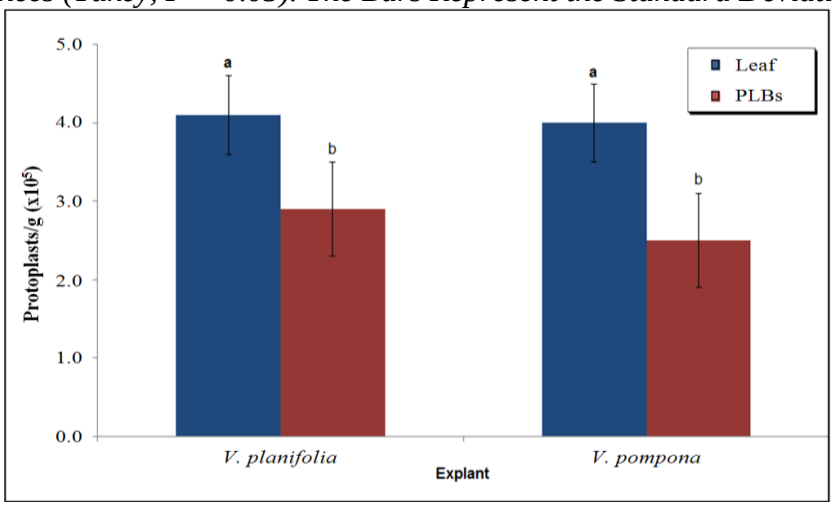
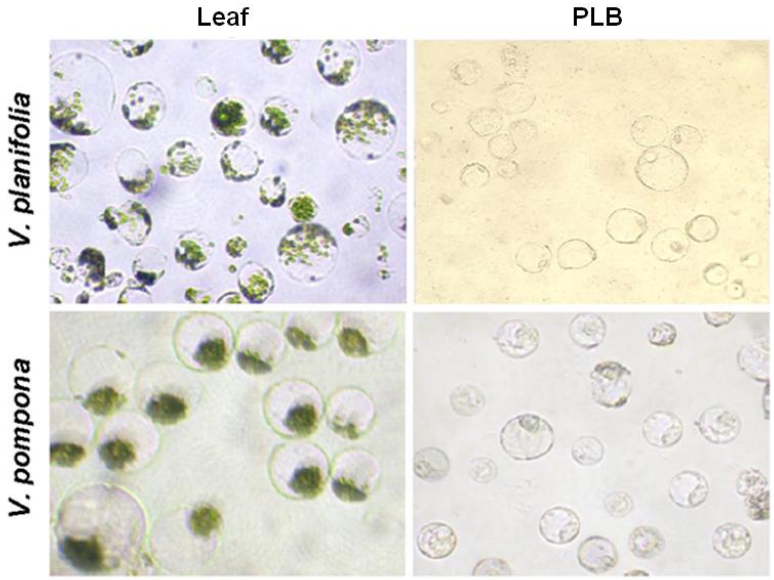


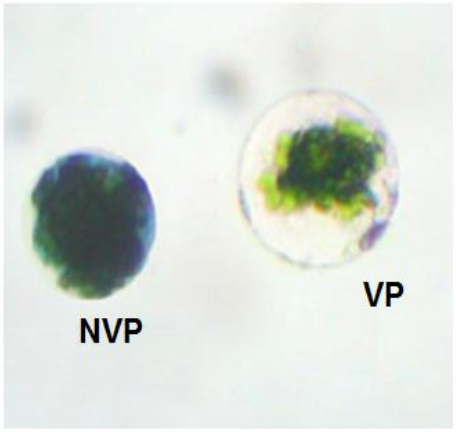
Figure 5. *Protoplasts Obtained from Two Explants (Leaf and PLB) in the Two Vanilla Species Tested (After 3 Hour Digestion with Enzyme Solution B, 100X)*



Viability Tests

Viability tests were performed by applying 0.01% Evans´ blue as described by Gaff and Okong'o-Omgola (1971). In this test the dye enters the cytoplasm of isolated protoplasts. After half an hour, the dye was removed from viable protoplasts, while non-viable protoplasts remain with a darker blue staining (see Figure 6).

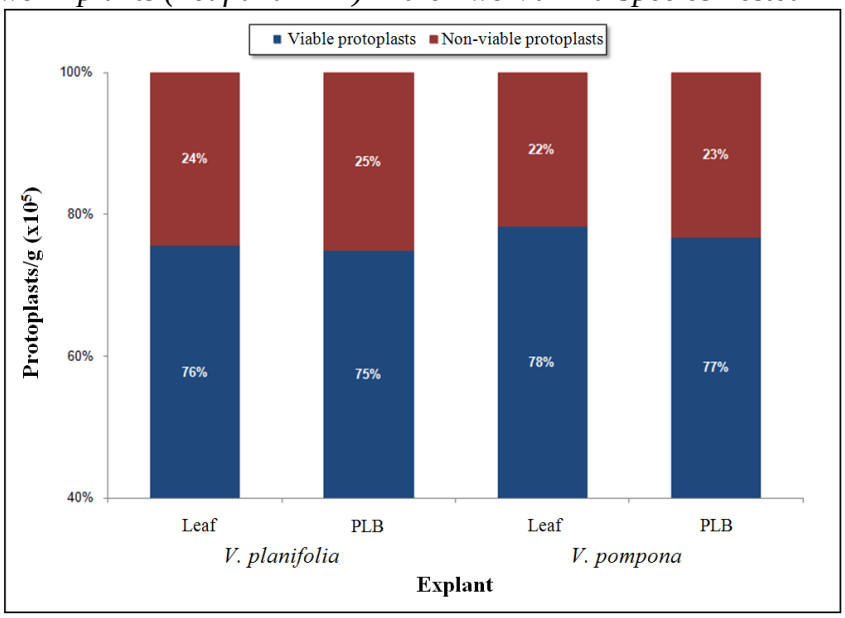
Figure 6. *Viability Testing Performed with 0.01% Evans´ Blue on Vanilla Purified Protoplasts (VP: Viable Protoplast; NVP: Non-Viable Protoplast; 400X)*



It was determined that the viability exhibited by leaf protoplasts was 76% for *V. planifolia* and 78% for *V. pompona*. For PLBs, the viability exhibited was 75% and 77%, respectively (see Figure 7).

The viability measured by 0.01% Evans´ blue depends primarily on the ability of plasmalemma of living or viable protoplasts to expel the dye from the cytoplasm, while non-viable or dead protoplasts retain the blue color inside (Gaff and Okong'o-Omgola 1971). High viability ratios increase the possibility of higher numbers of protoplasts regenerating their cell walls, which is a prerequisite for cell division under culture (Yasugi 1989). According to Divakaran *et al.* (2008), viability evaluated by fluorescein diacetate (FDA) showed a value of 72% for *V. planifolia* protoplasts, whereas for *V. andamica* it was 55%. The data obtained in this study shows great similarity with viability measured in a previous study (Montero 2009) for *V. planifolia* and *V. pompona* (between 75% and 81%).

Figure 7. *Viability Measured with 0.01% Evans´ Blue on Purified Protoplasts from Two Explants (Leaf and PLB) in the Two Vanilla Species Tested*



Protoplasts Electrofusion

Prior to fusion, alignment tests were performed in order to evaluate the effect of voltage on the alignment rate (see Figure 8). All voltage parameters evaluated on hipoosmolar solution showed alignment events. The 8 V test presented the highest number of alignments (21.87%) compared to other voltages evaluated (see Figure 9).

Figure 8. *Alignment of Purified Protoplasts from Two Explants (V. planifolia Leaf and V. pompona PLB, 40X)*

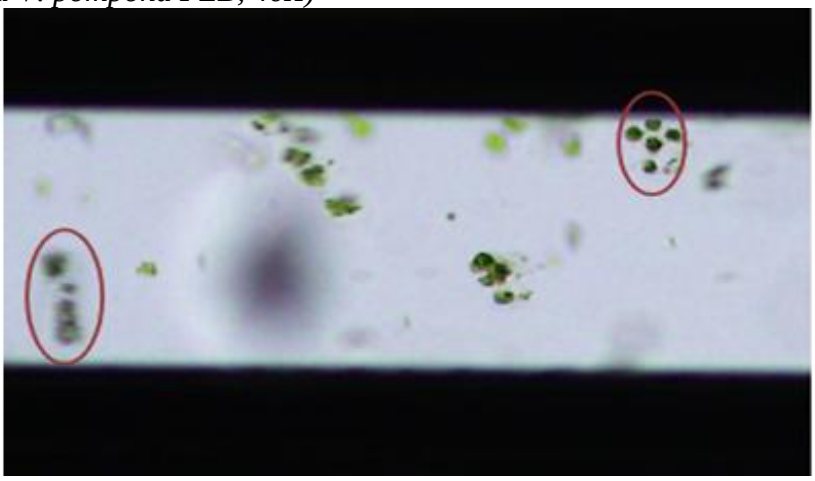
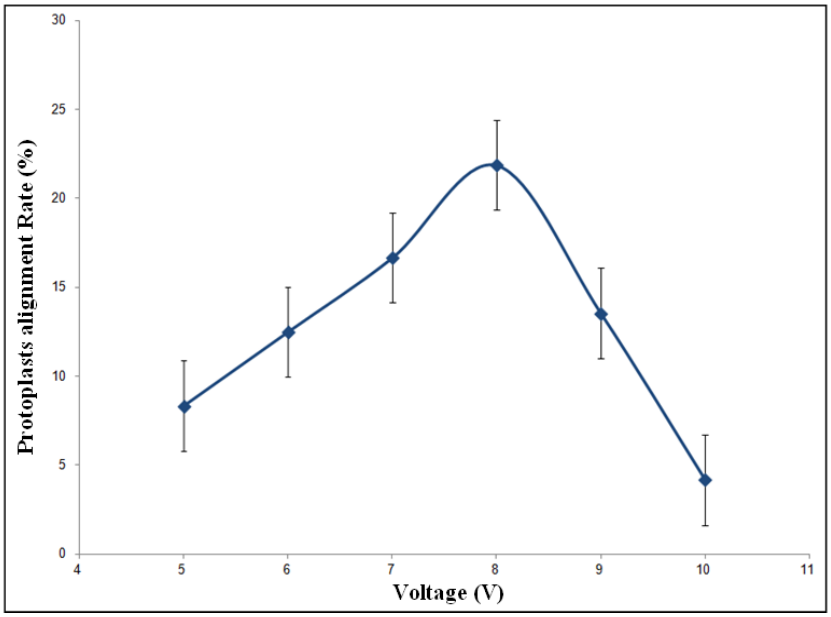


Figure 9. *Alignment Rate of Protoplasts Between Two Explants (V. planifolia Leaf and V. pompona PLB). Bars Represent the Standard Deviation*

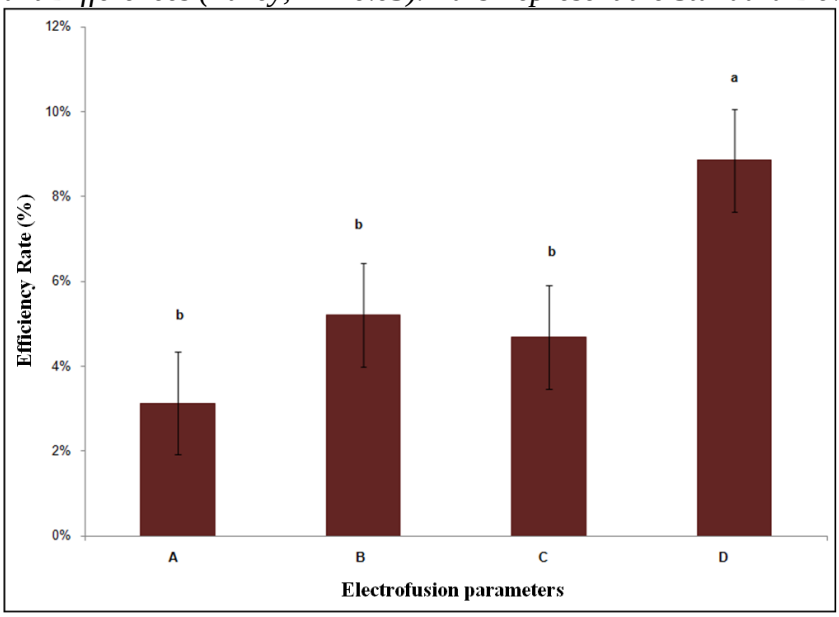


Once alignment voltage was established, electrofusion tests were conducted. Within these tests, alignment (U1) and post-alignment (U2) parameters were set to 8 V under standardized 60 seconds, in order to define the electrofusion parameters assay (Un, see Table 2). Among the electrofusion conditions evaluated, the highest of fusion events rate (8.9%) was observed with the D parameters (Un = 170 V, 30 microseconds, n 3) compared to the other treatments evaluated (see Figure 10).

During the alignment process, the protoplasts migrated due to bipolar charge generated by the alternating current applied; forming chains of aligned protoplasts (see Figure 8). Standardized periods of alignment and post-alignment allowed formation of higher number of chains, which facilitated the

process of electrofusion. By applying a direct current to the formed chains (Un), the plasmalems regions from different protoplasts that were in contact suffered localized electroporation due to this voltage (Biedinger *et al.* 1990, Van Wert and Saunders 1992, Blackhall *et al.* 1996). In a low conductivity medium, such as the hypoosmolar solution, media prevents that the voltage exceed the critical electric field needed for electrofusion, preventing the destruction of protoplasts (Jaroszeski *et al.* 1994).

Figure 10. *Electrofusion Efficiency Observed when Evaluating Different Electrofusion Parameters with Purified Protoplasts from Two Explants (Leaf and PLB) for Both Vanilla Species Studied. Similar Letters Indicate Absence of Significant Differences (Tukey, P < 0.05). Bars Represent the Standard Deviation*



Culture and Regeneration of Fusion Events

Preliminary studies (see Montero 2009) allowed optimization of the culture conditions for fusion events. Therefore, fusion events were directly plated on the agar medium described below and maintained with diffuse light. Fusion parameters D (A = 170 V, 30 µs, n 3) showed the highest microcalli number observed (9.4%), while fusion parameters A (A = 170 V, 25 µs, n 2) produced the lowest number of microcalli (see Figure 11). Microcalli grew constantly for 12 weeks after been plated (see Figure 12).

To culture fusion events obtained from *V. planifolia* and *V. andamica*, Divakaran *et al.* (2008) proposed the use of liquid MS salts and vitamins, supplemented with 0.5 mg/L of BA, 0.5 mg/L of indole-3-butyl acid (IBA), 7% mannitol and 3% sucrose (pH 5.7), under agitation. Yasugi (1989) reported the importance of supplying plant growth regulators (NAA, BA and 2,4-D) to stimulate the growth of microcalli obtained by fusion between *Dendrobium*, *Epidendrum*, *Paphiopedilum* and *Cattleya*.

Figure 11. *Regeneration Efficiency for Protoplast Fusion Events between Two Explants (V. planifolia Leaf and V. pompona PLB). Similar Letters Indicate Absence of Significant Differences (Tukey, $P < 0.05$). Bars Represent the Standard Deviation*

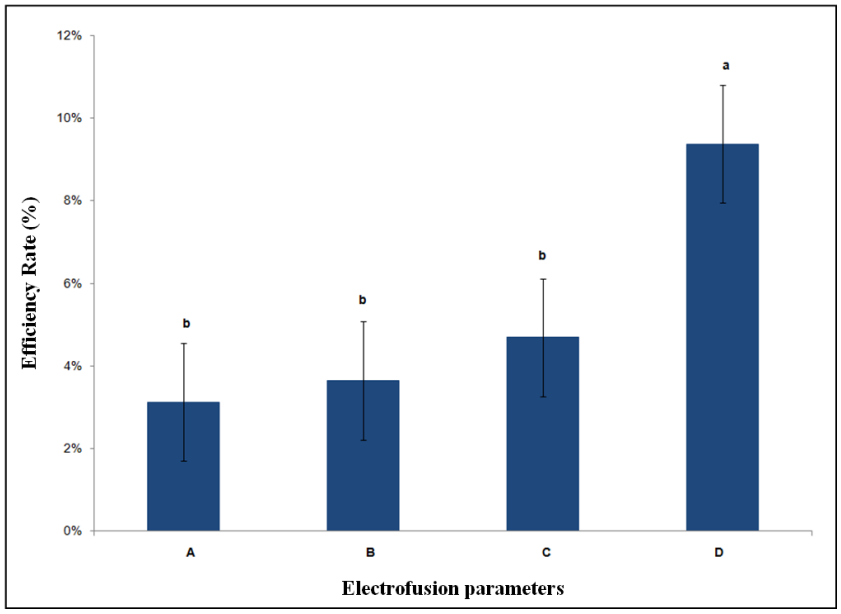
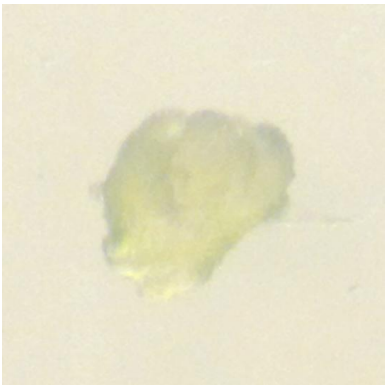


Figure 12. *Developed Microcallus from Fusion Events between V. planifolia Leaf Protoplasts and V. pompona PLB Protoplasts (12 Weeks After Been Plated, 40X)*



In this study, microcalli were cultured on a 50% MS semi-solid medium supplemented with 50% MS vitamins, 1% $CaCl_2$, 1 mg/L BA, 1 mg/L 2,4-D, 0.2 M mannitol, 0.03 M MES, 1 g/L CH, 20 g/L sucrose and 6.2 g/L of agar as gelling agent (pH 5.7) in order to stimulate their regeneration. The gradual reduction of osmotic potential by adding fresh medium allowed microcalli growth. The microcalli resulted from fusion events are currently being maintained at the Plant Biotechnology Laboratory of the CIDASTH to promote their growth and development, looking at their regeneration, and evaluation to determine the presence of somatic hybrids.

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