Vanilla (*Vanilla planifolia* Jacks.) cell suspension cultures: establishment, characterization, and applications

Marco A. Ramírez-Mosqueda¹ · Lourdes G. Iglesias-Andreu¹

Received: 3 February 2017 / Accepted: 6 July 2017 / Published online: 13 July 2017
© Springer-Verlag GmbH Germany 2017

Abstract The establishment and characterization of cell suspension cultures are an in vitro culture technique very useful for various plant biotechnological applications (production of secondary metabolites, mass micropropagation, protoplast isolation and fusion, gene transfer and the investigation of cell pathways). The objective of this study was to establish and characterization of cell suspension cultures of *V. planifolia* by inducing friable calluses. For that, friable calluses were obtained from immature seeds cultivated in MS medium supplemented with 0.45 μ M thidiazuron (TDZ). The effect of benzyladenine (BA) in different concentrations was evaluated. Cultures were incubated under photoperiod at continuous stirring at 120 rpm on an orbital shaker. The optimal condition found for biomass growth in suspension cultures was 0.5 g of inoculum density (fresh weight) in MS liquid, supplemented with 8.88 μ M BA. The growth kinetics of the cell suspension culture revealed a maximum cell growth (exponential growth phase) at day 16 and an 80% cell viability. The establishment and characterization of cell suspension cultures of *V. planifolia* constitute the bases of future studies and above all a better biotechnological use of this crop.

Keywords Friable calluses · Suspension culture · Cell viability · Growth kinetics

Introduction

The vanilla culture (*Vanilla planifolia* Jacks.) is economically important because of vanillin, an organic compound extracted from fruits that is highly appreciated in the food and cosmetic industry (Ramírez-Mosqueda and Iglesias-Andreu 2015). There are a limited amount of biotechnology studies on *V. planifolia* that allow a better use of this species (Retheesh and Bhat 2011; Ramírez-Mosqueda and Iglesias-Andreu 2015). The optimal establishment of cell suspension cultures (CSC) in this spice comprises the characterization and growth kinetics of CSC (Mathur and Shekhawat 2013; Kshirsagar et al. 2015). However, there is no efficient protocol for the establishment and characterization of cell suspension cultures in *V. planifolia*. Therefore, there is no adequate biotechnological use in this crop of high commercial interest.

In this context, CSC are an effective tool for various biotechnological applications, such as the production of secondary metabolites (Xu et al. 2011; Dwivedi et al. 2016a, b; Giri, Zaheer, 2016), in vitro selection of cells resistant to biotic or abiotic stress (Mahlanza et al. 2013; Naliwajski and Skłodowska 2013; Barbasz et al. 2016), massive micropropagation (Morais-Lino et al. 2016), gene transfer, and investigations on cell pathways (Dubrovina et al. 2016), protoplast isolation and fusion (Cai and Kang 2014; Mujib et al. 2014).

On the other hand, friable callus was reported as an ideal source of explants for the establishment of CSC of various plant species, since these disintegrate freely (Cai and Kang 2014; Mujib et al. 2014; Dwivedi et al. 2016a, b; Kumar et al.

✉ Marco A. Ramírez-Mosqueda
marcoa.rm.07@gmail.com

✉ Lourdes G. Iglesias-Andreu
liglesias@uv.mx

¹ Instituto de Biotecnología y Ecología Aplicada (INBIOTECA), Universidad Veracruzana, Av. de las Culturas Veracruzanos No. 101, Campus para la Cultura, las Artes y el Deporte, Col. Emiliano Zapata, 91090 Xalapa, Veracruz, Mexico

2016). Currently, the establishment of CSC of *V. planifolia* was limited because only to have protocols for obtaining callus with compact appearance (Janarthanam and Seshadri 2008; Tan et al. 2011, 2013). However, although protocols for the production of friable calluses from *V. planifolia* are currently unavailable, there are reports about the establishment of cell suspension cultures (Westcott et al. 1994; Havkin-Frenkel et al. 1996). To note, some of these protocols make no reference to the type of explant used, while others do not use friable calluses. In addition, these studies were aimed only on obtaining secondary metabolites (vanillin) and have not considered the possibility of selecting cell lines with specific characteristics.

Ramírez-Mosqueda and Iglesias-Andreu (2015) succeeded in broadening the genetic base of this crop through the induction and regeneration of friable calluses. As a continuation and use of these findings (obtaining of friable callus), this investigation aims to develop a protocol for the establishment and characterization of cell suspension cultures from friable calluses of *V. planifolia* to contribute to biotechnology studies of this crop.

Materials and methods

Plant material

For that immature seeds (approximately 7 months of maturity) are used as explants. Before to in vitro establishment, capsules of *V. planifolia* (Mansa morphotype) were collected from the Papantla region, Veracruz, Mexico. Subsequently they were washed with tap water and liquid detergent for 10 min. Then, in preparation for planting, capsules were cleaned with 75% ethanol four times in a laminar flow hood; afterwards, these were cut transversally and immature seeds were transferred to the callus induction medium using sterile scalpels.

Callus induction

Friable callus was induced following the methodology proposed by Ramírez-Mosqueda and Iglesias-Andreu (2015). The MS (Murashige and Skoog 1962) culture medium supplemented with 50 mg L⁻¹ L-cysteine hydrochloride, 100 mg L⁻¹ ascorbic acid, and 30 g L⁻¹ sucrose was used; 2.2 g L⁻¹ Phytigel® as gelling agent was used; 0.45 µM thidiazuron (TDZ) was used as plant growth regulator (PGR). The medium pH was adjusted to 5.8 ± 0.2 and culture flasks were autoclaved at 1.5 kg cm⁻² and 121 °C for 15 min. Cultures were incubated at 25 ± 2 °C under an irradiation of 50 µmol m⁻² s⁻¹ provided by fluorescent lamps, under photoperiod (16 h light/8 h dark).

Establishment of cell suspension cultures

0.5 g per flask (10 g L⁻¹) of fresh (30-day-old) friable calluses was disintegrated in 250 mL Erlenmeyer flasks. Flasks containing 50 mL of liquid MS medium supplemented with 250 mg L⁻¹ L-cysteine hydrochloride, 100 mg L⁻¹ ascorbic acid, and 20 g L⁻¹ sucrose were used. The effect of different concentrations of benzyladenine (BA) (0, 6.66, 8.88 and 11.11 µM) was evaluated. The medium pH was adjusted to 5.8 ± 0.2 and culture flasks were autoclaved at 1.5 kg cm⁻² and 121 °C for 15 min. Cultures were incubated at 25 ± 2 °C under an irradiation of 50 µmol m⁻² s⁻¹ provided by fluorescent lamps, under photoperiod (16-h light/8-h dark). Cell cultures were maintained under continuous stirring at 120 rpm on an orbital shaker.

For each treatment, the fresh and dry weight of cell suspension cultures was measured at day 14 of culture. Furthermore, the density of cell aggregates was determined (number of cells mL). To determine fresh weight (FW), each flask was centrifuged at 12,000 rpm for 10 min, and then cells were separated from the medium by filtration using Whatman No. 1 filter paper, and weighed. Afterwards, cells were dried to constant weight at 60 °C for 24 h, and cell dry weight (DW) was recorded. The density of cell aggregates was determined by counting in a Neubauer chamber. Additionally, cell viability was determined with the Evans blue dye test (Rodríguez-Monroy and Galindo 1999).

Growth kinetics of cell suspension cultures

Cultures were maintained in the culture media for 6 months inside the growth chamber, under conditions standardized for optimal cell growth. Subcultures were performed every 28 days using a 10% (v/v) cell inoculum in 150 mL Erlenmeyer flasks containing 50 mL of cultured medium.

The growth kinetics of cell suspension cultures was determined by disintegrating 0.5 g of friable callus in 250 mL Erlenmeyer flasks. Flasks contained 50 mL of liquid MS medium supplemented with 8.88 µM BA, 250 mg L⁻¹ L-cysteine hydrochloride, and 20 g L⁻¹ sucrose. The medium pH was adjusted to 5.8 ± 0.2 and culture flasks were autoclaved at 1.5 kg cm⁻² and 121 °C for 15 min. Cultures were incubated under the same conditions described above. Fresh weight (FW), dry weight (DW) and density of cell aggregates were subsequently evaluated according to the above techniques, at four-day intervals over the 28 days of cultivation. In addition, cell viability was determined by the Evans blue dye test (Mathur and Shekhawat 2013).

To check for anomalies or abnormalities in cultured cells, these were examined after 28 days of cultivation. To observe cells in the suspension culture, one drop (10 µL) of

liquid suspension was transferred directly to a slide and observed under a Leica® DM300 compound microscope.

Statistical analyzes

All experiments were established using a completely randomized design with five replicates. Each replicate consisted of a 250 mL Erlenmeyer flask containing 50 mL of MS medium and a 0.5 g inoculum of friable callus. All analyzes were performed in duplicate. The data obtained were processed statistically with the software IBM SPSS Statistics (version 21). An analysis of variance (ANOVA) followed by Tukey's test ($p \leq 0.05$) was performed. Data were tested for normality and variance homogeneity using the Kolmogorov–Smirnov and Levene tests, respectively. Variables that did not meet these assumptions were log-transformed (natural logarithm, ln).

Results

Callus induction and establishment of cell suspension cultures

A friable callus was obtained after 12 weeks of culture (Fig. 1a) according to the protocol of Ramírez-Mosqueda and Iglesias-Andreu (2015). Significant differences between treatments were observed at day 14 of cell culture. Cultures appeared yellow/white in color and displayed a slow growth (Fig. 1b, d). The higher increase in biomass, expressed in fresh weight (32.0 g L^{-1}) and dry weight (0.70 g L^{-1}), was observed when the culture medium was supplemented with $8.88 \mu\text{M}$ BA, followed by $11.11 \mu\text{M}$ BA, with 28.96 and 0.58 g L^{-1} fresh and dry weight, respectively (Table 1). Fresh and dry weight did not increase significantly in the control treatment (Table 1). As to the density of cell aggregates, a larger number of cells per mL (186.4×10^4) was observed in medium supplemented with $8.88 \mu\text{M}$ BA. In addition, this BA concentration led to a higher viability (84%) of cultured cells (Fig. 1D).

Growth kinetics of cell suspension cultures

Vanilla CSC was established (Fig. 1B). The growth curve of the *V. planifolia* CSC is shown in Fig. 2. Significant differences were observed in all variables with respect to the number of days of subculture (Fig. 2). The CSC was characterized by a 4-day lag phase (Fig. 1C), during which biomass reached only 12.6 g L^{-1} FW and 0.07 g L^{-1} DW (Fig. 2A, B). Subsequently, the cells entered an exponential growth phase (Fig. 1D), which continued until day 16 of culture. During this phase, the cultured cells attained the maximum growth, and an increase in biomass

accumulation (34.9 g L^{-1} FW and 0.68 g L^{-1} DW) was observed (Fig. 2A, B). The stationary phase was followed by a steady reduction in the variables assessed and a lower cell density after 16 days of culture.

The higher number of cells per mL was observed at day 16 of culture (1.99×10^6) during the exponential phase (Fig. 2d). Cell density declined gradually in the stationary phase. Furthermore, cell viability remained around 82% throughout the 16 days of culture (Fig. 2D).

Microscopically, no anomalies or abnormalities in cultured cells were observed (Fig. 1f). Initially cells were clustered in aggregates, but became subsequently disaggregated in the culture medium (Fig. 1c). Finally, after 6 months cultures appeared colored and cells showed a slow growth, indicative of a declining phase (Fig. 1e).

Discussion

This study achieved the establishment of cell suspension cultures of *V. planifolia* from friable calluses, with the purpose to contribute to further biotechnology research on this species. It is worth highlighting that previous reports about the establishment of cell suspension cultures for this species were not based on friable calluses (or at least this is not mentioned) as a source of explants, or were conducted to obtain secondary metabolites (Westcott et al. 1994; Havkin-Frenkel et al. 1996).

Our study ensured a successful establishment of CSC by inducing friable calluses. This is the explant most commonly used for the establishment of cell suspension cultures in various plant species. Examples of other studies include *Stevia* (*Stevia rebaudiana*) (Mathur and Shekhawat 2013), *Rhodiola* (*Rhodiola crenulata*) (Shi et al. 2013), coriander (*Coriandrum sativum*) (Mujib et al. 2014) and wild or red sage (*Lantana camara*) (Kumar et al. 2016). In addition, our study contrasts with the work by Westcott et al. (1994), who established cell suspension cultures from organized aerial roots of *V. planifolia*.

In this study, an aspect worth underlying is that optimum cell growth and development were attained in MS medium supplemented with BA. These results are consistent with those reported by Dwivedi et al. (2016a, b), who set CSC of *Stevia rebaudiana* in MS medium added with BAP and NAA. However, most studies on the establishment of CSC indicate that the use of 2,4-dichloro phenoxyacetic acid (2,4-D) is essential as PGR (Mathur and Shekhawat 2013; Kumar et al. 2016; Morais-Lino et al. 2016). In addition, our study contrasts with the report by Havkin-Frenkel et al. (1996), who used the V and W medium (Vacin and Went 1949) in the establishment of CSC in *V. planifolia*.

The success in the establishment of CSC is determined by the increase in the accumulation of biomass, expressed

Fig. 1 Establishment of a *Vanilla planifolia* cell suspension culture. **A** Callus from immature seeds, **B** *Vanilla planifolia* suspension culture grown in flasks, **C** Cell aggregates at the bottom of flask, lag

phase, **D** Exponential and stationary phase, **E** Declining phase, **F** Photomicrograph of a cell suspension culture ($\times 40$) Evans blue dye

Table 1 Effect of different concentrations of BA in the establishment of cell suspension cultures of *V. planifolia* after 14 days of culture

PGR (BA)	Fresh weight (g L ⁻¹)	Dry weight (g L ⁻¹)	Density (N ° cells/mL)	Viability (%)
0	10.88 $\pm$ 1.10 ^c	0.34 $\pm$ 0.01 ^c	50.7 $\times$ 10 ^{4d}	60 $\pm$ 2.1 ^c
6.66	21.86 $\pm$ 1.35 ^b	0.46 $\pm$ 0.01 ^{bc}	66.0 $\times$ 10 ^{4c}	55 $\pm$ 1.8 ^c
8.88	32.00 $\pm$ 1.05 ^a	0.70 $\pm$ 0.02 ^a	186.4 $\times$ 10 ^{4a}	84 $\pm$ 1.1 ^a
11.11	28.96 $\pm$ 1.74 ^a	0.58 $\pm$ 0.01 ^{bc}	156.7 $\times$ 10 ^{4b}	78 $\pm$ 1.5 ^{ab}

These values represent the mean $\pm$ SE (standard error). Means with different letters are significantly different (Tukey, $p \leq 0.05$)

as fresh weight and dry weight, number of cells per liter of medium, and percent cell viability. In our study, we observed significant differences in these variables relative to the control treatment, which indicate an optimal cell growth. These variables were assessed for the establishment of CSC of various plant species (Mathur and Shekhawat 2013; Döring and Petersen 2014; Kshirsagar et al. 2015; Dwivedi et al. 2016a).

The determination of the cell growth curve is a key element in the establishment of CSC (Mathur and

Shekhawat 2013; Kshirsagar et al. 2015). This study investigated the growth kinetics of cell suspension cultures of *V. planifolia*. In addition, the different cell growth phases were identified (4-day lag, exponential phase at day 16, stationary phase at day 20 and declining phase at day 24).

Cell cultures are fast-growing, and in several plant species the largest increase in biomass (exponential phase) occurs at Days 14–18 (Mathur and Shekhawat 2013; Kshirsagar et al. 2015). Our results showed the maximum

Fig. 2 Growth kinetics of cell suspension cultures of *V. planifolia*. **A** Fresh weight, **B** Dry weight, **C** Density of cell aggregates, and **D** Cell viability

growth and increase in biomass of the cell culture at day 16 of culture. Similar to our study, Mathur and Shekhawat (2013) recorded the exponential phase at day 14 of culture in CSC of *S. rebaudiana*, and Kshirsagar et al. (2015) at day 15 of CSC of *Swertia lawii*. However, some CSC needs a longer culture period (Shi et al. 2013; Wong et al. 2013; Qi et al. 2014). Separately, cell viability was stable at around 82% over the 16 days of culture. When cell viability is less than 50%, it is considered that the establishment of the CSC has failed (Mathur and Shekhawat 2013).

Conclusion

In conclusion, cell suspension cultures of *V. planifolia* were successfully established. Optimum cell growth and development depend on the type and concentration of PGR used, as well as on the time of subcultures. The establishment of CSC in this species may contribute to the development of other biotechnological applications such as the production of secondary metabolites, in vitro selection of cells resistant to biotic or abiotic stress, massive micropropagation, protoplast isolation and fusion, gene transfer, and investigations on cell pathways. In particular, these results are being used

for the production and regeneration of *V. planifolia* cell lines resistant to *F. oxysporum* f. sp. *vanilla*.

Authors' contributions LGIA and MARM conceived and designed research. MARM conducted experiments. MARM analyzed and reviewed the statistical analysis. MARM wrote the manuscript. LGIA and MARM read and approved the manuscript.

Acknowledgments The authors would like to thank the "Programa para el Desarrollo Profesional Docente (PRODEP)" for financial support provided for the project "Biotechnological Basis for the Genetic Improvement of *Vanilla planifolia*" within the "Conservation, Management and Plant Breeding network. MARM thanks the Consejo Nacional de Ciencia y Tecnología (CONACyT) for the grant scholarship No. 275736, which allows the realization of this work.

Conflict of interest The author(s) declare that they have no conflicting interests.

References

- Barbasz A, Kreczmer B, Oćwieja M (2016) Effects of exposure of callus cells of two wheat varieties to silver nanoparticles and silver salt (AgNO₃). *Acta Physiol Plant* 38:76
- Cai X, Kang XY (2014) Plant regeneration from cell suspension-derived protoplasts of *Populus × beijingensis*. *In Vitro Cell Dev Biol*. 50:92–98

- Döring AS, Petersen M (2014) Production of caffeic, chlorogenic and rosmarinic acids in plants and suspension cultures of *Glechoma hederacea*. *Phytochem Lett* 10:111–117
- Dubrovina AS, Aleynova OA, Kiselev KV (2016) Influence of overexpression of the true and false alternative transcripts of calcium-dependent protein kinase CPK9 and CPK3a genes on the growth, stress tolerance, and resveratrol content in *Vitis amurensis* cell cultures. *Acta Physiol Plant* 38:78
- Dwivedi S, Alam A, Shekhawat GS (2016a) Antioxidant response of *Stevia rebaudiana* (Bertoni) Bertoni (Angiosperms; Asteraceae) during developing phase of suspension cell culture. *Plant Sci Today* 3:115–123
- Dwivedi S, Alam A, Shekhawat GS (2016b) Relative production and quantification of stevioside from in vitro generated shoots, callus, suspension culture and synseeds of *Stevia rebaudiana* (bertoni) bertoni. *Plant cell Biotechnol Mol Biol* 17:3–4
- Giri CC, Zaheer M (2016) Chemical elicitors versus secondary metabolite production in vitro using plant cell, tissue and organ cultures: recent trends and a sky eye view appraisal. *Plant Cell Tiss Organ Cult* 126:1–18
- Havkin-Frenkel D, Podstolski A, Knorr D (1996) Effect of light on vanillin precursors formation by in vitro cultures of *Vanilla planifolia*. *Plant Cell Tiss Organ Cult* 45:133–136
- Janarthanam B, Seshadri S (2008) Plantlet regeneration from leaf derived callus of *Vanilla planifolia* Andr. *In Vitro Cell Dev Biol—Plant* 44:84–89
- Kshirsagar PR, Chavan JJ, Umdale SD, Nimbalkar MS, Dixit GB, Gaikwad NB (2015) Highly efficient in vitro regeneration, establishment of callus and cell suspension cultures and RAPD analysis of regenerants of *Swertia lawii* Burkill. *Biotechnol Reports* 6:79–84
- Kumar P, Chaturvedi R, Sundar D, Bisaria VS (2016) Piriformospora indica enhances the production of pentacyclic triterpenoids in *Lantana camara* L. suspension cultures. *Plant Cell Tiss Organ Cult* 125:23–29
- Mahlanza T, Rutherford RS, Snyman SJ, Watt MP (2013) *In vitro* generation of somaclonal variant plants of sugarcane for tolerance to *Fusarium sacchari*. *Plant Cell Rep* 32:249–262
- Mathur S, Shekhawat GS (2013) Establishment and characterization of *Stevia rebaudiana* (Bertoni) cell suspension culture: an in vitro approach for production of stevioside. *Acta Physiol Plant* 35:931–939
- Morais-Lino LS, Santos-Serejo JA, Amorim EP, Ferreira de Santana JR, Pasqual M, e Silva SDO (2016) Somatic embryogenesis, cell suspension, and genetic stability of banana cultivars. *In Vitro Cell Dev Biol* 52:99–106
- Mujib A, Tonk D, Ali M (2014) Plant regeneration from protoplasts in Indian local *Coriandrum sativum* L.: scanning electron microscopy and histological evidences for somatic embryogenesis. *Plant Cell Tiss Organ Cult* 117:323–334
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol Plant* 15:473–497
- Naliwajski MR, Skłodowska M (2013) Proline and its metabolism enzymes in cucumber cell cultures during acclimation to salinity. *Protoplasma* 251:201–209
- Qi Y, Du L, Quan Y, Tian F, Liu Y, Wang Y (2014) Agrobacterium-mediated transformation of embryogenic cell suspension cultures and plant regeneration in *Lilium tenuifolium* oriental 3 trumpet ‘Robina’. *Acta Physiol Plant* 36:2047–2057
- Ramírez-Mosqueda MA, Iglesias-Andreu LG (2015) Indirect organogenesis and assessment of somaclonal variation in plantlets of *Vanilla planifolia* Jacks. *Plant Cell Tiss Organ Cult* 123:657–664
- Retheesh ST, Bhat AI (2011) Genetic transformation and regeneration of transgenic plants from protocorm-like bodies of vanilla (*Vanilla planifolia* Andrews) using *Agrobacterium tumefaciens*. *J Plant Biochem Biot* 20:262–269
- Rodríguez-Monroy M, Galindo E (1999) Broth rheology, growth and metabolite production of *Beta vulgaris* suspension culture: a comparative study between cultures grown in shake flasks and in stirred tank. *Enz Microb Technol* 24:687–693
- Shi L, Wang C, Zhou X, Zhang Y, Liu Y, Ma C (2013) Production of salidroside and tyrosol in cell suspension cultures of *Rhodiola crenulata*. *Plant Cell, Tissue Organ Cult* 114:295–303
- Tan BC, Chin CF, Alderson P (2011) Optimisation of plantlet regeneration from leaf and nodal derived callus of *Vanilla planifolia* Andrews. *Plant Cell, Tissue Organ Cult* 105:457–463
- Tan BC, Chin CF, Alderson P (2013) Effects of sodium nitroprusside on shoot multiplication and regeneration of *Vanilla planifolia* Andrews. *Plant Cell, Tissue Organ Cult* 105:457–463
- Vacin EF, Went EW (1949) Some pH changes in nutrient solutions. *Bot Gaz* 110:605–613
- Westcott RJ, Chetham PSJ, Barraclough AJ (1994) Use of organized viable *Vanilla* plant aerial roots for the production of vanillin. *Phytochemistry* 35:135–138
- Wong SM, Salim N, Harikrishna JA, Khalid N (2013) Highly efficient plant regeneration via somatic embryogenesis from cell suspension cultures of *Boesenbergia rotunda*. *In Vitro Cell Dev Biol* 49:665–673
- Xu J, Ge X, Dolan MC (2011) Towards high-yield production of pharmaceutical proteins with plant cell suspension cultures. *Biotechnol Adv* 29:278–299