Cellular Forms in Cultivation in Suspension of Bougainvillea glabra Choisy Variety Surprise

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Abstract: The objective of this work was to describe the predominant cell forms in the phases of growth kinetics in a suspension culture of the Bougainvillea glabra Choisy variety Surprise. Treatments in suspension with Murashige and Skoog (MS) basal culture medium were supplemented with six different concentrations and combinations of vegetable growth regulator (VGR) of type auxin (2,4-D and NAA) and cytokinin (BAP) (0.2-5.3 mg/L) and a control. Friable callus was obtained from leaf explants ex vitro to in vitro culture in solid MS medium using Phytagel™ as gelling agent to 2.2 g/L. A portion of callus (1.0 g) was used as cellular inoculum and grown under controlled conditions (50 mL, 120 rpm, 25 °C and luminous intensity of 48 μmol m²/s). The best treatment with significant differences (p ≤ 0.05) were with a hormonal relation of 2:1 of BAP and NAA, respectively, with a fresh weight yield ranging from 1.7905 g to 5.8340 g, which represents around 70%. An adaptation phase was observed from day 0 to day 14 on the curve of fresh weight; an exponential phase at day 14 to day 19 and a declination phase at day 21. Cellular forms in the adaptation phase were elongated cells, a few globular and a few with kidney shape. In the exponential phase, these cells formed small aggregates of globular cells. In the death phase, brown, elongated, damaged and fragmented cells were found. With this data obtained it is possible to establish the subculture time in fresh medium.

Key words: Bougainvillea, kinetics of cell growth, in vitro culture.

1. Introduction

Plants have the capacity to produce secondary metabolites that play an important relationship between the plant and the environment that surrounds them, either as a defense or as a product of some environmental alteration or attraction to pollinate [1]. The Bougainvillea glabra Choisy variety Surprise of the genus Bougainvillea and family Nyctaginaceae [2] is native to the tropical and subtropical regions of South America (Brazil and Peru). It grows in areas of warm, semi-warm and mild weather [3], with different varieties that are distinguished by the habit of development, colors of the leaves and colored bracts (purple, white and yellow, among others) [4]. Besides ornamental, it is used in traditional medicine against respiratory diseases [5-7], gastric ulcers or antimicrobial activity [8, 9] and in some places, bracts and flowers are used as part of the diet [10]. The bracts contain betacyanins (red-violet pigments) that make Bougainvillea an alternative to obtain secondary metabolites of industrial interest [9], besides being used in the ornamental industry [11].
medicinal and ornamental importance of this plant, it is desirable to create biotechnological tools for the production of cell lines by means of cell cultures in suspension where it is to obtain pigment from callus tissue [12]. This type of culture allows us to investigate the different aspects of cell lines such as their metabolic, physiological and biochemical behavior, as well as learn how to control and optimize the conditions of the crop for the production of biomass in an in vitro culture and/or the production of secondary metabolites using subcultures that allow to extend the life of the cell lines for their use [13]. In vitro culture has the advantage of allowing a regeneration of complete plant from any tissue can, even grow from one cell; phenomenon known as “totipotencia”. Also, using these techniques, it is possible to spread plants in small quantities with adequate phytosanitary and nutritional quality [12, 14], while minimizing fungal contamination [15]. The cultivation of cells in suspension requires an initial process that includes the induction to form friable callous tissue and subsequently the establishment of cell suspension culture [16, 17]. To maintain a culture of cells in suspension in continuous growth, it is necessary to identify the appropriate moment in which the components of the culture medium are exhausted and must be renewed [18]. The appropriate stage is determined from a cell growth curve with respect to time [19]. In the cell growth curve, three phases can be observed at different growth rates [20]. The differences in the type of cell growth curves depend on the amount of inoculum, type of culture medium, agitation and the species. For example, in culture of cells in Uncaria tormentosa suspension a very short adaptation phase was identified [21]. The most used method to determine the cell growth curve is fresh weight and dry weight [22]. During the culture of cells in suspension, the production of secondary metabolites and the formation of compact callus aggregates (CCA) have been recorded [23]. There is also the hypothesis that the shape of the cells is a function of the species and may be associated with the production of secondary metabolites [1]. In this study the aim was to evaluate the predominant cellular forms during the phases of growth kinetics in suspension culture of B. glabra Choisy variety Surprise to estimate the subculture time in a fresh culture medium enhanced with vegetable growth regulator (VGR).

2. Materials and Methods

2.1 General Aspects

The research was performed at the Laboratory of Vegetable Tissue Cultures of the Centro de Desarrollo de Productos Bióticos of Instituto Politécnico Nacional (CEPROBI-IPN), located in San Isidro colony, with coordinates: latitude 18°49'44.278” N, longitude 99°5’34.296” W, altitude 1,064 m.a.s.l., Yautepec, Morelos, Mexico. The plants were grown under greenhouse conditions of 25 °C and 55% relative humidity.

2.2 Vegetal Material Treatment and Callous Induction

Explants used for the development of friable calluses were obtained from young leaves of B. glabra Choisy variety Surprise which is a species of Bougainvillea with presence of white bracts, roses or the presence of both colors as shown in Fig. 1. The explants were washed with a soapy solution (1% w/v) and were subsequently rinsed three times with distilled water. With the help of a metal perforator previously sterilized, the cuts of the explants were made 0.5 cm in diameter and washed for 20 min in a commercial 1.25% sodium hypochlorite solution (Cloralex®) in a laminar flow hood (VECO, Model: GVFL-B12, Morelos, México) [24]. The explants were incubated at 25 °C, under a photoperiod of 16 light hours for 8 h of darkness and
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**Fig. 1** Different bract colors of *Bougainvillea glabra* variety Surprise: a) pink, b) white and c) pink and white in the same bract.

**Table 1** Composition* of MS culture medium for callus induction.

<table>
<thead>
<tr>
<th>Component (macronutrients)</th>
<th>Component (micronutrients)</th>
<th>Component (vitamins &amp; aminoacids)</th>
<th>mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄NO₃</td>
<td>MnSO₄·4H₂O</td>
<td>Myoinositol</td>
<td>1,650</td>
</tr>
<tr>
<td>KNO₃</td>
<td>ZnSO₄·4H₂O</td>
<td>Thiamin</td>
<td>1,900</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>H₂BO₃</td>
<td>Nicotinic acid</td>
<td>440</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>KI</td>
<td>Riboflavin</td>
<td>370</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>FeSO₄·7H₂O</td>
<td>Glycine</td>
<td>170</td>
</tr>
<tr>
<td>-</td>
<td>Na₂EDTA</td>
<td></td>
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</tr>
</tbody>
</table>

*MS culture medium, Phytagel™ (as gelling agent 2.2 g/L), sucrose (30 g/L), pH 5.7. MS: Murashing and Skoog to 100 %(4.4 g/L).

**Table 2** Combination of growth regulators for the induction of callus in leaf explants of *Bougainvillea glabra* Choisy variety Surprise.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>VGR* (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BAP</td>
</tr>
<tr>
<td>T₁</td>
<td>1.0</td>
</tr>
<tr>
<td>T₂</td>
<td>2.0</td>
</tr>
<tr>
<td>T₃</td>
<td>2.7</td>
</tr>
<tr>
<td>T₄</td>
<td>2.7</td>
</tr>
<tr>
<td>T₅</td>
<td>5.3</td>
</tr>
<tr>
<td>T₆</td>
<td>-</td>
</tr>
</tbody>
</table>

*VGR: vegetable growth regulators; BAP: 6-benzylaminopurine; 2,4-D: 2,4-dichlorophenoxyacetic acid; NAA: 1-naphthaleneacetic acid; T₁: treatments (with different VGR combination); T₆: control (without VGR).

luminous intensity of 48 μmol m⁻²/s for 7 d without VGR. Afterwards, the explants were developed in 100% MS (4.4 g/L) culture medium with added sucrose (30 g/L), VGR for each treatment (Table 2) and phytagel™ (2.2 g/L) to pH 5.7 as measured with a potentiometer (JENCO, Electronics, LTD; Model 1671, San Diego, CA, USA).

2.3 Suspension Culture and Growth Kinetics

The suspended culture was established from the generated callus, the friable callus was formed in the culture medium without gelling agent. One gram of
Friable callus was inoculated with 50 mL of culture medium, in 125 mL Erlenmeyer flasks. The culture was maintained under the same incubation conditions as before (100% of MS culture medium, sucrose 30 g/L and VGR), with a constant stirring of 120 rpm in an orbital shaker (SEV, model AGO 60-40, Puebla, México) for a month. To determine the phases in the cell growth curve the contents of the flasks was leaked and fresh weight and dry weight was obtained. Whatman filter paper no. 1 was placed in a filtration funnel to collect the solids which were dried at 100 °C to determine the amount of biomass produced. The sample was monitoring every 2 h for 2 d until reaching a constant weight [26].

2.4 Morphology by Photonic Microscopy of Suspension Cells

The samples of the supernatant to determine the amount of biomass were stored in refrigeration at 5 °C inside Eppendorf tubes for observation under a photonic microscope (Eclipse 80i, Nikon®, Japan). A suspension of cells in deionized water was formed and used for the separation of cellular aggregates and elimination of the culture medium. Then the samples were placed in a slide and observed in a photon microscope, the structures present per field were evaluated at an increase of 4×, 10×, 20× and 40×, with three repetitions per sample [27].

2.5 Statistical Analysis

Statistical analysis of results was carried out using the MINITAB® 17 statistical package release 14.12.0 [28]. A one-way ANOVA analysis was performed using the Turkey method at a confidence level of 95%. There were nine samplings every 3 d with three repetitions.

3. Results and Discussion

3.1 Initial Assessment Criterion

The treatments with increased callus proliferation were selected using the evaluation criteria subjective of: null (-) 0%, little (+) 33.3%, moderate (++) 66.6%, abundant (+++) 100%. From the best treatments of where there was induced callus proliferation which was T2, it was continued with the second stage of the experiment, where the establishment of in vitro culture of explants from young leaf was of little in B. glabra Choisy variety Surprise as shown in Table 3.

3.2 Induction Callus Response

It was observed that treatment two and treatment three presented the best callus growth (66% and 100% induction), so treatment two was selected for further tests. It was observed that in all treatments there was necrosis which caused a slow proliferation of callus, with the exception of treatment two, which consisted of the addition of VGR mixture of BAP and NAA in concentration of 2.0 mg/L and 1.0 mg/L, respectively.

Initially, in all treatments on average it was obtained callus formation at day 26 after replanting (first isolation). In the treatment (T3) with BAP (2.7 mg/L) and 2,4-dichlorophenoxyacetic acid (2,4-D) (0.3 mg/L) there was proliferation of callus, but necrosis
was observed in the callused mass. Duhoky and Al-Mizory [29], reported that at six weeks (42 d) with BAP and NAA at a concentration of 2.0 mg/L and 0.2 mg/L, respectively, they obtained the highest percentage of callus induction (71.43%) in three species of Bougainvillea (B. buttian, B. spectabilis and B. glabra). However, these researchers did not report the varieties studied.

The treatment with 2 mg BAP and 1 mg NAA (T2), after 26 d in semi-solid culture, generated friable callus; at 40 d a yellow-amber coloration was observed (Fig. 2a). The changes in color during the generation of callus in the different stages of the growth curve presented variation in the pigments from the adaptation phase in the culture medium changed from yellow-amber to coffee color (Figs. 2b and 2c).

After sowing in suspension culture, also appears a yellow-amber coloration (Fig. 2d). This must have been mainly because the conditions of the donor plant should not present any symptom of disease and oxidation may occur during the preparation of the explants. Oxidation caused the slow proliferation of callus tissue, not so in T2, where there was also oxidation but in which an induction of callus was achieved after sowing. Difference in callus coloration may be due to the presence of cytokines in the culture medium, because these molecules promote synthesis of chlorophyll [30].

3.3 Kinetic Parameters on Growth Curve of Fresh and Dry Weight

The adaptation phase was identified qualitatively
from the growth curve as being from day 0 to day 12, where an increase in the production of biomass began to register more significantly (Fig. 3). The ANOVA analysis shows a significant difference between first two samples ($p < 0.05$), although at first glance it is not appreciable in the graph of cell growth curve. On days 5, 7 and 10 there were not significant differences, so it was considered that it was part of adaptation process to the nutritional conditions of the environment. The exponential growth phase started on day 12 and continued for 7 d more. The statistical analysis had a highly significant difference between day 12 and day 19 in fresh and dry weight ($p < 0.05$).

Trejo et al. [31] and Fierros et al. [18] reported that, it is because the components of the culture medium are spent. Similar results in the behavior in this phase were observed in suspension cultures of Solanum chrysotrichum [31], Uncaria tomentosa [21] and Medicago sativa [32].

The stationary phase (stable line) was very short in U. tomentosa (Willd.) D. C. [21] observed that the number of initial cells is related to the stationary phase which showed that a higher initial cell biomass causes short growth kinetics and when the initial cell number is low, generating a more extensive growth curve. In this study, 0.5 g of cells was used; possibly with a larger flask and volume of culture medium, greater availability of nutrients is favored [33]. In the stage of death, cellular fragments were observed, some with dark color and deformed. In the death phase [34], the cells showed damage to the cell membrane that caused loss of turgor pressure, showing an irregular wrinkled appearance with a deformed cell wall. The formation of aggregates is of great importance since large cell aggregates tend to change the metabolic characteristics of the culture such as the rheology of the broth [35].

3.4 Cellular Morphology of Cell Culture in Suspension

In the adaptation phase (14 d) of cell growth kinetics, elongated cells were mostly observed. Small globular aggregates were also seen, it is noteworthy that only in this phase the presence of kidney-shaped cells was observed (Fig. 4). In the phase of exponential growth it was determined that the cells in suspension formed large cell aggregates. Formation of aggregates is great importance, since large cell aggregates

![Fig. 3 Growth kinetics in fresh weight of cells of B. glabra Choisy variety Surprise.](image)

Stockings that do not share a letter are significantly different ($p < 0.05$).
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Fig. 4  Growth kinetics in dry weight and typical cells of *B. glabra* Choisy variety Surprise.
Stockings that do not share a letter are significantly different \( p < 0.05 \).

Fig. 5  Cell morphology in suspension of *B. glabra* Choisy variety Surprise: a) elongated cell; b) cell aggregate; c) and d) kidney-shaped cells with yellowish internal content; e) and f) cellular fragments. Micrographs to 10x.

Tend to change metabolic characteristics of the culture as well as rheology of the broth and culture medium with secreted metabolites [36].

In the fall of the curve, the cells presented physical damage, due to loss in turgor pressure, showing irregular cells, with swollen appearance, probably with deformed cell walls (Fig. 5). Trejo and Monroy [1] claim that this behavior is typical of cells in the death stage, and is reflected in a decrease in fresh and dry weight. The cells can form reactive oxygen
species; these cause oxidative stress in the cells and activate oxidases enzymes. They also release phenols causing a toxic effect that prevents proliferation, and if they are not removed from the culture medium they cause cell death [36, 37].

4. Conclusions

With leaf explants of B. glabra Choisy variety Surprise, with growth regulators the formation of friable callus was induced. And in the suspension culture, three phases of cell growth were identified with each growth phase; cellular forms in the adaptation phase were elongated cells, a few globular and a few with kidney shape. In the exponential phase, these cells formed small aggregates of globular cells. In the death phase, brown, elongated, damaged and fragmented cells were found. With these data obtained it is possible to establish the subculture time in fresh medium, which is estimated between 15 d and 17 d.

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