Oxidation and contamination control in the in vitro establishment of rosa chinensis

Controle de oxidação e contaminação no estabelecimento in vitro da Rosa chinensia

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ABSTRACT
Introduction and objectives: Rose is one of the most harvested and demanded species as ornamental flower; its traditional propagation is limited due to the spread of pests and diseases; for that reason, in vitro culture has turned out to be an alternative to propagate healthy plants. However, microbial contamination and tissue oxidation are critical problems that can limit in vitro plant establishment. The aim of this research was to evaluate disinfection protocols and culture conditions for in vitro regeneration of Rosa chinensis with low contamination and oxidation percentages. M&M: The explants were washed with different disinfectant agents and seeded in a basal medium interacting with different factors such as activated charcoal (1 g/L) and temporary darkness (3 days). Results: protocol 2 presented the lowest percentage of bacterial (18.75 %) and fungal (12.5 %) contamination in the disinfection phase; while treatment T3 (activated charcoal 1 g/L + 3 days of darkness) obtained the best results in the introduction phase, and the explants presented the lowest phenolization level (0.88), favoring the induction of buds and...
callus in the explants, and reaching averages of 81.25 and 31.25 %, respectively.

**Keywords:** morphogenesis, chinese Rose, activated charcoal, darkness period, tetracycline.

### 1 INTRODUCTION

*Rosa chinensis*, a plant species native to southwest China [1], is cultivated and sold worldwide as a cut flower or garden plant [2].

Roses can be propagated in two ways, the first involves the fusion of haploid gametes (sexual), and the second requires grafting, budding or rooted cuttings (asexual); however, these propagation systems may present some limitations, such as spread of pests and diseases (due to poor agricultural practices), slow and costly production, among others [3].

Plant tissue culture is a technique that allows rapid propagation by cloning seedlings with the best morphological and physiological characteristics through the organogenesis system [4, 5]; however, problems related with microbial contamination and oxidation in the tissues can threaten the success of *in vitro* culture.
The establishment and multiplication of explants can be limited by the presence of contaminating microorganisms [6], which can be introduced with the initial plant material (mother plant), due to its direct contact with the soil in field conditions [7]; during handling in the laboratory (poor conditions or aseptic techniques) or due to inefficient sterilization of plant material, culture media, instruments, among others [8, 9]. On the other hand, contaminating microorganisms can act as pathogens causing plant death, because they compete for space and nutrients [10]; some authors have proposed different alternatives to reduce their incidence, such as performing pre-treatments to the mother plant, using antibiotic or antifungal substances in the disinfection protocol [11], or adding antibiotics in the medium [12].

Oxidation happens because plants can synthesize a large amount of primary and secondary metabolites [13], among the latter are phenolic compounds that when oxidized by the action of the polyphenoloxidase enzyme (PPO) produce quinones (reactive species), which are responsible for generating a brownish color in plant tissues, and can even cause cell death [14]. To control oxidative problems, Azofeita [15] recommends using young explants, reducing the intensity of light, keeping the culture media in darkness for 2-3 days, using antioxidants (citric acid, ascorbic acid and cysteine), adding activated charcoal to the culture media, substituting disinfectant agents, among others.

The aim of this research was to evaluate disinfection protocols and culture conditions for the in vitro regeneration of R. chinensis, with low contamination and oxidation percentages.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL

The initial plant material consisted of Rosa chinensis plants in field conditions; their age, health and the highest number of buds per stem were considered for their selection.

2.2 DISINFECTION OF PLANT MATERIAL

Two disinfection protocols, combining different disinfecting agents, were evaluated for the disinfection of stems (Table 1).
Table 1. Disinfection protocols (P) applied to *R. chinensis* explants.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Protocol 1</th>
<th>Protocol 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration</td>
<td>Time</td>
</tr>
<tr>
<td>Captan (pre-treatments)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Water and soap</td>
<td>1%</td>
<td>3 min</td>
</tr>
<tr>
<td>Sodium hypochlorite</td>
<td>3%</td>
<td>1 min</td>
</tr>
<tr>
<td>Alcohol</td>
<td>80%</td>
<td>1 min</td>
</tr>
<tr>
<td>Captan</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.05%</td>
<td>1 min</td>
</tr>
</tbody>
</table>

The variables evaluated were percentage of bacterial contamination (formation of bacterial colonies on the explant and/or culture medium) and fungal contamination (fungal growth on the explant and/or culture medium).

2.3 INTRODUCTION PHASE

In this phase, axillary buds of *R. chinensis* were introduced in a plantain flour medium (30 g/L), supplemented with sucrose (30 g/L) and gelling agent (30 g/L). Three treatments were evaluated to determine the optimal culture conditions for the establishment of explants with low phenolization levels: T1= Control, T2= activated charcoal (1 g/L) and T3= activated charcoal (1 g/L) + darkness (dark period for 3 days).

Once the explants were inoculated, cultures were placed in a growth room under controlled conditions with a temperature of 24±2 °C, with 18 watts of white fluorescent light and a photoperiod of 16 hours of light and 8 hours of darkness (T1 and T2) and temporary darkness of 72 hours (T3). The variables evaluated were: phenolization level, using the following scale: (1) low level of phenolic oxidation (10 - 30 % of explant volume), (2) moderate phenolic oxidation (30 - 60 % of explant volume), (3) high phenolic oxidation (> 60 % of explant volume); shooting and callogenesis percentage.

The experimental unit consisted of a test tube with culture medium and a rose bud, each treatment with twelve replicates.
2.4 EXPERIMENTAL DESIGN

A completely randomized design (CRD) was applied with five replicates per treatment, both for the disinfection phase and the introduction phase. A linear (relationship between phenolic oxidation level and shooting) and polynomial (relationship between phenolic oxidation level and callogenesis) regression analysis was performed, with a significance level of \( p \leq 0.05 \).

3 RESULTS AND DISCUSSION

3.1 DISINFECTION PROTOCOL

3.1.1 Bacterial and fungal contamination

When analyzing bacterial and fungal contamination in the disinfection process, it was observed that protocol 2 presented lower bacterial (18.8 %) and fungal (12.5 %) contamination; unlike protocol 1, which presented a high percentage of bacterial (62.5 %) and fungal (90.6 %) contamination (Fig. 1).

![Fig. 1. Bacterial and fungal contamination in *R. chinensis* explants subjected to disinfection protocol 1 (P1) and protocol 2 (P2).](image)

The disinfection performed in protocol P1 showed a high contamination percentage because some chemical agents used only disinfect superficially the tissues [16]. However, researchers suggest disinfection of explants with different chemical agents such as alcohol, sodium hypochlorite (NaOCl), among others [17].

While protocol P2, by including a fungicide in both the pre-treatment and disinfection protocols, and an antibiotic in the disinfection process, even eliminated...
endogenous contaminants, which can be found latently in the internal tissues of the plant material and activated when the conditions of the medium are optimal [18]. The results are in agreement with several researchers who suggest pretreatment of donor plants with fungicides or pesticides in the prepropagation phase (phase 0), in order to attenuate in vitro contamination without affecting the viability of explants [19]; they also propose the addition of fungicides, bactericides and antibiotics with different action spectra [8].

The use of the active ingredient Captan during the disinfection phase decreased fungal contamination by approximately 78%. Captan is a protective and curative fungicide, it effectively controls low infection levels, it hinders fungal growth and development, as it interferes in several chemical reactions, inhibiting the process of cellular respiration and spore germination [20]. Some authors point out that the high concentration of contaminating microorganisms in explants is related to the phytosanitary status of the donor plant, for that reason, they indicate that the application of fungal substances in the pre-propagation phase (pretreatment) has a positive effect, since they decrease the infection processes and the efficiency of this phase increases [21, 22].

The use of tetracycline as an antibiotic treatment in this assay reduced bacterial contamination by approximately 44%, maybe because of the antimicrobial activity of tetracyclines, which prevent the assembly of peptides and cause bacterial cell death by binding to the 30s subunit [23]. Tetracyclines are the best example of broad-spectrum antibiotics, with activity on Gram-negative and Gram-positive bacteria [24], favoring the elimination of endogenous contaminants [25].

Because the use of antibiotics in plant cell and tissue culture is highly debated, Tewelde et al. [26] suggest determining the optimal concentrations and exposure times of explants to avoid phytotoxic effects on tissues. For example, in the experiment conducted by Habiba et al. [27], explants treated with tetracycline did not produce healthy shoots and the seedlings turned yellow; opposite results to those obtained in this investigation, which is related to the species and concentration of the antimicrobial agent.
3.2 INTRODUCTION PHASE

3.2.1 Phenolization level

The explants in all treatments showed phenolization symptoms, but not in equal intensity. Buds with the highest level of phenolic oxidation were observed in treatment T1 (2.94 on average, greater than 60% of the explant); treatment T2 presented buds with moderate phenolic oxidation (2 on average, less than 60% of the explant); while the level of phenolic oxidation in the explants was minimal in treatment T3 (0.88 on average, less than 30% of the explant) (Fig. 2).

Fig. 2. Phenolization curves in explants of *R. chinensis* in the introduction phase.

The growth of plant tissues during *in vitro* establishment can be limited by the occurrence of lethal browning in explants and/or culture medium [15]. This problem is related to oxidative and nitrosative stress in plant tissue cells, a phenomenon caused by the instability of reactive oxygen and nitrogen species which by being highly reactive molecules can oxidize different cellular components and cause oxidative destruction of cells [15]. The lowest levels of phenolic oxidation were obtained in the T3 treatment, that corresponded to explants grown in medium with activated charcoal (1 g/L) and exposed to temporary darkness for 3 days (Fig. 2). There are studies that indicate that polyphenol exudation decreases when using activated charcoal at concentrations of 0.5 - 1 % and incubating the explants in dark conditions for a few days [28, 29].

The addition of activated charcoal to the culture medium reduces tissue browning and favors the morphogenetic process by absorbing phenolic
compounds excreted by growing cells and inactivates oxidases responsible for the death of explants [15, 30, 31]. Moreover, a great diversity of molecules can adhere to their surface due to their high adsorption capacity [32]. However, the addition of activated charcoal in some trials was not sufficient to avoid oxidation problems [33], which is consistent with the results obtained in this assay, since explants in treatment T2 presented moderate phenolic oxidation despite the addition of activated charcoal (Fig. 2).

On the other hand, several authors suggest modifying the light factor, because light can activate enzymes involved in the synthesis and oxidation of phenols, causing the death of plant tissues [15], and can also generate free radicals such as hydroxyl radical (OH), which are highly oxidizing [34].

3.2.2 Shooting

Regarding bud shooting and comparing the treatments evaluated with the control, treatment T3 showed the highest shooting percentage of explants (81.25 %), while treatment T1 did not show shooting, because of the high phenolization level of explants (Fig. 3A).

The relationship between the variables phenolization level (x) and shooting percentage (y) was explained with a polynomial model, \( y = 0.1743x^2 - 1.0601x + 1.6099 \), \( R^2 = 1 \), where the maximum shooting occurred when explants presented the lowest phenolization level and decreased in explants that presented the maximum phenolization level (Fig. 3b).
Activated charcoal, despite not being a growth regulator, promotes morphogenesis, maybe due to its capacity to absorb compounds secreted by the explants, decreasing the accumulation of toxic metabolites in the culture medium [35]. Treatment T2 presented a low percentage of shooted explants (18.7 %), which could be because activated charcoal is not able to completely absorb the phenolic compounds responsible for inhibiting explant growth. In contrast, the factors evaluated in treatment T3 (activated charcoal + temporary darkness) favored shoot production by up to 80%, due to the minimal phenolization of the explants (Fig. 3a). Some authors report that the physiological development of plants, shoot formation and elongation, and therefore their survival were favored by the addition of activated charcoal in the medium, and by temporary darkness conditions [16, 36, 37].

### 3.2.3 Callogenesis

The highest callus cell formation in explants was obtained with treatment T3 (31.45 %), while the percentages were 18.75 and 25 % in treatments T1 and T2 respectively (Fig. 4A and Fig. 5).

The regression analysis in Fig. 4B shows a linear correlation between the variables phenolization level (x) and callogenesis percentage (y), with a R²=0.9975, p=0.0321. The relationship was inversely proportional, since as phenolization in explants decreased, callus cell formation increased.
The formation of callus in all treatments could be because the explants presented an endogenous level of auxins, which are responsible for cellular differentiation and proliferation [38]. However, the incubation of explants in different environmental conditions may interfere with this response. Jimenez [39] point out that callus proliferation can increase under dark conditions, since light does not degrade auxins and generate photo-oxidation problems. The same occurs in the results obtained in T3 treatment and in Kryvenki [40] who obtained a high callus formation with better characteristics when the explants (nodal segments and leaves) were incubated in dark conditions.

Fig. 5: Callogenesis and shooting in an explant of *R. chinensis* grown *in vitro*. Scale = 5 mm.

On the other hand, some authors point out that the formation of disorganized cell masses (callus) can be stimulated by cuttings in plant tissues, as a natural defensive reaction to protect against infections caused by pathogens, prevent water loss and act as cell sources for organogenesis [41]. In all treatments evaluated, callus tissue formation occurred in the cut zone, agreeing with what was reported by Gallardo *et al.* [42], who observed a breakdown of the protective tissue formed in the cut zone and subsequent callus growth.

4 CONCLUSIONS

Regarding the explant disinfection process, the addition of captan and tetracycline in the disinfection protocol (P2), allowed obtaining the lowest levels of bacterial and fungal contamination, with averages below 20 %, thus favoring the *in vitro* establishment of the species studied.

The addition of activated charcoal with incubation in temporary darkness
days), prevented the explants from presenting a high phenolization level, an effect that favored the morphogenetic response in shoot and callus formation by 81.25 and 31.25 %, respectively.

5 AUTHORS' CONTRIBUTION

IV conceptualization and coordination of the project, JA and MP research development. All authors participated in the research design, data interpretation and writing of the manuscript.

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