Efficiency of ampicillin and benomyl at controlling contamination of Annonaceae leaf segments cultured *in vitro*

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Efficiency of ampicillin and benomyl at controlling contamination of Annonaceae leaf segments cultured *in vitro.*

Abstract — Introduction. For the micropropagation of woody species, contamination of *in vitro* cultured explants is a constant problem, which can compromise the development of the technique. The aim of our study was to evaluate the effects of an antibiotic and a fungicide on surface and endophytic microorganisms associated with *Annona cauliflora, A. bahiensis* and *A. glabra* (Annonaceae) tissue culture. **Materials and methods**. Our work made it possible to compare a standard disinfection of *in vitro* cultured explants and the use of either a commercial fungicide, Benlate 500 (50% of benomyl) at (0.0, 1.0, 2.0 and 4.0) $g \cdot L^{-1}$, or an antibiotic, ampicillin at (0.0, 1.0, 2.0 and 4.0) $mg \cdot L^{-1}$. **Results**. The study revealed considerable variations in the infection rates within species and according to the concentrations of benomyl and ampicillin used. Benomyl was effective at cleaning leaf segments, and, at a concentration of 1.0 $g \cdot L^{-1}$, this fungicide was sufficient to eliminate all fungi. Ampicillin treatments at (0.0 to 4.0) $mg \cdot L^{-1}$ were ineffective at controlling bacterial contamination. **Conclusion**. Referring to the difficulty in obtaining an aseptic *in vitro* culture starting from a woody species, the average number of healthy explants obtained after the disinfection of foliar explants of *Annona* sp. using the antimicrobial substances tested was significant. Further studies to evaluate the effects of the concentration of chemicals on *in vitro* plant regeneration for *Annona* species are needed to clarify the relationship between the concentration and phytotoxic effect of chemicals.

Brazil / Annonaceae / plant propagation / in vitro culture / disinfection / fungicides / antibiotics

Efficacité de l'ampicilline et du bénomyl pour contrôler la contamination d'explants de feuilles d'Annonacées cultivées *in vitro*.

Résumé — Introduction. Pour la micropropagation d'espèces ligneuses, l'état sanitaire des explants cultivés *in vitro* est un problème récurrent qui peut compromettre le développement de la technique. Le but de notre étude a été d'évaluer les effets d'un antibiotique et d'un fongicide sur les micro-organismes de surface ou endophytes, associés à la culture de tissus de *Annona cauliflora, A. bahiensis* et *A. glabra*. **Matériel et méthodes**. Nos travaux ont permis de comparer une désinfection standard des tissus mis en culture et l'utilisation soit d'un fongicide commercial, le Benlate 500 (50 % de bénomyl) à (0,0, 1,0, 2,0 et 4,0) $g \cdot L^{-1}$, soit d'un antibiotique, l'ampicilline à (0,0, 1,0, 2,0 et 4,0) $mg \cdot L^{-1}$. **Résultats**. L'étude a révélé de fortes variations des taux d'infection en fonction des espèces mises en culture et des concentrations de bénomyl et d'ampicilline utilisées. Le bénomyl a été efficace pour aseptiser les explants de feuille, et, à une concentration de 1,0 $g \cdot L^{-1}$, ce fongicide a été suffisant pour éliminer tous les champignons. Les traitements à l'ampicilline (0,0 à 4,0) $mg \cdot L^{-1}$ ont été inefficaces pour contrôler les contaminations bactériennes. **Conclusion**. Si l'on se réfère à la difficulté d'obtenir une culture *in vitro* aseptique à partir d'une espèce ligneuse, le nombre moyen d'explants sains obtenus après la désinfection des explants foliaires d'*Annona* sp. à l'aide des substances antimicrobiennes testées a été significatif. D'autres études seront nécessaires pour évaluer les effets de la concentration des produits chimiques sur la régénération *in vitro* de plants du genre *Annona* afin de clarifier le rapport entre la concentration et l'effet phytotoxique des produits chimiques.

Brésil / Annonaceae / multiplication des plantes / culture in vitro / désinfection des tissus / fongicide / antibiotique

1. Introduction

The application of an *in vitro* vegetative propagation technique to arborescent species is frequently confronted with the problem of contamination at the beginning of the culture [1]. These difficulties are more serious in humid tropical areas, where the climate encourages the proliferation of microorganisms [2]. Contamination by endophytic microorganisms is the most serious problem in plant tissue culture, especially in tropical species [3]. A good example is the case of *Annonaceae* in Brazil, where the explant contamination problem is a serious limiting factor to the *in vitro* propagation of the family species.

For tissue culture, the most common disinfectant is sodium hypochlorite used diluted with water to about 1% of its original strength. To improve wetting of the tissue surface, treatments with hypochlorite are often preceded by a detergent or alcohol wash. Ethanol partially removes hydrophobic waxes and resins, which protect microorganisms from contact with aqueous disinfectants [4].

When traditional sterilization is ineffective, improved sterilization is often obtained with fungicides and bactericides. The use of antibiotics and fungicides can help with disinfection of explants. However, the antimicrobials must show some desirable features to be used on plant tissue culture, such as solubility and stability; they have to be unaffected by pH and by media, with reduced side effects and a broad spectrum of bactericidal activity, suitable in combination, showing reduced chance of resistance, and with low cost. It is unlikely that any single drug will fulfil all these criteria, but it is essential that the user is fully aware of any adverse effects that the media might have [5].

The objective of this study was to evaluate the effects of an antibiotic and a fungicide on surface and endophytic microorganisms associated with *Annonaceae* tissue culture.

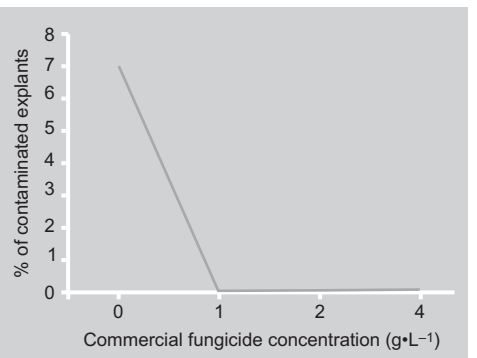

Figure 1.
Percentage of fungal contamination of *Annona glabra*, *A. bahiensis* and *A. cauliflora* leaf segments after exposure to a commercial fungicide, Benlate 500 (50% of benomyl), at various doses.

2. Materials and methods

2.1. Plant material

The experimental work was carried out in the Laboratory of Plant Tissue Culture of the Department of Biology of the Federal University of Lavras, Minas Gerais, Brazil.

Young expanding leaves were collected from 3-year-old plants of *Annona cauliflora, A. babiensis* and *A. glabra*. The explants were (1.5 to 2.5) cm long with 1-cm-wide leaf segments.

2.2. Disinfectants and antimicrobial agents

Leaves were briefly washed with a soap solution, then soaked in 70% (v/v) ethanol 95° for 30 s. The next phase consisted of soaking for 15 min in a disinfectant solution (sodium hypochlorite) to which a wetting agent (0.25 mL Tween 20) was added. This solution consisted of 25% (standard decontamination), 50%, and 100% of NaOCl (2–2.5% active chlorine). Explants were then rinsed three times in sterile distilled water, sectioned and inoculated.

Since disinfection with hypochlorite was found to be inadequate, antimicrobial substances (antibiotic and fungicide) were incorporated into the culture medium. After standard decontamination, the explants were inoculated on WPM [6] medium solidified with 0.7% (w/v) agar (Sigma) and supplemented with 3% sucrose and the combinations of different concentrations of the commercial fungicide Benlate 500 (50% of

benomyl) at (0.0, 1.0, 2.0 or 4.0) $g \cdot L^{-1}$ and ampicillin antibiotic at (0.0, 1.0, 2.0 or 4.0) $mg \cdot L^{-1}$. The benomyl was added to the medium prior to autoclavation. The ampicillin was filter-sterilized (Millipore 0.22 µm) and introduced to the medium after autoclaving.

The cultures were maintained at (25 ± 3) °C under a 16 h photoperiod with a photosynthetic photon flux density of (45 to 56) $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by fluorescent light. The treatments consisted of 10 replications with 10 explants per replication.

The results were expressed as a percentage of healthy explants 30 days after inoculation.

3. Results

The study revealed highly significant ($p < 0.05$) differences in the percentages of aseptic material within the *Annona* species studied (*table I*). At the concentration of 1 $g \cdot L^{-1}$ of the commercial fungicide Benlate 500, it was possible to achieve the total elimination of fungus contaminants (*figure 1*). On the other hand, the ampicillin at the concentrations used was ineffective at eliminating bacteria from the cultures (*table II*).

In all species used, aging of tissue was accompanied by an increase in microbial infection, often aggravated by surface wounds. Explants taken from leaves from the last season (3 months old) gave more healthy specimens than explants from leaves taken from an earlier growth unit (data not shown). This result confirms the deterioration of the health of the plant material during the aging process.

4. Discussion

The average number of healthy explants obtained after disinfection of leaf tissue with antimicrobial substances was significant, considering the difficulty of obtaining an aseptic *in vitro* establishment of arborescent plants.

The origin of the plant material removed to be cultured also appeared to be a decisive factor during the explant culture. Due to rapid growth, the upper part of the shoot is less colonized by microorganisms, favoring

Table I.
Percentage of aseptic material from leaf and nodal segments of Annonaceae family plants, in function of the species cultured.

Species	Aseptic material (%)
Annona glabra	91.5 a
Annona bahiensis	54.1 b
Annona cauliflora	37.7 c

Means followed by the same letter in a column are not significantly different by Tukey's multiple range test at $p < 0.05$.

Table II.
Percentage of bacterial contamination (%) of *Annona glabra*, *A. bahiensis* and *A. cauliflora* leaf segments cultured *in vitro*, in function of the ampicillin dose used to disinfect the tissues.

Species	Ampicillin concentration ($mg \cdot L^{-1}$)				Mean
	0	1	2	4	
Annona cauliflora	64.1 a	63.7 a	60.0 a	60.0 a	61.9 a
Annona bahiensis	45.0 b	42.5 b	40.0 b	40.0 b	41.8 b
Annona glabra	8.7 c	8.7 c	6.8 c	8.7 c	8.2 c

Means followed by the same letter in a same column are not significantly different by Tukey's multiple range test at $p < 0.05$.

better explant aseptic conditions. It has been reported that cabendazime, fendedazole and imazalil provide a broad spectrum of antifungal activity, without being toxic to plant material (*Nicotiana* protoplast, roots, callus and germinating seeds) [7]. *Camellia sinensis* and *C. japonica* shoot tip explants were exposed to the fungicide benomyl and the antibiotic rifampicin for 24 h after sterilization in hypochlorite. This treatment reduced contamination rates and had no phytotoxic effects [8]. Similar results were reported by Viana *et al.* [9] with rifampicin (50 $mg \cdot L^{-1}$) on *Carica papaya* shoot tip explants and the authors concluded that rifampicin did not modify the explant development and no toxic effect was observed. Kritzinger *et al.* [10] pretreated *Zantedeschia aethiopica* rhizomes with a mixture of broad-sprectrum commercial fungicides, Captab 500 WP (5 $g \cdot L^{-1}$) and Dithane (5 $g \cdot L^{-1}$), belonging to the same group of benomyl, and the result showed that a fungicide pretreatment of at least 24 h was necessary in the disinfection procedure to obtain maximum contamination control.

The concentration and disinfection time of the antibiotic used was probably not sufficient to clean the explants. Scortichini and Chiariotti [11] employed three antibiotics, streptomycin sulphate (S), piperacillin (P) and rifampicin (R) at (25 to 200) $mg \cdot L^{-1}$, in order to test the antibacterial and phytotoxic effects of these compounds on shoot tip explants of *Prunus persica*. The authors found that the antibiotics used, even though presenting a broad spectrum, partially eliminated contaminants (30.0% of explants escaped their action) and clearly showed phytotoxicity with rifampicin and streptomycin sulphate. Reed *et al.* [12] reported that internal bacterial contaminants in tissue-cultured hazelnuts were eliminated by antibiotic treatment. Single antibiotics were ineffective, but a combination of two or more of them eliminated most contaminants. Streptomycin combined with timentin or gentamicin killed all of the isolated bacteria tested. The authors also found that, in plant tissues, antibiotics with a concentration 3–4 times higher than those effective on isolated bacteria were needed to eliminate internal bacteria.

Controlling aseptic conditions in primary culture requires protection of mother plants with regard to the environment. Thus, further studies to evaluate the effects of the concentration of chemicals on *in vitro* plant regeneration for *Annona* species are needed to clarify the relationship between the concentration and phytotoxic effect of chemicals.

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Eficacia de la ampicilina y del benomil para controlar la contaminación de explantes de hojas de Anonáceas cultivadas *in vitro*.

Resumen — Introducción. Para la micropropagación de especies leñosas, el estado sanitario de los explantes cultivados *in vitro* es un problema recurrente que puede comprometer el desarrollo de la técnica. El objetivo de nuestro estudio fue evaluar los efectos de un antibiótico y de un fungicida sobre los microorganismos de superficie o endófitos, asociados al cultivo de tejidos de *Annona cauliflora, A. bahiensis* y *A. glabra*. **Material y métodos**. Nuestros trabajos permitieron comparar una desinfección normal de tejidos puestos en cultivo y el empleo, bien de un fungicida comercial, el Benlate 500 (50% de benomil) a [(0,0, 1,0, 2,0 y 4,0) $g \cdot L^{-1}$], bien de un antibiótico, la ampicilina a [(0,0, 1,0, 2,0 y 4,0) $mg \cdot L^{-1}$. **Resultados**. El estudio reveló fuertes variaciones de las tasas de infección en función de las especies puestas en cultivo y según las concentraciones de benomil y de ampicilina utilizadas. El benomil se mostró eficaz para aseptizar los explantes de hoja y, con una concentración de 1,0 $g \cdot L^{-1}$, dicho fungicida fue suficiente para eliminar todos los hongos. Los tratamientos con ampicilina (0,0 à 4,0) $mg \cdot L^{-1}$ fueron ineficaces para controlar las contaminaciones bacterianas. **Conclusión**. Si nos referimos a la dificultad de obtener un cultivo *in vitro* aséptico a partir de una especie leñosa, el número medio de explantes sanos obtenidos, tras desinfección de los explantes foliares de *Annona* sp. mediante las sustancias antimicrobianas probadas, fue significativo. Serán necesarios más estudios para evaluar los efectos de la concentración de los productos químicos sobre la regeneración *in vitro* de plantas del género *Annona* con el fin de aclarar la relación entre la concentración y el efecto fitotóxico de los productos químicos.

Brasil / Annonaceae / propagación de plantas / cultivo in vitro / desinfección / fungicidas / antibióticos