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Original Article

Soursop contamination and sprouting in function of the fungicide in the culture medium

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Abstract

The present research was conducted to verify the effectiveness of adding different doses of fungicide to the culture medium, in the contamination of *in vitro* culture of the explants of soursop. The experiment was conducted using explants collected from seedlings of soursop that were kept on lath house conditions (50% brightness), underwent three treatments, consisting in doses of fungicide carbendazim added to the MS culture medium: 5 ml per liter of culture medium, 10 ml/L, 15 mL /L, more a control treatment. The study lasted for 42 days and were evaluated daily number of explants with contamination and with budding. There was no beneficial effect of fungicide in reducing contamination, nor in increased sprouting, for the *in vitro* cultivation of soursop.

Key words: *Annona muricata* L., *in vitro* propagation, carbendazim

Contaminação e brotação de gravioleira em função do uso de fungicida no meio de cultura

Resumo

Este trabalho foi realizado objetivando verificar a eficácia da adição de diferentes doses de fungicida ao meio de cultura, na contaminação de explantes de gravioleira cultivados *in vitro*. O experimento foi realizado usando explantes coletados de mudas recém-formadas de gravioleira, que estavam mantidas em condições de ripado (50% de luminosidade), submetidos a três tratamentos, compostos por doses do fungicida carbendazim acrescidos ao meio de cultura MS: 5 mL por litro de meio de cultura; 10 mL/L; 15 mL/L, além de um tratamento testemunha. O estudo teve a duração de 42 dias e foram avaliados o número de explantes com contaminação e com brotação. Não houve efeito benéfico do fungicida na redução de contaminação, nem no aumento de brotação, para o cultivo *in vitro* da gravioleira.

Palavras-chave: *Annona muricata* L., propagação *in vitro*, carbendazim

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Introduction

The soursop (*Annona muricata* L.) is a fruit of the family Annonaceae and widely cultivated in Brazil, especially in the North and Northeast regions (Kitamura & Lemos, 2004), presenting a great prospect for export (Ledo & Cabanelas, 1996), and growing demand by industries manufacturing pulp, juices, ice creams and jellies (Oliveira et al., 2009).

The production of seedlings can be realized using seed (sexual), or vegetative method (especially grafting), and according to Costa et al. (2005), the recommendation of seed propagation has been more restricted to obtaining rootstocks, although, in spite of leading to the formation of non uniform orchards, many seedlings are still produced in this way, generating more vigorous plants with root system and abundant depth, longevity, besides providing obtaining new varieties and training genebanks (Manica et al., 2003).

In addition to these methods to obtaining plants, there is a possibility of the use of micropropagation, as have been done for other fruit and some researchs have been performed, with the most diverse species of Annonaceae, trying to success, from the acquisition protocol suitable for *in vitro* cultivation, beyond the elimination or control of contamination, which is still a very important factor and causes damage to the process (Santana et al., 2003).

The micropropagation, or plant cell culture, comprises a set of techniques, in which each explant (cell, tissue or organ) is isolated under aseptic conditions in a nutrient medium. It is based on the principle of totipotency, that is, each cell has all the genetic information necessary, conferring the ability to generate a new plant, with full maintenance of the characteristics of the mother plant (Crocomo & Gallo, 1995; Caldas et al. 1990)

The *in vitro* culture has been considered an almost indispensable tool to accelerate conventional breeding techniques, and is an alternative to traditional vegetative propagation and aims to achieve large scale and in a short period of time, free of pathogens and plants identical to the original, in other words, to realize with aseptically perform a plant cloning, which is defined as an asexual propagation of cells or organisms, to obtain new individuals, maintaining the genotype identical to the common ancestor (Torres et al., 2000).

During *in vitro* multiplication, the ability of the explants to survive, develop and multiply is the result of several factors, including the genetic, physiological state and environmental conditions of cultivation (Kozay et al., 1997).

To micropropagate an specie, the first step is the selection of the most suitable explants (Erig & Schuch, 2005) and also the decontamination protocol. According Fachinello et al. (1995), most studies involving micropropagation of fruit, the explants chosen are obtained from apical or axillary buds.

In the establishment phase of cultivation, contamination can compromise the work of micropropagation. When is exogenous, the possibility of control of the main contaminants (fungi and bacteria) is significant, however, when contamination is endogenous, the consequences can be limiting and may cause loss of time, financial resources and genetic material (Souza et al. 2006). Contaminating microorganisms compete for nutrients with the explants in the culture medium and cause direct and indirect damage by colonization of their tissues, and can eliminate in the medium, metabolites toxic to plants (Montarroyos, 2000).

One of the biggest obstacles for *in vitro* establishment of some species is the difficulty to obtaining tissue free from contamination caused by fungi and bacteria and also by oxidation caused by the release of phenolic compounds (Thorpe et al., 1991; Grattapaglia & Machado, 1998). The disinfestation of nodes is the first step to be considered for the realization of a great establishment *in vitro* (Pierik, 1990).

Protocols for disinfestations must, therefore, always be developed and tested for each species that is to be propagated using tissue culture. Thus, this research aimed to verify the efficacy of adding different doses of fungicide to the culture medium, in the contamination of soursop explants cultured *in vitro*, evaluating the percentage of contamination, as well as the percentage of sprouting by the explants, obtaining information about the viability of its

use in a protocol for this species and infer about the possibility of the formation of plants by this method of vegetative propagation.

Material and Methods

The experiment was conducted in Micropropagation Laboratory, located in the Department of Plant Production of the "Faculdade de Ciências Agrárias e Veterinárias/ UNESP", Campus of Jaboticabal city, São Paulo State, Brazil, using explants (lateral buds) collected by newly formed soursop seedlings, which were kept in lath house conditions (50% of brightness).

After collecting the material, the explants were subjected to a disinfestations procedure, comprising the following: 1) wash in running water for 15 minutes; 2) immersion in neutral detergent (Tween 20) for 10 minutes; 3) 70% alcohol for 1 minute; 4) 1% sodium hypochlorite for 15 minutes; and 5) washing in distilled and autoclaved water for 5 times. During the disinfestations, the explants were kept in Becker, sealed with gauze, in order to avoid contact with the environment, being this gauze removed only in a laminar flow, where was made the micropropagation procedure (cut of the explants - reducing them as much as possible, avoiding exposed tissue, that could be the source and cause of contamination).

The explants were submitted to three treatments, composed by the doses of the fungicide carbendazim added to MS (Murashige & Skoog, 1962) medium: 5 ml per liter of culture medium; 10 mL/L; 15 mL / L; and control treatment, without the use of carbendazim.

The delineation was completely randomized and for each treatment was realized four replicates with 10 explants in each one, being used one explant for glass containing approximately 50 mL of culture medium, which was sterilized by autoclaving vertical at a temperature of 120°C, under pressure of 1 atm, for 20 minutes. Besides the carbendazim, to the MS medium was added 2 grams/liter of activated coal, avoiding the oxidation of the explants.

The vials of glass containing the culture medium and the explants were sealed with foil autoclaved, masking tape and plastic wrap. The explants were kept in a growth room with a temperature of 27±2°C, 16 hours of photoperiod and light intensity of approximately 75μmol m^{-2} s^{-1} provided by fluorescent lamps of 40W.

The experiment lasted for 42 days and were evaluated, daily, the number of explants with contamination and the number of explants with budding (leaf developed). The results, expressed in percentages, were transformed in $arcsen\sqrt{x/100}$, subjected to analysis of variance and the means for the Tukey test at 5% of probability.

Results and discussion

Evaluating the effect of the fungicide carbendazim in the reducing of the contamination of the explants to *in vitro* cultivation of soursop, can be observed that, although there was more contamination in the control treatment, there was no statistically significant difference between the used treatments (Table 1). Likewise, despite being checked a highest sprouting in explants subjected to higher dose of carbendazim (57%), not existed a significant difference between the treatments. However, the occurrence of sprouting in the rate that was verified, can be considered positive and including can serve to explant in successive crops, starting with material free from contamination.

Table 1. Results of statistical analysis for contamination rate and sprouting explants of soursop as a function of dose of carbendazim

Doses of Carbendazim (DC) – mL/L	Contamination (%)	Sprouting (%)
0	54.215	38.950
5	51.330	51.050
10	47.885	53.935
15	48.165	57.100
TEST F		
DC	0.287 NS	3.486 NS
CV (%)	22.12	16.91

In each column, for each factor, means followed by the same capital letter do not differ among themselves by "Tukey test," the 5% probability. NS: not significant (P <0.05) *: Significant (P <0.05) **: Significant (P <0.01). CV%: Coefficient of variation.

Was not observed, thus, efficiency of the fungicide to avoid contamination, which was considered high and can be by several factors, including the source of the material (although it has not been collected in field conditions, where contamination is major) and the immersion time of the explants in the different products used in the disinfestations procedure, the proper fungicide that was used, in addition to dealing with contamination endogenous and not exogenous.

Cid & Zimmermann (2006) reported that the use of chemicals, such as sodium hypochlorite, ethanol and fungicides, as used in the present research, in the doses and certain times, can be effective to control fungal contamination in explants collected in the field, although for Annonaceae the *in vitro* multiplication and in a large scale, still have been confronted with some limitations and, among them, endogenous contamination of the explants (Santana et al., 2003), highlighting the importance of more further research, in order to define a protocol for disinfestations and use of chemicals (especially fungicides) added to the culture medium.

Other important information, as cited by Londe et al. (2007), is that the MS medium, commonly used in *in vitro* cultures, contains relatively high concentrations of nutrients, which can promote and explain the high rate of contamination found in the present research, even after a thorough and careful disinfestations procedure and material handling. These same authors, in work aimed to study the effect of benomyl on MS medium for contamination control in micropropagation cajuí (*Anacardium humile*), also found no significant effect of the use of fungicide to control contamination.

Have in the literature, however, works that relate the use of benomyl in the culture medium, but assessing its positive relationship with budding, not to mention the occurrence of contamination, as performed by Moreira (1993) with *Citrus sunki* and Da Silva et al . (2002), in *Ananas comosus*.

The levels of contamination, in accordance with Medeiros (1999), tend to be higher when the mother plant used as a source of explants are derived from the field, which was observed by Rodrigues et al. (2003) when study the *in vitro* cultivation of different varieties of *Prunus*, observing that cultivars Mirabolano, Okinawa, Nemaguard, Diamond and Aldrighi showed higher contamination rate, attributing to the phytosanitary conditions of the plant material collected, since the plants were kept in orchards and not under controlled conditions (screened and greenhouses).

However, even plants subjected to strict phytosanitary control and kept at a nursery or greenhouse (as was the case of the soursop explants used in this research), are potential sources of microorganisms, which can become limiting to the procedures of cultivation *in vitro* (Medeiros, 1999). Thus, even using aseptic internally and proper handling at all stages of the micropropagation procedure, the contamination can occur. An alternative might be performing a prior treatment in plants donor of explants, for a period before being carried the collecting of material to be used.

In this research, it was also observed, even without significant difference between the treatments, an increased in the percentage of sprouting due to the increase of the dose of the fungicide used. Many papers referring to the action of benomyl favorably to the occurrence of sprouting, as indeed observed here was somewhat expected, since carbendazim is also a compound that belongs to the family of benzimidazoles and when degrade, generates two compounds, which are benomyl and thiophanate-methyl (Anastassiades et al. 1,998), two fungicides also for systemic use.

Conclusions

There was no beneficial effect of fungicide used in this research to reduce contamination, or to increase the sprouting, for the *in vitro* cultivation of soursop.

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