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Control of *in vitro* contamination during the establishment of *Pyrus communis* explants using Plant Preservative Mixture™

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ABSTRACT

The presence of fungal and bacterial contamination in *in vitro* propagation is a determinant of the culture establishment. The biocide PPM (Plant Preservative Mixture™) has been utilized to control *in vitro* contamination. According to the manufacture's label, it is a heat-stable, broad-spectrum product, non-selective, that affects directly microorganisms without causing damage to plant cells. The purpose of this study was to evaluate the influence of PPM asepsis on the control of contamination during the *in vitro* establishment and survival of *Pyrus communis* rootstock explants. Six compositions were tested for asepsis and explants from two locations (field and greenhouse). The results showed that asepsis with Alcohol 70% (1 minute) + NaOCl 2.5% + Tween 20 (15 minutes), plus 4.0 ml L^{-1} PPM added to media performed on explants from greenhouse plants and asepsis, with PPM 5% solution bath and PPM 5% solution bath plus 2.0 ml L^{-1} PPM added to media performed on explants from field-grown plants presented a good microbial control and good rate of survival.

Index terms: Biocide; tissue culture; micropropagation; pear tree; PPM.

INTRODUCTION

During micropropagation of woody plants, the presence of fungal or bacterial contamination represents a great obstacle in the *in vitro* establishment of culture. The contamination present on the surface of leaves tissue and buds can be controlled with chemical substances such as alcohol and sodium and calcium hypochlorite. In addition to surface contamination, it is frequent to find endogenous contamination resident in the interior of the tissues. Endogenous contamination is mainly found in explants from field-grown trees (Teixeira, 2001). Kaluzna et al., (2013) state that in a survey of tissue cultures originating from eight laboratories in Poland found the presence of multiple bacterial taxa, with genera such as *Bacillus, Pseudomonas, Methylobacterium, Corynebacterium, Staphylococcus* and *Agrobacterium* being ubiquitous.

Many species of endogenous fungi and bacteria can function in the establishment and development of plants during the tissue culture process. Due to their rapid growth features in the media, numerous bacteria have posed a significant risk to *in vitro* cultures, by the way, the efficiency of the *in vitro* sterilization procedure has a direct impact on the establishment and maintenance of plants in *in vitro* cultures (Babu et al., 2022). To control endogenous contamination there are methods such as antibiotic and fungicide treatments, but despite their effectiveness, they can be phytotoxic and should be used only for specific contaminants, otherwise they can reduce the sensitivity of microorganisms to *in vitro* treatments (Leifert; Ritchie; Waites, 1991).

The broad-spectrum biocide PPM (Plant Preservative Mixture™) has been successfully applied to control *in vitro* contaminations of exogenous and endogenous sources. The composition of PPM includes methilisothiazolinone, magnesium nitrate, potassium sorbate, and sodium benzoate. It is heat-stable, can be sterilized, and effectively reduces *in vitro* microorganisms. The use of PPM in tissue culture has been growing, with several studies demonstrating its efficiency in the micropropagation of diverse cultures, such as walnuts (Kushnarenko et al., 2022), gobiroba (Machado et al, 2020), lily (SONG et al., 2019) pera (LOTFI et al., 2020),

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reflecting the expression of microorganisms and preventing their growth.

The manufacture's recommendation of PPM is 0.5 to 2.0 ml L^{-1} PPM (Plant Cell Technology, 2012). Higher concentrations at 10 – 20 ml L^{-1} cause severe phytotoxicity to woody plant explants as *Citrus sinensis* (Niedz; Bausher, 2002).

The purpose of this study was to evaluate the influence of PPM asepsis on the control of contamination during the *in vitro* establishment and survival of *Pyrus communis* rootstock explants.

MATERIAL AND METHODS

The study was carried out in the Laboratory of Plant Micropropagation of the College of Agronomy and Veterinary from Santa Catarina State University, in Lages -SC.

This study was conducted with explants from plants grown in the field and from plants grown in a greenhouse with controlled temperature and humidity (25 °C and 70%, respectively). Both field plants and greenhouse plants did not received any kind of phytosanitary treatment during the two months prior the explant collection. The greenhouse plants received irrigation daily, at mornings, while the field plants did not have irrigation system. The branches selected as explant donors were harvest early in the morning, under mild temperatures, and transported to the laboratory inside a Becker with water. After the branches were harvested from the field plants and greenhouse plants, their leaves were trimmed and branches were washed with tap water for 15 minutes. Then, the branches were cut into small segments containing on bud, representing an explant. The explants were taken to the laminar flow hood for asepsis. It was tested six types of asepsis, displayed on Table 1.

Following the asepsis bath, explants were washed with autoclaved distilled water for 3 times and inoculated in culture media. The culture media utilized was MS (Murashige; Skoog, 1962) plus 30 g L^{-1} sucrose, without growth regulators. The pH was adjusted to 5.6 before the addition of 6.5 g L^{-1} agar and the media was autoclaved at 121 °C and 1.5 atm for 20 minutes. Tubes (15x2cm) were used with 10 ml of culture media. Explants were cultured in the dark for 72 hours and then transferred for a 16-hour light photoperiod with a temperature of 25 ± 2 °C. The results were analyzed after 3 weeks.

The experimental design used was completely randomized, arranged in a 2 x 6 factorial (explant origin x asepsis) with 12 replications per treatment. After three weeks the following variables were analyzed: bacterial contamination, fungal contamination, and explant survival. Survival was considered when explants (side buds) sprouted. The percentage data were transformed into arc sin $\sqrt{x}$ /100, submitted to analysis of variance (ANOVA), and the means were compared by Tukey at a significance level $p < 0.05$, with the help of the SAS 9.1 program.

RESULTS AND DISCUSSION

The ANOVA for bacterial contamination was significant for the interaction origin of explants x asepsis. Bacterial contamination occurred with more incidence on explants from field-grown plants, treated with A1 and A2 asepsis, presenting 100 and 74.9% of contamination, respectively (Figure 1).

The explants from plants grown in a greenhouse, in general, presented low bacterial contamination, and the lowest values (8.3%) were observed with A3 and A6 (Table 2).

Table 1: Asepsis treatments used on *Pyrus communis* explants.

Asepsis	Bath in	Culture media with
A1	Alcohol 70% (1 minute) + NaOCl 2.5% + Twen 20 (15 minutes)	0 ml L^{-1} PPM
A2	Alcohol 70% (1 minute) + NaOCl 2.5% + Twen 20 (15 minutes)	2.0 ml L^{-1} PPM
A3	Alcohol 70% (1 minute) + NaOCl 2.5% + Twen 20 (15 minutes)	4.0 ml L^{-1} PPM
A4	Stirred in PPM 5% solution for four hours	0 ml L^{-1} PPM
A5	Stirred in PPM 5% solution for four hours	2.0 ml L^{-1} PPM
A6	Stirred in PPM 5% solution for four hours	4.0 ml L^{-1} PPM

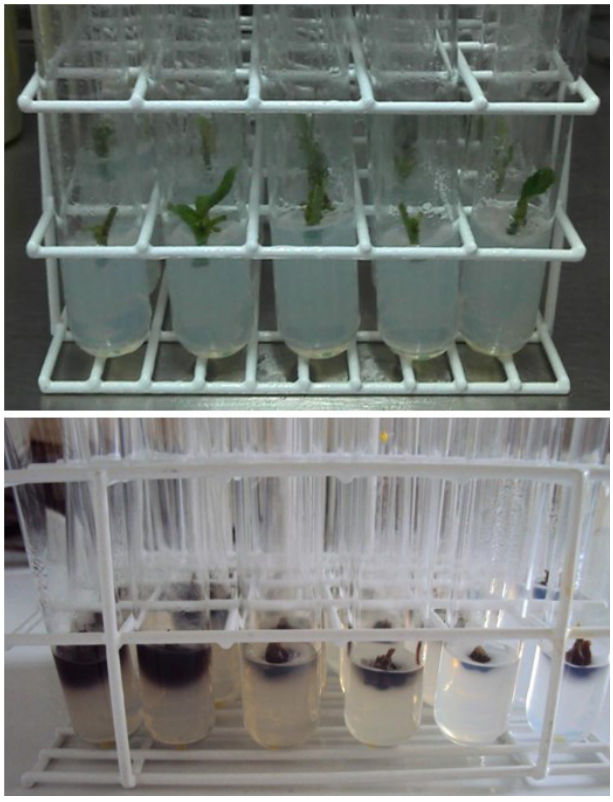

Figure 1: Contamination level of *Pyrus communis* explants, from greenhouse plants (A) and field plants (B), treated with A1 asepsis.

Table 2: Bacterial contamination rate of *Pyrus communis* explants originated from field-grown plants and growth chamber plants, after 21 days of *in vitro* establishment.

Asepsis	Origin of explant	
	Field plants	Greenhouse plants
	Bacterial contamination (%)	
A1	100 Aa	33.2 Ab
A2	74.9 Aa	24.9 Ab
A3	58.3 Ba	8.3 Bb
A4	24.9 Ca	33.2 Aa
A5	16.8 Ca	16.8 Aa
A6	0 Ca	8.3 Ba

Means followed by the same uppercase letters (column) and lowercase letters (row) do not differ by Tukey's test ($p > 0.05$).

This data agrees with what Dantas et al. (2002) observed on *in vitro* establishment of pear trees, that explants from buds of field-grown plants presented the highest bacterial contamination in relation to greenhouse plants. Kushnarenko et al., (2022) also observed that PPM added to culture media was more effective in controlling contamination from greenhouse plants and did not obtain satisfactory results related to explants from field-grown plants. On the other hand, there are studies that show signs of phytotoxicity for some species or bacterial and fungal resistance, and it should be necessary to adjust the doses for each type of explant and species (Kushnarenko et al., 2022; Thomas; Agrawal; Bharathkumar, 2017). For explants of field-grown plants, the control of contamination was effectively reached with 20 ml L^{-1} of PPM in the media, though phytotoxicity was very severe at this level.

The ANOVA for fungal contamination was significant for origin of explants and for type of asepsis, but without interaction among these factors. The asepsis that presented major fungal contamination were A1 and A4, with 33.4 and 12.45%, respectively (Table 3). This two asepsis did not have PPM added to the culture media. In general, explants from field-grown plants presented higher fungal contamination than explants from greenhouse plants (Table 3).

Table 3: Fungal contamination of *Pyrus communis* explants treated with different assepsis, after 21 days of *in vitro* establishment.

Asepsis	Fungal contamination (%)
A1	33.4 A
A2	4.61 B
A3	4.61 B
A4	12.45 AB
A5	0 B
A6	0 B
Origin of explant	
Field plants	13.8 A
Greenhouse plants	2.7 B

Means followed by the same letter do not differ by Tukey's test ($p > 0.05$).

This result was expected since the field-grown plants are more exposed to superficial contamination (Teixeira, 2001). Contamination may include many microorganisms, such as bacteria, fungi, molds, or yeasts and this factor

affect in the loss of time and effort related to plant tissue culture (Abdalla et al., 2022). External contamination results from the laboratories and used materials (media; glassware; culture vessels, tools, explants), whereas internal contamination is related to the endophytic microbes in mother plants (Andújar et al., 2020). The explants treated with A5 and A6 asepsis did not showed any contamination by fungi. As observed in this study, the fungal contamination was better controlled with PPM then the bacterial contamination. The PPM at recommended concentrations has been more effective in controlling air and water-borne fungi and bacteria at low inoculum densities, but when exposed to higher inoculum densities more concentrations of PPM are required (Niedz, 1998).

The ANOVA for the survival of explants was significant for the interaction origin of explants x asepsis. The asepsis that presented more survival rates for explants from field-grown plants were A4 an A5, with 75.1 and 91.6%, respectively (Table 4).

Table 4: Survival rate of *Pyrus communis* explants originated from field-grown plants and growth chamber plants, after 21 days of *in vitro* establishment.

Asepsis	Origin of explant	
	Field plants	Greenhouse plants
	Survival rate (%)	
A1	0 Cb	75 Aa
A2	50 Bb	83.4 Aa
A3	16.7 Cb	100 Aa
A4	75.1 ABa	83.5 Aa
A5	91.6 Aa	83.4 Aa
A6	41.6 Ba	41.6 Ba

Means followed by the same uppercase letters (column) and lowercase letters (row) do not differ by Tukey's test ($p > 0.05$).

These two asepsis had the explants stirred in 5% PPM solution with 0 or 2.0 ml L^{-1} of PPM in the media. Moreover, the A1 asepsis, which used none PPM, was not efficient to control the microbial contamination in explants from field-grown plants, resulting in none survival for this treatment. The explants from greenhouse plants presented high survival in all treatments, excepting the A6 asepsis (Figure 2).

The higher PPM concentration of the A6 asepsis, which had the explants stirred in 5% PPM solution with 4.0 ml L^{-1} of PPM in the media, may have affected the shoot development. This behavior was also observed by Compton and Koch (2001) in petunia explants, where the number of shoots and buds were exponentially reduced with the increase of PPM concentration in the media. George and Tripepi (2001) also observed a linear decrease of shoot regeneration as the concentration of PPM in the media was increased. On bryophytes species, the increase of PPM in the media reduced protonemal dimensions, in both non contaminated and contaminated cultures (Rowntree, 2006). Babaoglu and Yorgancilar (2000) observed that 2.0 ml L^{-1} of PPM in the culture media is enough to control air-borne contamination without affecting seed germination and explant development of *Poterium sanguisorba*.

Figure 2: Survival of a *Pyrus communis* explant, from greenhouse plants, without fungal or bacterial contaminants, treated with A3 asepsis.

The A4 and A5 asepsis showed high survival rates and were able to control efficiently the bacterial and fungal contamination for explants from field-grown plants. For explants from greenhouse plants the A2, A3, A4 and A5 asepsis showed high survival and high control of fungal contamination, but A3 asepsis was more efficient controlling bacterial contamination.

CONCLUSIONS

The Plant Preservative Mixture™ is an effective biocide to control microbial contamination, working successfully in both ways tested, as explant bath and as media content. Results showed that for greenhouse plants is not necessary to do the PPM bath, which represents cost reduction, with asepsis A3 being the best choice. However, for field plants the PPM bath is very important to controll contamination and at lower concentrations does not affect the ability of shoot development.

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