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Aseptic manipulation of cultures outside the laminar flow hood

Juliana Martins Ribeiro[1], Joselita Cardoso de Souza[2], Brenda Lima Ribeiro[3], Alessandro Rosa Nascimento[3], Cínthia Carolinne de Souza Ferreira[3], Jefferson dos Santos Caxias de Souza[3] and Silvio Lopes Teixeira[4]

[1]Research at Embrapa Semi-Arid Region, Brazil
[2]University of the State of Bahia, UNEB, Brazil
[3]Agronomy at University of the State of Bahia, Brazil
[4]Retired professor, Federal University of Viçosa (UFV), Brazil

Corresponding author: juliana.ribeiro@embrapa.br

Abstract

In the present study, laminar flow hoods were used as air filters in inoculation rooms. This study was developed in two stages. First, on a bench inside the laboratory inoculation room, with surfaces that had been cleaned with water and bleach; and second with air filtered by laminar flow hoods inside the room. In the first stage, the growth medium was sterilized by autoclaving and contamination was analyzed in culture flasks without explants. The tested media remained open on a bench for 10 minutes in an environment where laminar flow hoods were previously operated at different time ranges (2, 4, 6 and 8 hours). This stage was performed to find out the approximate time required for the air in the room to be decontaminated. In the second stage, the growth medium was sterilized both by autoclaving and by adding sodium hypochlorite (0,003%), and contamination was evaluated in culture flasks, with explants, in an environment previously sterilized by laminar flow hoods at different time ranges (4, 6 and 8 hours). The results showed no contamination by fungi or bacteria in the growth medium that was chemically sterilized with sodium hypochlorite (0,003%), in an environment where laminar flow hoods were previously operated for four hours. In conclusion, manipulating aseptic cultures outside the laminar flow hood is possible using sodium hypochlorite as a chemical sterilizer of the growth medium, where an appropriate air filtration system is adopted.

Keywords: Air purification; bacteria; cost reduction; contamination; fungi; micropropagation.
Abbreviations: MS_Murashige and Skoog; UNEB_ State University of Bahia; l.a.f. hood_laminar airflow hood.

Introduction

Commercial plant propagation in the laboratory started in the 1970's, with estimated global production of 500,000,000 plants per year (Ribeiro et al., 2013a). In Brazil, the commercial production of seedlings in the laboratory is carried out by institutions such as Biofábrica de Cacau (Cocoa Biofactory) and Campo Biotecnologia (Biotechnology Field), specialized in the production of banana microplants, both located in Bahia; by the Biofactory of the Northeastern Strategic Technology Center (CETENE), in Pernambuco, specialized in sugarcane micropropagation; Bioteca, in Mato Grosso, specialized in *in vitro* teak (*Tectonagrandis*) propagation; Aracruz Celulose (Aracruz Cellulose), in Espírito Santo, specialized in eucalyptus micropropagation; and Campo Biotecnologia Vegetal (Plant Biotechnology Field), in Paracatu, Minas Gerais, which was created to foster national fruit production through the production of high-quality propagating materials, among others (Ribeiro et al., 2013a).

Even though this technology brings many advantages, the *in vitro* production of seedlings still has high cost, requiring research for alternatives to reduce the final cost of microplant production. Some initiatives have already been tested in this regard, such as replacing the autoclaving technique with the chemical sterilization of the growth medium (Pais et al., 2016, Ribeiro and Teixeira, 2008a; Ribeiro et al., 2009, Ribeiro et al., 2011; Ribeiro et al., 2013; Teixeira et al., 2006; Teixeira et al., 2008), the use of sunlight as source of light in the plant growth room (Silva et al., 2014; Braga et al., 2010; Braga et al., 2019; Dignart et al., 2009), and replacing high-cost pro-analysis (PA) reagents with lower cost options (Ribeiro and Teixeira, 2008b; Ribeiro et al., 2012; Ribeiro et al., 2013b; Ribeiro et al., 2014).

As mentioned, a great deal of research has been conducted in order to reduce costs in plant micropropagation. However, there is no information available on alternative methods at inoculation rooms in plant tissue culture laboratories to eliminate application of laminar airflow hood for aseptic handling of cultures. Laminar airflow hoods are expensive pieces of equipment that require electricity for the entire operation. Furthermore, they are relatively large, and need big room spaces, restricting working spaces, which renders it necessary to have one piece of equipment for each worker.

On the other hand, using air filters, positive pressure systems, or air purifiers in the laboratory inoculation room to replace laminar airflow hoods would significantly reduce

Table 1. Number of flasks contaminated by fungi or bacteria, and number of uncontaminated flasks, according to different operating time ranges of the laminar airflow hood.

Treatments (Laminar air flow hood)	Number of contaminated flasks		Number of non-contaminated flasks	Total
	Fungi	Bacteria		
T1 (off)	10 **a**	6 **ab**	4 **b**	20
T2 (running for 2 hours)	3 **ab**	7 **a**	10 **ab**	20
T3 (running for 4 hours)	2 **ab**	2 **ab**	16 **ab**	20
T4 (running for 6 hours)	3 **ab**	1 **ab**	16 **ab**	20
T5 (running for 8 hours)	0 **b**	0 **b**	20 **a**	20

Data followed by the same letter do not differ according to Dunn's test at 5% significance.

acquisition and maintenance costs, since not only they are cheaper, but also their operation is also less energy consuming. Moreover, this replacement would open more space for benches, and consequently, allow an increased number of operators per square meter, who would work under more comfortable conditions, resulting in increased production.

In this context, the search for options to reduce microplant production costs has encouraged Embrapa Semiárido (CPATSA) and State University of Bahia (UNEB) to carry out a joint research aiming to cause this cost reduction to become a reality. This study checked the possibility of using air filters in inoculation rooms for the aseptic handling of cultures outside the laminar airflow hood.

Results and discussion

Table 1 shows data from the first stage of the research, which corresponds to the number of flasks contaminated by fungi or bacteria in autoclaved media and without explant inoculation, according to different air filtration time ranges in the inoculation room.

In the inoculation room, flasks that were left open while laminar airflow hoods were turned off (T1) exhibited higher fungal contamination compared to the treatment in which laminar air flow hoods were operational for eight hours before (T5). As for contamination by bacteria, there was no significant difference between the treatment in which the laminar airflow hoods were disconnected and those with different operating time ranges.

Regarding data observed in treatments with connected laminar airflow hoods (T2 to T5), there was no statistical difference between the numbers of flasks contaminated by fungi with different operating time ranges. However, regarding the number of flasks contaminated by bacteria, a higher number of contaminated flasks was observed among those manipulated two hours after the beginning of equipment operation (T2), compared to those manipulated eight hours after the laminar airflow hood was connected (T5).

Based on data obtained in the first experiment stage, time ranges starting at 4 hours (4, 6, or 8 hours) were set to be analyzed in the second stage, since there was no significant difference in the number of flasks contaminated by fungi and bacteria at different evaluated air filtration time ranges after 4 hours. In this stage, the explants were introduced into either chemically sterilized or autoclaved growth media.

Table 2 shows data from the second stage of the study, which corresponds to the number of flasks contaminated by either fungi or bacteria in thermally or chemically sterilized media, without explant inoculation, according to different air filtration time ranges in the transfer room.

The most frequent contaminants observed in *in vitro* plant cultures are filamentous fungi, bacteria, and yeasts (Oliveira et al., 2008). Cultures contaminated by fungi can be removed a few days after growing, as they are easily perceived in the growth medium (Leifert and Woodward, 1998). However, cultures contaminated by bacteria or yeasts are not always evident at the beginning of the culture, which enables their dissemination among materials during different stages of micropropagation (Montarroyos, 2000).

The introduction of the above-mentioned contaminants in aseptic cultures may occur from four main sources, namely: 1) the explant, 2) the nutrient medium, 3) manipulation of cultures by the operator, and 4) ambient room air (Souza et al., 2007). Among the mentioned sources, contamination derived from the aseptic manipulation of cultures by operators can be easily solved by delegating such activity to properly trained and experienced professionals.

The control of *in vitro* contamination by explants has been reported in literature, by disinfestation in sodium hypochlorite solution (Pereira et al., 2015, Golle et al., 2013,), antibiotics (Pereira et al., 2014,), PPM (Plant Preservative Mixture), and mercury chloride, among others.

As for alternatives to reduce contamination of cultures by the growth medium, studies have reported the efficiency of chemical sterilization using sodium hypochlorite (Pais et al., 2016, Ribeiro and Teixeira, 2008a; Ribeiro et al., 2009, Ribeiro et al., 2011; Ribeiro et al., 2013; Teixeira et al., 2006; Teixeira et al., 2008), and replacing thermal sterilization with autoclaving. This method is not only efficient, but is also an alternative that has been proven more cost effective than autoclave for the same purpose.

Filamentous fungi, bacteria, and yeasts can be spread in nature via atmospheric air (Cartaxo et al., 2007). Nonetheless, there is no information in literature on methods other than laminar airflow hood, with proven efficiency and cost reduction, for filtering air in inoculation rooms of plant tissue culture laboratories. Laminar airflow hoods are expensive pieces of equipment that require regular maintenance. Moreover, they are relatively large and provide restricted working space, which limits their use to just one operator.

This study demonstrated the possibility of aseptic manipulation of cultures outside the laminar airflow hoods, provided an efficient air filtering system is adopted in the inoculation rooms. This filtration could be performed by installing air filters, positive pressure systems, or air purifiers to replace laminar flow hoods, which would not only result in cost reductions regarding equipment acquisition and maintenance, but also in more free space for the installation of benches and, consequently, an increased number of operators per square meter, resulting in increased production.

It is worth mentioning that handling sterile cultures outside the laminar air flow hood, using an appropriate air filtration

Table 2. Number and percentage of flasks contaminated by fungi or bacteria according to different operating times of the laminar airflow hood and different growth medium sterilization methods.

Treatments		Contaminated flasks (%)	
Laminar air flow hood	**Sterilization procedure**	**Fungi**	**Bacteria**
T1 running for 4 hours	Autoclaved medium	1 (6.67%)	1 (6.67%)
T2 running for 4 hours	Chemically sterilized medium	0	0
T3 running for 6 hours	Autoclaved medium	0	0
T4 running for 6 hours	Chemically sterilized medium	0	0
T5 running for 8 hours	Autoclaved medium	1 (6.67%)	0
T6 running for 8 hours	Chemically sterilized medium	0	0
T7 inside l.a.f. hood	Autoclaved medium (control 1)	0	0
T8 inside l.a.f. hood	Chemically sterilized medium (control 2)	0	0

system, associated with sodium hypochlorite as a chemical sterilizer in the growth medium proved to be quite efficient, since no type of contamination was observed in cultures that were chemically sterilized (Table 2). Unlike thermal sterilization, sodium hypochlorite (in the chemical sterilization of nutrients) in the growth medium not only sterilizes upon addition, but also continues to act thereafter. This allows for a more efficient and cost-effective control of the appearance of endophytic bacteria, which are potential contaminants in plant tissue culture, or the appearance of other late contaminations, than conventional procedures currently adopted in plant tissue culture laboratories.

Materials and Methods

The experiment was developed in the biotechnology laboratory of the Department of Technology and Social Sciences (DTCS) of the University of the State of Bahia (UNEB) (latitude -9 ° 25'12" and longitude -40 ° 29'10"). The environmental experiments were carried out to check the feasibility of aseptic handling of cultures outside the laminar flow hood consisted of: a 3.5 x 3.5-m room (Figure 1 A) containing three Pachane PCR3 laminar airflow hoods, which were used as air filters in the working environment (Figure 1 B), and two 0.65 x 1.40 x 0.70 m (H x W x D) benches placed side by side (Figure 1C). The room floor was previously cleaned with a cloth with water and bleach.

The experiment was conducted in two stages. In the first stage, contamination in growth flasks containing MS medium (Murashige and Skoog, 1962) was analyzed, sterilized in an autoclave (thermal sterilization) and without explants. Treatments were as follows: **T1:** aseptic flasks opened in a non-sterile environment; **T2:** aseptic flasks opened in an environment with laminar airflow hoods that were turned on two hours earlier; **T3:** aseptic flasks opened in an environment with laminar airflow hoods turned on four hours earlier; **T4:** aseptic flasks opened in an environment with laminar airflow hoods turned on six hours earlier; **T5:** aseptic flasks opened in an environment with laminar airflow hoods turned on eight hours earlier. In the control treatment, culture flasks were opened on the workbench (Figure 1B) for 10 minutes. After that, they were closed with PVC film. In the other treatments, laminar airflow hoods were turned on, and after 2, 4, 6, and 8 hours, flasks were placed on the workbench without a PVC plastic cover. After 10 minutes, they were closed as described above. Flasks were taken to the growth room for a 16-hour photoperiod and temperature of 26^{o} C, and after 30 days, treatments were evaluated for contamination by fungi and bacteria.

The first stage of the experiment was conducted in a completely randomized design with five replications with four plots (flasks), totaling 20 flasks per treatment. The data were tested using Kruskal-Wallis non-parametric statistics, and when proven significant, they were compared using Dunn's test at 5% significance.

In the second stage of the experiment, laminar airflow hoods were used as air filter in the work environment, using the time ranges that resulted in less contamination in the previous stage. Treatments consisted of intervals of environment sterilization, combined with thermal or chemical sterilization of the growth medium, according to Ribeiro et al. (2008). Treatments were as follows: **T1:** flasks containing autoclaved MS medium (thermal sterilization), handled in an environment with laminar airflow hoods turned on four hours earlier; **T2:** flasks containing MS medium with addition of 0.003% NaClO (chemical sterilization), handled in an environment with laminar airflow hoods turned on four hours earlier; **T3:** flasks containing autoclaved MS medium (thermal sterilization), handled in an environment with laminar airflow hoods turned on six hours earlier; **T4:** flasks containing MS medium with 0.003% NaClO (chemical sterilization), handled in an environment with laminar airflow hoods turned on six hours earlier; **T5:** flasks containing autoclaved MS medium (thermal sterilization), handled in an environment with laminar airflow hoods turned on eight hours earlier; **T6:** flasks containing MS medium with 0.003% NaClO (chemical sterilization), handled in an environment with laminar airflow hoods turned on eight hours earlier; **T7:** Control 1 - flasks containing autoclaved MS medium (thermal sterilization), handled inside a working laminar airflow hood; and **T8:** Control 2 - flasks containing autoclaved MS medium with 0.003% NaClO (chemical sterilization), handled inside the laminar flow hood.

In all treatments, *in vitro* generated shoots of *Oppuntia* sp, cv. "Orelha de Elefante", were used as explants. After inoculated with explants, flasks were sealed with PVC film and taken to the growth room, with a 16-hour photoperiod at a temperature of 26º C. The four operators (Figure 1A) who handled the sterile flasks wore lab coats, masks, caps, and non-sterile latex gloves.

The second stage of the experiment was conducted in a completely randomized design with five replications of three plots (flasks), totaling 15 flasks per treatment. Due to the nature of the data obtained, no statistical analysis was performed and results were presented as a percentage.

Conclusions

Aseptic handling of cultures is possible in inoculation rooms outside the laminar airflow hood, provided that an adequate room air filtration system is adopted. Aseptic handling of cultures outside the laminar airflow hood, using an appropriate air filtration system, combined with sodium

Figure 1: Environment where experiments were carried out to check the feasibility of aseptic handling of cultures outside the laminar flow hood. **A:** Overview of the inoculation room, with operators working outside the laminar flow hoods; **B:** Laminar flow hoods used as environment filters; **C:** Benches on which flasks were handled (Pictures: Juliana Martins Ribeiro).

hypochlorite as a chemical sterilizer of the growth medium prevents its contamination. Air filtration in the transfer room for a period of at least 4 hours is enough to provide a sterile environment required to adopt the technique of aseptic handling of tissue cultures outside the laminar airflow hood.

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