Different methods for surface sterilization of *Pyrostegia venusta* (Ker Gawl.) Miers (Bignoniaceae) leaf explants

Diferentes métodos para a esterilização superficial de explantes foliares de *Pyrostegia venusta* (Ker Gawl.) Miers (Bignoniaceae)

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ABSTRACT

A fast, simple, economical and effective method of sterilization using ethanol and sodium hypochlorite in presented. Eighteen different surface sterilization methods were evaluated using leaf explants of *Pyrostegia venusta* growing in fragments of the Brazilian Cerrado. All protocols used sought to combine technical routine in order to determine the fastest and cheapest method of asepsis. Our results showed that methods named A13 and A17, which uses two immersion procedures: ethanol 70% for 1 minute + sodium hypochlorite 1% for 5 minutes and ethanol 70% for 1 minute + sodium hypoclorite 3% for 10 minutes, respectively, were the most effectives. The search for a simple, rapid and economical method of sterilizing explants for tissues culture causes us to recommend the A13 protocol for presenting reduced contamination and oxidation values of explants.

Keywords: Surface sterilization, leaf explant, asepsis.

RESUMO

Um método simples, rápido, econômico e eficaz para desinfestação superficial, utilizando etanol e hipoclorito de sódio é apresentado. Dezoito diferentes métodos de esterilização foram avaliados em folhas de *Pyrostegia venusta* que crescem em fragmentos de cerrado brasileiro. Todos os protocolos testados combinaram técnicas rotineiras, a fim de determinar o método mais simples e barato para assepsia. Nossos resultados mostraram que os métodos nomeados A13 e A17 que consistem em dois procedimentos de imersão: etanol 70% por 1 minuto + hipoclorito de sódio 1% por 5 minutos e etanol 70% por 1 minuto + hipoclorito de sódio 3% por 10 minutos, respectivamente, foram os mais efetivos. A busca por um método simples, rápido, efetivo e econômico de explantes para a cultura de tecidos vegetais, nos faz recomendar o protocolo A13 por apresentar valores reduzidos de contaminação e oxidação dos explantes.

Palavras chave: Esterilização superficial, explante foliar, assepsia.

INTRODUCTION

Pyrostegia venusta (Ker Gawl.) Miers, also known as orange trumpet or "cipó-de-São-João", is a liana creeper with flowering and fruiting seasonal found in southern and southeastern Brazil (Rossato and Kolb, 2010). Braga, Coimbra and Castro (2015) emphasize its high ornamental potential due to abundant production of orange flowers. From the medical point of view, pharmaceutical preparations of *P. venusta* exhibit antinociceptive, anti-inflammatory, antioxidant, antimicrobial and wound healing activities (Roy et al., 2011; Silva et al., 2011; Roy et al., 2012; Veloso et al., 2012), improvement of symptoms of cold and flu in animal models (Veloso et al., 2010), stimulating melanogenesis (Moreira et al., 2012), as well as the treatment of inflammation and metabolic disorders induced by diets carbohydrate (Veloso et al., 2014).

The *in vitro* propagation proved to be a promising technique for studies related to the production of disease-free plants, somaclonal variation, embryogenesis and somatic hybridization, as well as culture of protoplasts and meristems. The success of these techniques is directly related to the aseptic conditions of the environment in which the explants are cultured. Contamination by microorganisms is considered one of the most important reasons for the low productivity of plant tissue cultures (Oyebanji et al., 2009), considering that these agents actively compete for water and nutrients available in the culture medium, causing deficits such as: increased mortality and variation in growth rate of cultures; necrotic tissue and reduction in the proliferation of shoots and roots (Pereira, Matos and Fortes, 2003).

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Several techniques for disinfection of plant tissues, such as the use of paraformaldehyde (Fior, Prestes and Rodrigues, 2005), copper sulphate (Rajmohan et al., 2010), Tween-20 (Couto et al., 2004), Tween-80 (Pereira, Matos and Fortes, 2003), sodium hypochlorite (NaClO) (Miche and Balandreau, 2001; Donini et al., 2005) and ethanol (Ahroni et al., 1997; Zuker et al., 1997), all in different concentrations and exposure time have been employed with the to reduce costs, time and dispose of potentially polluting solutions in water bodies. Thinking about it, this paper proposed several protocols for sterilization in order to reduce contamination from leaf explants *P. venusta* which is a source for the production of bioactive compounds with potential use in the pharmaceutical industry (Loredo-Carillo et al. 2013; Braga, Coimbra and Castro, 2015).

MATERIAL AND METHODS

Plant material

Young leaves of *Pyrostegia venusta* (Ker) Gawl. Miers. (Bignoniaceae) for these experiments were collected from the *stricto sensu* Brazilian savanna (Cerrado) fragments located near the *Campus* Centro-Oeste Dona Lindu of Federal University of São João Del Rei (UFSJ), in the municipality of Divinópolis, Minas Gerais State, Brazil (S20º10’45.9”; W44º55’07.2”). Samples of plant material were collect and herbalized. The herbarium specimens were identified, cataloged and deposited in the EPAMIG Herbarium, Company of Agricultural Research of Minas Gerais, under registration number PAMG 56307.

Chemicals

Ethanol was obtained from the company’s Tec-Lab®, Brazil. Sodium hypochlorite 3-5% used was acquired from the company Vetec®, instead of the products with high market price traded.

Sterilization of explants

Table 1 describes the sterilization protocols using leaf discs (6 mm). Absence of surface sterilization was the control. MS medium (Murashige and Skoog, 1962), containing 30 g L^{-1} sucrose and solidified with 7 g L^{-1} agar, was used as basal medium. Further, pH was adjusted to 5.8 ± 0.1 and medium was sterilized at 120 °C (1.37×105 Pa) for 20 min. The explants were maintained at 27 ± 2 °C under 16:8 h light/dark regime, with a light intensity of 40 µmol m^{-2} s^{-1}. Explants´ contamination and oxidation were evaluated on the 5th, 10th and 15th day after the start of the culture. The experimental design was totally randomized, with 4 replicates and 10 plants each. The results were submitted to ANOVA and the averange compared by Scott-Knott test ($p < 0.05$). The experiment was repeated twice.

RESULTS AND DISCUSSION

Explants´ contamination varied from 1 to 100 % and total contamination was only reported in the absence of surface sterilization (A0) (Table 1). The lowest contamination rates (1, 5 and 11%, respectively) occurred in explants treated with 70% ethanol for 1 min + 1 or 3% NaClO for 5 or 10 min (A13 and A17, respectively) and 70% ethanol for 10 min (A9). Considering a 10% contamination rate as a frequency with good results in terms of *in vitro* tissue culture (Donini et al., 2005), the A13 and A17 treatments promoted efficient sterilization.

The contamination percentage from treatment A13 to A15 increased according to the increase in NaClO concentration (Table 1). Sodium hypochlorite is a powerful germicide, with a broad antimicrobial spectrum and harmless to humans in commercial concentrations. Whereas its major advantage is the persistence of residual activity, its disadvantages comprise inactivation by organic matter, metal corrosion activity and bleaching effects. When there is considerable residue of organic and/or mineral matter, these combine to the chlorine solution and produce combined chlorine with low disinfectant activity (Rutala and Weber, 1997).

The oxidation rate was relatively low in *P. venusta* explants (Table 1). Highest rates were reported with treatments A8, A9 and A12 (total oxidation) when 70% ethanol and 1% NaClO were used and A4, A5 and A11 (partial oxidation) with tap water and 1% NaClO for 5 min. Treatments A1 to A3 (H_2O at 52°C for 5, 10 and 15 min, respectively) and A6 (tap water for 24 hours) did not cause oxidation of explants, similar to control (A0), although an efficient surface sterilization failed to occur. Oxidation of explants (which usually starts at the edges) may occur for various reasons, such as time of explant exposure to the disinfecting agent, donor plant age, tissue type, genotype,

explant type and others (Dumas et al., 2003) and cause a serious issue for establishing *in vitro* cultures (Plazek and Dubert, 2010). Polyphenol oxidase, peroxidase and its products (such as quinones) oxidize phenolic compounds. They are the cause of the brown color in cultures, with growth inhibition and death of explants of a large number of species (Wu and Lin, 2002). The occurrence of oxidation on explants of woody plants is a common problem (Jaskani et al., 2008) which also affects *P. venusta*. More developed explants are less predisposed towards oxidation than younger ones (Werner et al., 2009).

The absence of pigments (Figure 1B) was observed mainly in the explants treated with water heated to 52º C (which sought to reduce bacterial contamination - Rodrigues et al., 2009) and 70% ethanol for 10 minutes. The initial stage of chlorophyll degradation is influenced by external factors such as drought stress, light, thermal changes, increased levels of ethylene or a combination of these factors (Heaton and Marangoni, 1996) and the lack of photosynthetic pigments can cause physiological deficiencies in plants (Mullet, 1993).

Treatment A13 was selected for the sterilization of explants surfaces used in the induction of *P. venusta* calli due to its use of low NaClO concentration, exposure time to the disinfectant agent and relatively low oxidation rates. The protocol did not produce any apparent damage on tissues. The use of ethanol or ethanol + other disinfectants, such as NaClO, is very common for the sterilization of materials subjected to *in vitro* cultures, that are low-cost, fast, simple and effective for the surface sterilization of leaf explants (Couto et al., 2004; Donini et al., 2005).

Table 1 – Percentage of explants contaminated, fully and partially oxidized after 15 days of incubation.

Treatments		Variables			References
		Contamination (%)	Total Oxidation (%)	Partial Oxidation (%)	
A0	Absence surface sterilization	100 ** a	0 ** c	0 ** d	-
A1	H_2O at 52ºC for 5 min	64 ± 5.72 b	0 ** c	3 ± 0.72d	Hol and Van Der Linde 1992; Rodrigues et al. 2009
A2	H_2O at 52ºC for 10 min	35 ± 3.68 c	0 ** c	0 ** d	
A3	H_2O at 52ºC for 15 min	44 ± 4.38 b	0 ** c	0 ** d	
A4	Tap water at room temperature for 6 hours	79 ± 5.44 a	8 ± 1.61 b	53 ± 3.15 a	Santos et al. 2009
A5	Tap water at room temperature for 12 hours	71 ± 4.72 a	13 ± 5.45 b	51 ± 4.13 a	
A6	Tap water at room temperature for 24 hours	68 ± 5.64 a	0 ** c	23 ± 5.91 b	
A7	70% EtOH for 1 min	50 ± 6.37 b	15 ± 4.21 b	30 ± 2.28 b	Pereira, Matos and Fortes, 2003; Rodrigues et al. 2009
A8	70% EtOH for 5 min	31 ± 4.72 c	28 ± 3.31 a	18 ± 1.25 c	
A9	70% EtOH for 10 min	11 ± 2.13 d	21 ± 2.77 a	14 ± 1.88 c	
A10	1% NaClO for 1 min	61 ± 4.00 b	9 ± 1.57 b	30 ± 5.10 b	Couto et al. 2004
A11	1% NaClO for 5 min	46 ± 4.83 b	5 ± 1.77 b	43 ± 2.17 a	
A12	1% NaClO for 10 min	50 ± 1.02 b	28 ± 2.98 a	31 ± 2.13 b	
A13	70% EtOH for 1 min + NaClO 1% for 5 min	1 ± 0.60 d	10 ± 1.77 b	20 ± 2.28 b	Pereira, Matos and Fortes, 2003; Donini et al. 2005; Diniz et al. 2008; Kielse et al.2009
A14	70% EtOH for 1 min + NaClO 3% for 5 min	29 ± 4.13 c	11 ± 2.58 b	24 ± 2.58 b	
A15	70% EtOH for 1 min + NaClO 5% for 5 min	51 ± 1.20 b	14 ± 1.88 b	23 ± 2.98 b	
A16	70% EtOH for 1 min + NaClO 1% for 10 min	30 ± 4.56 c	10 ± 1.77 b	24 ± 2.13 b	
A17	70% EtOH for 1 min + NaClO 3% for 10 min	5 ± 1.44 d	11 ± 1.20 b	18 ± 2.60 c	
A18	70% EtOH for 1 min + NaClO 5% for 10 min	30 ± 8.72 c	9 ± 2.13 b	21 ± 4.25 b	

Legend: EtOH = ethanol, NaClO = sodium hypochlorite. Results represent the mean of 80 replicates of each variable. *Means in each column followed by the same letter are not significantly different at $p \leq 0.05$ by the Scott-Knott test. ** Standard deviation rates for these data were omitted.

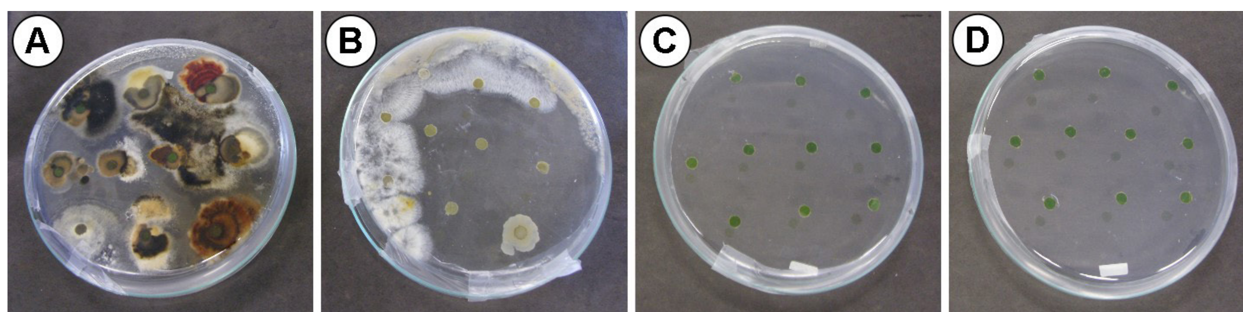

Figure 1 – General aspects of leaf explants of *P. venusta* (Ker Gawl.) Miers. after 15 days of incubation. **A** – Control (A0). **B** – H_2O at 52ºC for 10 minutes (A2). **C –** 70% Ethanol for 1 minute + 1% NaClO for 5 minutes (A13). **D –** Ethanol 70% for 1 minute + 3% NaClO for 10 minutes (A17).

CONCLUSION

Results this study have demonstrated that the use of two simple procedures immersion in ethanol 70% for 1 minute, followed by soaking in sodium hypochlorite 1% for 5 minutes is sufficient for the surface sterilization leaf explants of *P. venusta* collected in Cerrado environment.

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