

Assays for the *in vitro* establishment of *Swietenia macrophylla* and *Cedrela odorata*

Ensayos para el establecimiento *in vitro* de *Swietenia macrophylla* y *Cedrela odorata*

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Abstract

Recalcitrance and contamination in Mahogany (*Swietenia macrophylla* King) and Spanish cedar (*Cedrela odorata* L.) stem tissues are the main causes of its ineffective *in vitro* propagation. The objectives of this research were: a) to evaluate sodium hypochlorite (NaOCl) and plant preservative mixture (PPM®) as surface disinfectants and/or added to the culture medium for the *in vitro* establishment of nodal explants taken from 10-year-old Mahogany and Spanish cedar plants, and b) to evaluate the *in vitro* response of such explants treated with N6-benzylaminopurine (BAP) (0, 2.2, 4.4, 8.8, 17.7 µM), silver nitrate (AgNO₃) (0, 3 mg l⁻¹), activated charcoal (0, 1 g l⁻¹) and vented caps. All the experiments were arranged in a completely randomized design. The NaOCl at 15%, for 20 min, as a surface sterilization or PPM at 2 ml l⁻¹ into the culture medium, were the best treatments to reduce contamination for both species. For Mahogany explants, BAP at 17.7 µM resulted in higher percentages of bud breaks than Spanish cedar (64% and 25%, respectively). Leaves on elongated shoots dropped off by 20 days after starting the explants in culture and neither the activated charcoal nor the AgNO₃ alone or combined prevented leaf abscission. The AgNO₃ decreased contamination, but also increased leaf abscission. Bud breaks was two-fold higher for nodal explants established in vessels with vented caps than with normal caps. Mahogany nodal explants were easier to surface sterilize and more buds broke from BAP treated explants than Spanish cedar treated explants in the *in vitro* establishment.

Key words: spanish cedar, mahogany, mature plants, surface sterilization, *in vitro* response

Resumen

La contaminación y la recalcitrancia de tejidos de tallo de Caoba (*Swietenia macrophylla* King) y Cedro español (*Cedrela odorata* L.) son las causas principales de su inefectiva micro-propagación. Los objetivos de la investigación fueron: a) evaluar el hipoclorito de sodio (NaClO) y una mezcla preservadora de plantas (PPM®) como desinfectantes superficiales y/o agregados al medio de cultivo para el establecimiento *in vitro* de explantes nodales de Caoba y Cedro español de 10 años de edad; b) evaluar la respuesta *in vitro* de tales explantes tratados con N6-benzylaminopurine (BAP) (0, 2.2, 4.4, 8.8, 17.7 µM), nitrato de plata (AgNO₃) (0, 3 mg l⁻¹), carbón activado (0, 1 g l⁻¹) y tapas porosas. Los experimentos fueron establecidos bajo un diseño completamente al azar. La contaminación se redujo en ambas especies con NaOCl al 15% durante 20 min como desinfección superficial o con PPM® (2 ml l⁻¹) agregado al medio de cultivo. El mayor porcentaje de brotación de explantes se obtuvo con BAP a 17.7 µM en caoba (64%) comparado con cedro (25%). Los brotes se defoliaron a los 20 días de cultivo y ni el carbón activado ni el AgNO₃, solos o combinados evitaron la defoliación. El AgNO₃ disminuyó la contaminación, pero incrementó la defoliación. La brotación fue dos veces mayor en los explantes nodales establecidos en recipientes con tapas porosas que cuando se utilizaron tapas normales. Los explantes nodales de Caoba respondieron mejor a la desinfección superficial y a los tratamientos de BAP comparados con los de Cedro español en el establecimiento *in vitro*.

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Introduction

Mahogany *Swietenia macrophylla* King and Spanish cedar *Cedrela odorata* L. (Meliaceae) are included in the CITES list as endangered species. Many attempts have been done to micropropagate mature individuals of both species without success. Such absence of response is known as recalcitrance which is characterized by lack of ability to break bud, absence or negligible growth of sprouted buds, leaf abscission on new shoots and presence of only one elongated petiole (Lee and Rao, 1988). In addition, contamination is a problem that must be overcome in order to micropropagate older forest trees successfully (Maruyama *et al.* 1989). Specifically, for Mahogany and Spanish cedar the difficulty in obtaining microbe-free cultures starting with explants from mature trees has been related to surface and internal contamination in donor plants (Maruyama 2006).

Contamination appeared to be absent from initial culture, but contamination was present after the fifth or later subcultures of mahogany shoots from pruned 3-year-old trees (Maruyama 2006). During micropropagation of *Toona ciliata* (Meliaceae) nodal explants from 2-year-old plants, between 17 and 35% of the cultures were contaminated during establishment, even though explants were surface sterilized by soaking in 70% ethanol for 1 min followed by immersion in 1.8% w/v sodium hypochlorite (NaOCl) for 30 min (Mroginski *et al.* 2003). For Mahogany nodal explants from 2-year-old plants, losses due to contamination during *in vitro* establishment were 44% even after double surface sterilization in calcium hypochlorite (Ca(OCl)₂) at 10% w/v for 20 min and then 8% w/v for 10 min (Flores 2001). Contamination of explants can also be prevented by adding NaOCl to the culture medium (Teixeira *et al.* 2006). These researchers report that the addition of NaOCl (0.0003% active chlorine) sterilized the culture media, doubled biomass and the number of new shoots on *Ananas comosus* explants.

Niedz (1998) suggests Plant Preservative Mixture (PPM™, Plant Cell Technology, Washington DC, USA) be used to reduce endogenous and surface contamination of explants. This product is a heat stable biocide, which kills bacteria and fungi cells, prevents germination of their spores, and potentially eliminates endogenous contaminants without impairing the response of

explants in tissue culture (Guri and Patel 1998). These authors state that the active ingredients (isothiazolones—methylchloroisothiazolinone and methylisothiazolinone) in PPM™ interfere the citric acid cycle and the electron transport chain and also inhibit transport of monosaccharides and amino acids in fungal and bacterial cells, improving the response of explants.

The response of explants *in vitro* also could be improved by adding plant growth regulators (PGR) and other substances to the culture medium and modifying the atmosphere inside the vessels. N6-benzylaminopurine (BAP) is the typical PGR used to induce bud break (Krikorian 1994), whereas activated charcoal and silver nitrate (AgNO₃) have been used to improve bud break of explants by modifying both the culture medium and the atmosphere in culture vessels (Kitaya *et al.* 2005). Silver nitrate is a regulator of ethylene biosynthesis, one of the main gases affecting explant response in culture vessels (Fuentes *et al.* 2000), and has bactericidal activity (Kuvshinov *et al.* 1999). Activated charcoal is often added to the culture medium to absorb inhibitory substances released by the explant into the medium and vessel headspace and has the additional effect for enhancing air movement in the culture vessels (Kitaya *et al.* 2005).

The atmosphere in vessels also can be modified by air exchange as is practiced for photoautotrophic micropropagation (Zobayed *et al.* 2004). Air exchange inside of the vessels depends on the type of sealant and closures used. For genetic transformation of *Brassica rappa*, cultures sealed with Micropore 3M paper tape increased regeneration from 0 to 5% up to 60 to 80% compared to parafilm mainly by allowing air interchange (Kuvshinov *et al.* 1999). Recalcitrance and contamination of Mahogany and Spanish cedar stem tissues are the main causes of ineffective micropropagation of both species *in vitro* by nodal explants excised from mature donor plants. Therefore, the objectives of this research were: a) to evaluate NaOCl and PPM™ as surface disinfectants and/or added to the culture medium for the *in vitro* establishment of nodal explants taken from 10-year-old Mahogany and Spanish cedar plants, and b) to evaluate the *in vitro* response of such explants treated with BAP, AgNO₃, activated charcoal, vented caps and Micropore paper.

Materials and methods

Plant material and explant preparation

Single nodal explants were taken from stems of 10-year-old Mahogany and Spanish cedar plants, which were maintained in a greenhouse. The shoots were cut early in the morning (ca. 6 a.m.) to obtain turgid tissues. Inside the greenhouse, leaves were severed, and shoots were put in a plastic bag and taken to the biotechnology laboratory at the Tropical Agricultural Research and Higher Education Center (CATIE, Costa Rica). There, plant material was washed with soap 0.03% (FADIS-Quimisol, Costa Rica), and placed under running water for 15 min, before cutting single nodal explants of about 2 cm in length. Nodal explants were then pre-disinfected as follows unless stated otherwise: 1 hour in Benomyl (Piscis, Costa Rica; 2 g l⁻¹) plus Manzate (Pfizer, México; 3 g l⁻¹) and Agrimycin (Pfizer, México; 1 g l⁻¹); 20 min in Ca(OCl)₂ at 10% w/v and 15 min in Ca(OCl)₂ at 8% w/v. Explants were treated with Ca(OCl)₂ inside the laminar flow hood. Explants were rinsed three times for 30 seconds each with sterile double-distilled water after surface sterilization. Fungicides, bactericides and Ca(OCl)₂ treatments were supplemented with Tween 20 (Sigma, St. Louis, MO, USA) one drop for 100 ml of solution.

1. Establishment of aseptic cultures

1.1 Use of NaOCl for surface sterilization and added to the culture media

- a) Pre-disinfection in Ca(OCl)₂ was changed by using NaOCl (commercial bleach at 3.5% active ingredient) at 15, 30, or 45% (v/v) for 10 or 20 min and then used in combination with NaOCl at 0, 2 or 4% v/v added to the medium. Each one of the 18 treatments consisted of three replications, each one with 10 Spanish cedar nodal explants.
- b) For Mahogany nodal explants, NaOCl at 15% (v/v) for pre-disinfection procedure for 10 or 20 min was combined with NaOCl at 2 or 4% (v/v) added to the medium. Each treatment consisted of five replications, each one with 10 explants.
- c) From the two latter experiments, the best combination reducing explant contamination and increasing bud break was NaOCl at 2% (v/v) added to the medium and NaOCl 15% (v/v) for 10 min during surface sterilization.

Therefore, another experiment consisting of three Mahogany assays of nodal explants 15 days after each other was completed in order to confirm the effect of such treatment. Each assay consisted of four replications. The number of explants introduced depended on the availability of plant material. Therefore, the first assay had 25 nodal explants per replication, the second had 70, and the third one had 45 nodal explants per replication.

In the three experiments, the medium used was Schenk and Hildebrandt (1972, SH medium) supplemented with BAP 1 mg l⁻¹. Containers and caps were autoclaved at 1.46 kg cm⁻² for 20 min, and the culture medium boiled for 5 to 10 min after heating for 10 min per liter in a microwave before dispensing into the containers. This procedure of culture media sterilization was used for all experiments since media containing NaOCl failed to gell when autoclaved. To be sure that media were free of microbial contamination non-inoculated media were allowed to sit for 10 d in sterile vessels. For the experiments with PPMTM, media and containers were autoclaved at 1.46 kg cm⁻² for 20 min.

1.2 Use of PPMTM added to the culture media and combined to the pre-disinfection treatment.

- a) In order to test the effect of PPMTM added to the culture medium, media were supplemented with 0, 1, 2, 3, or 4 ml l⁻¹ of this biocide and were placed in a chamber. Each dose (treatment) contained five replications, with 10 explants in each one. b) In a parallel experiment, the pre-disinfection in Ca(OCl)₂ was changed by rinsing nodal explants in PPMTM at 0 or 2 ml l⁻¹; later, explants were rinsed with sterile double distilled water and inoculated directly into the culture medium containing PPMTM at 0, 0.5, 1.0, 1.5, or 2 ml l⁻¹. Each treatment had five replications, with five explants in each one.

1.3 Use of PPMTM instead of the pre-disinfection treatment

To reduce internal contamination in Spanish cedar nodal explants, PPMTM at 5% v/v was added to Murashige and Skoog (MS, Murashige and Skoog, 1962) salts instead of the pre-disinfection treatment (Benomyl + Agrimycin, and then Ca(OCl)₂). Nodal explants were treated for 6, 12, 18, or 24 h, with the pre-disinfection treatment as control. Gentle shaking was applied

continuously during the PPMTM treatment in a shaker (GyromaxTM 721, HOTECH Instruments Corp., Taipei, Taiwan). Each one of the four treatments and the control included 10 replications, each one with 10 explants per replication. The culture medium was also supplemented with indolebutyric acid (IBA) 0.5 mg l⁻¹, isopentenyladenine (2-iP) 1 mg l⁻¹ and PPMTM 2 ml l⁻¹. Control explants were rinsed three times in sterile double-distilled water after surface sterilization; explants treated with PPMTM at 5% (v/v) were not rinsed.

2. Response of Spanish cedar and Mahogany to *in vitro* culture

2.1 Effect of BAP on bud break

Spanish cedar and Mahogany nodal explants were surface sterilized by rinsing in fungicides as described above and then treated for 10 min in NaOCl 15% (v/v). One experiment was completed to evaluate the effect of BAP at 0, 2.2, 4.4, 8.8, or 17.7 μM on bud break by both species. Each one of these five treatments had four replications for Spanish cedar and five for Mahogany, each one with 10 explants. The MS culture medium was supplemented with NaOCl 2% (v/v).

2.2 Improvement of the culture atmosphere

a) Use of silver nitrate and activated charcoal.

The objective of the experiment was to prevent leaf abscission by shoots from Spanish cedar nodal explants, by adding activated charcoal or AgNO₃ to the culture medium. The explants used had been introduced 20 days before on half strength MS medium, supplemented with sucrose 1.5% w/v, 2-iP 3.6 μM, NaOCl 2% (v/v), and Agrimycin 1 g l⁻¹. Activated charcoal at 1 g l⁻¹ and AgNO₃ at 3 mg l⁻¹, were tested alone or combined together (three treatments) along with a control, which lacked these substances. Each one of these four treatments included five replications with 10 explants per replication.

b) Use of vented caps. The objective of the experiment was to compare the effects of normal versus vented caps (Magenta Corp., Sigma Chemical) on leaf abscission of Mahogany shoots during *in vitro* establishment. Three replications were used for each treatment (either a normal or a vented cap on a glass jar) with each replicate containing 50 explants.

c) Use of Micropore tape as a vessel sealant.

The objective of the experiment was to compare the effect of Micropore tape (3M Corporation, St. Paul, MN) as vessel sealant during *in vitro* establishment of Spanish cedar nodal explants compared to using plastic wrap (Glad, Costa Rica) which is a common sealant (control) used around the vessel lid. Six replications were used for both treatments, each one with 12 nodal explants.

Half-strength MS culture medium was used as described by Flores (2001) in the experiments for improving the culture vessel atmosphere of explants *in vitro*. Medium was supplemented with 1.8 μM 2-iP, 11.4 μM indoleacetic acid (IAA), sucrose 1.5% w/v, activated charcoal 1 g l⁻¹, streptomycin 300 mg l⁻¹, and NaOCl 2% (v/v). The variables evaluated for aseptic establishment of cultures were the percentages of contamination by fungi and bacteria as well as bud break (shoots with axillary buds or at least one expanded bud at least 3 mm long). The response of explants to *in vitro* culture was recorded as the percentage of leaf abscission (means of fallen leaflets divided per mean total leaves multiplied by 100).

Culture conditions. All culture media were supplemented with sucrose 3% (w/v) unless stated otherwise, and gelled with agar 7 g l⁻¹; the pH was adjusted to 6.0 or 5.8 depending on the addition or absence of NaOCl in the medium. Cultures were incubated at 25 °C under a 12 h photoperiod provided by Phillips cool-white fluorescent light tubes.

Experimental design. All experiments were arranged in a completely randomized design. The experimental unit was an individual nodal explant in a 50 ml vial with 10 ml of culture medium for establishing of aseptic cultures or in 200 ml glass jar with 30 ml of culture medium for the response of nodal explants to *in vitro* culture.

Statistical analysis. Data were examined for normal distribution and homogeneity of variances required for analysis of variance (ANOVA). Contamination and bud break data were transformed by $Y = \arcsin(\sqrt{Y})$, and shoot length was transformed by $Y = \sqrt{Y + 0.5}$ before statistical analysis. ANOVA was completed (Proc GLM; SAS, 2001) and, if the F test was significant, means were compared using the Duncan's multiple range test or the least square mean test at the 5% level.

Results and discussion

Although stock plants were maintained in a greenhouse and fungicides and bactericides were applied routi-

nely every 15 d, in a preliminary experiment for *in vitro* establishment the incidence of contamination was 90% for Spanish cedar and 84% for Mahogany nodal explants, whereas bud break was lower than 17% for explants from both species. Therefore, the following experiments were completed in an attempt to reduce culture contamination and increase explant bud break.

1. Establishment of aseptic cultures

1.1 Use of NaOCl during surface sterilization and added to the culture media

Contamination of Spanish cedar nodal explants differed among levels of NaOCl used during surface sterilization ($F_{17, 36} = 10.03$, $P < 0.0001$), between the two times of sterilization ($F_{1, 36} = 17.33$, $P = 0.0002$), and

among levels of NaOCl added to the culture medium ($F_{2, 36} = 66.32$, $P < 0.0001$) (table 1). However, a three-way interaction between the factors was absent ($P = 0.1$).

A two-way interaction between treatments time for surface sterilization (10 and 20 min) and NaOCl added to the medium (0, 2, 4%) was presented for bud break of explants ($F_{2, 36} = 3.45$, $P = 0.04$). The 20 min of exposure for surface sterilization and NaOCl at 4% (v/v) added to the medium reduced explant contamination, but bud break was similar among NaOCl concentrations (table 1). During the surface sterilization treatments, deep penetration of sterilizing agents into the explant tissue could have killed cells and affected tissues, which resulted in delayed and reduced growth responses by explants (Thakur and Sood 2006), such as bud break.

Table 1. Contamination and bud break percentages of Spanish cedar (*C. odorata*) nodal explants as affected by NaOCl used during surface sterilization and added to the culture medium

| Treatments | Percentage* | |
|---|---------------|-----------|
| | Contamination | Bud break |
| Time (min) of surface sterilization | | |
| NaOCl 10 | 57.4 a | 10.4 a |
| NaOCl 20 | 42.2 b | 7.4 a |
| Concentration (%) for surface sterilization | | |
| NaOCl 15 | 52.2 a | 11.1 a |
| NaOCl 30 | 52.8 a | 7.8 a |
| NaOCl 45 | 44.4 a | 7.8 a |
| Concentration (%) added to the culture medium | | |
| NaOCl 0 | 78.9 a | 5.6 a |
| NaOCl 2 | 40.6 b | 11.7 a |
| NaOCl 4 | 30.0 b | 9.4 a |

For each individual factor, mean separation within columns by Duncan test at 5% level ($n = 53$).

* For time of surface sterilization data were the average over all the NaOCl concentrations; for concentration for surface sterilization and added to the culture medium data were the average over the surface sterilization time.

Contamination of Mahogany nodal explants differed among the combination of NaOCl treatments ($F_{3, 16} = 4.15$, $P = 0.02$) (table 2). The NaOCl concentration in the medium affected explant contamination ($F_1 =$

12.09 , $P = 0.003$), and an interaction between the factors was absent ($F_1 = 0.25$, $P = 0.6$). The surface sterilization time either 10 or 20 min similarly affected both contamination ($F_1 = 0.25$, $P = 0.6$) and bud break ($F_1 =$

0.25, $P = 0.6$), and the lower time (10 min) combined with 2% NaOCl added to the medium was enough to reduce contamination to 14%. Combined NaOCl treatments also affected bud break differently ($F_{1,36} = 7.67$, $P = 0.0004$) (table 2). The NaOCl concentrations in the medium affected bud break ($F_1 = 28.06$, $P = 0.0001$), and an interaction between NaOCl treatment time during surface sterilization and concentration NaOCl in the medium was absent ($F_1 = 4.10$, $P = 0.06$).

The lower dose of NaOCl added to the medium favored the higher percentage of bud break. Therefore, the best combination for explant treatment was NaOCl at 2 ml l⁻¹ added to the medium and 15% NaOCl used for 10 min during surface sterilization. Compared to 3% for 10 and 30 min or 2% for 10, 20 and 30 min and in

terms of survival, contamination and necrotic explants, Collado *et al.* (2004) defined NaOCl at 3% for 20 min as the best sterilization treatment for nodal explants from mature *S. macrophylla* plants.

The least squares means indicated statistical differences among treatments for both contamination and bud break (table 2). The low NaOCl concentration in the medium reduced contamination and increased bud break significantly more than the high concentration in the medium. Such results agreed with those of Teixeira *et al.* (2006) who reported active chlorine concentration as low as 0.0003% used to sterilize the culture medium improved the response of *Ananas comusus* (pineapple) by doubling biomass and number of new shoots.

Table 2. Contamination and bud break percentages of Mahogany (*S. macrophylla*) nodal explants as affected by NaOCl used during surface sterilization and added to the culture medium

| NaOCl at 15% as surface sterilization | Percentage | |
|--|---------------|-----------|
| | Contamination | Bud break |
| For 10 min + NaOCl at 2% into the culture medium | 14.0 a | 50.0 a |
| For 20 min + NaOCl at 2% into the culture medium | 20.0 a | 42.0 a |
| For 10 min + NaOCl at 4% into the culture medium | 43.2 b | 18.6 b |
| For 20 min + NaOCl at 4% into the culture medium | 42.0 b | 26.0 b |

Mean separation within columns by least squares means at the 5% level ($n = 50$).

In the follow up study, the best combination of NaOCl treatments was used in three trials for starting Mahogany nodal explants in culture. From these trials, the overall contamination rate of nodal explants was low ($19.8\% \pm 1.8\%$), and bud break was $53\% \pm 3.5\%$. Although bud break was similar among the three trials ($F_{2,9} = 3.86$, $P = 0.06$), the percentages of explant contamination were 23%, 8.2% and 28.2%, respectively. The contamination differences among the trials were most clearly due to environmental conditions. High contamination rates in the first and third trials were probably due to taking nodal explants during periods of heavy rainfall, and the experiment was conducted in December when temperature was lower than in summer, although rainfall amounts were higher (Salas 2000). For the micropropagation of *Eucalyptus tereticornis* at various times throughout the year, incidence of endogenous fungal or bacterial contaminants was

most severe during the hot and humid months of late summer (Sharma and Ramamurty 2000).

1.2 Use of PPM™ added to the culture medium and then combined with PPM™ treatment during surface sterilization.

Various concentrations of PPM™ added to the medium failed to affect contamination ($F_{4,20} = 0.27$, $P = 0.9$) and bud break percentage ($F_{4,20} = 1.49$, $P = 0.2$) for Spanish cedar; the mean (\pm SE) for contamination and bud break were $49.6\% \pm 3.6\%$ and $71.6\% \pm 2.5\%$, respectively, and contamination of nodal explants was mainly due to bacteria. For Mahogany, PPM™ in the culture medium significantly reduced contamination and also significantly favored shoot elongation (table 3), but its effect on bud break was similar ($F_{4,20} = 0.55$, $P = 0.7$). The mean (\pm SE) bud break was 81.2%

± 2.4%. Contamination of nodal explants was mainly due to fungi. The difference of contaminants between Mahogany (32.4% fungi, 5.2% bacteria) and Spanish cedar (8.8% fungi, 40.8% bacteria), agreed with results from Leifert and Waites (1992) who showed different plant species grown *in vitro* create distinct environments inside the culture vessels, which in turn allow or prevent the growth of different contaminants.

Differences of contaminants between both species also could be attributed to different efficacy of PPM™ treatments. The PPM™ added to the medium worked better for Mahogany than for Spanish cedar reducing contamination and increasing bud break. Compared to the preliminary experiment, contamination was reduced by 46.4%, whereas bud break was increased by 68.2% for Mahogany. Besides reducing contamination ($F_{4, 20} = 4.07$, $P = 0.01$) and increasing bud break ($F_{4, 20} = 2.84$, $P = 0.05$), PPM™ at 2 ml l⁻¹ also favored the elongation of sprouted buds on Mahogany (table 3).

Table 3. Contamination percentages and subsequent shoot length of Mahogany (*S. macrophylla*) nodal explants as affected by various concentrations of PPM™ added to the culture medium

| PPM™ (ml l ⁻¹) | Contamination (%) | Shoot length (cm) |
|----------------------------|-------------------|-------------------|
| 0 | 54.00 a | 0.40 b |
| 1 | 44.00 ab | 0.60 ab |
| 2 | 32.00 bc | 0.70 a |
| 3 | 38.00 ab | 0.50 ab |
| 4 | 20.00 c | 0.50 ab |

Mean separation within columns by Duncan test at the 5% level (n = 25).

For Spanish cedar explants, PPM™ doses had similar effects as the control for contamination and bud break percentages. The mean contamination varied from 42% for PPM™ at 4 ml l⁻¹ to 54% for PPM™ at 3 ml l⁻¹. The bud break mean percentage varied from 64% on PPM™ at 3 ml l⁻¹ to 84% for PPM™ at 1 ml l⁻¹. For the control, contamination and bud break were 50% and 72%, respectively.

When PPM™ was used during surface sterilization and added to the culture medium, treatment effects on Spanish cedar nodal explants were similar to each

other for contamination ($F_{9, 40} = 1.05$, $P = 0.42$) and bud break ($F_{9, 40} = 0.65$, $P = 0.74$). Although the bud break (67.2% ± 3.3%) was similar to that of the previous experiment, the contamination was higher (79.2 ± 2.6%, with 71.5% explants contaminated by bacteria, and 7.7% by fungi). This result could be explained by incomplete pre-surface sterilization treatment for half of introduced explants because PPM™ at 0 or 2 ml l⁻¹ was applied instead of Ca(OCl)₂ in the surface sterilization procedure. Jiménez *et al.* (2006) reported that the use of pre-surface sterilization with NaOCl combined with PPM™ in the culture medium was an efficient way to establish *in vitro* explants of the neotropical giant bamboo (*Guadua angustifolia*). Another reason would be that three out of four concentrations of PPM™ added to the medium were lower than the level suggested by the manufacturer (2 ml l⁻¹), which also was the best in the previous experiment to reduce contamination, to increase bud break, and enhance shoot elongation for Mahogany.

For Mahogany, an interaction between surface sterilization with PPM™ and PPM™ in the medium affected explant contamination ($F_{9, 37} = 2.90$, $P = 0.01$) and bud break ($F_{9, 37} = 2.28$, $P = 0.03$) (table 4), but shoot elongation appeared unaffected ($F_{9, 37} = 1.01$, $P = 0.4$). The mean (± SE) shoot elongation was 0.5 cm ± 0.2 cm. If used alone, PPM™ at 1 or 2 ml l⁻¹ added to the medium, controlled contamination better and increased bud break compared to all the other treatments.

The combination of PPM™ used during surface sterilization and added to the culture medium increased contamination and reduced bud break, compared to PPM™ added to the culture medium (the above experiment). In this experiment, PPM™ again reduced contamination more for Mahogany than for Spanish cedar, maybe due to the different microbial contaminants in each species. Mahogany was contaminated by fungi and Spanish cedar by bacteria. Such specificity of contaminants could be attributed to plant species-specific bacteria being introduced into culture due to endogenous contamination within the explants (Fisse *et al.* 1988).

1.3 Use of PPM™ during surface sterilization.

Rinsing time with 5% PPM™ during explant surface sterilization affected explant contamination ($F_{4, 45} = 6.28$, $P = 0.0004$), bud break ($F_{4, 45} = 33.18$, $P < 0.0001$), and damage by necrosis ($F_{4, 45} = 45.55$, $P = 0.0001$) depending on the rinsing time (table 5). Compared to the control, PPM™ at 5% during surface sterilization failed to reduce contamination or improve bud break

but was toxic to subsequent shoot growth of Spanish cedar nodal explants. Toxicity increased as the PPM™ treatment time increased. Such toxicity could be due to PPM™ by being an acid and inducing necrosis (Guri and Patel 1998). So, PPM™ damaged the nodal explants preventing bud break and increased explant

susceptibility to contaminants. Nevertheless, methylisothiazolinone and methylchloroisothiazolinone, the biocides used in PPM™, are reported to be nonphyto-toxic at concentrations of 4 to 5% for 4 to 12 h for the prophylactic control of endogenous contaminants in plant tissue cultures for woody plants (Guri 2004).

Table 4. Contamination and bud break percentages of Mahogany (*S. macrophylla*) nodal explants as affected by PPM™ used during surface sterilization and added to the culture medium

| PPM™ concentration (ml l ⁻¹) | | Percentage | |
|--|-----------------|---------------|-----------|
| As surface sterilization | Into the medium | Contamination | Bud break |
| 0 | 0.0 | 44 abc | 52 b |
| 0 | 0.5 | 32 bc | 72 ab |
| 0 | 1.0 | 20 c | 96 a |
| 0 | 1.5 | 32 bc | 56 b |
| 0 | 2.0 | 20 c | 72 ab |
| 2 | 0.0 | 70 a | 55 b |
| 2 | 0.5 | 52 abc | 64 b |
| 2 | 1.0 | 70 a | 55 b |
| 2 | 1.5 | 60 ab | 50 b |
| 2 | 2.0 | 44 abc | 44 b |

Mean separation within columns by Duncan test at the 5% level (n = 47).

Table 5. Contamination, bud break and necrosis percentages of Spanish cedar (*C. odorata*) nodal explants as affected by rinsing time with 5% PPM™

| Rinsing time (h) with PPM™ 5% | Percentage | | |
|-------------------------------|-------------------|-----------|-----------------------|
| | Contamination | Bud break | Necrosis ^y |
| 0 | 56 b ^z | 30 a | 1 c |
| 6 | 80 a | 0 b | 4 c |
| 12 | 59 b | 1 b | 26 b |
| 18 | 45 b | 0 b | 45 a |
| 24 | 60 b | 1 b | 55 a |

^y A necrotic explant was considered when more than a half of its surface had a brown-blackish color.

^z Mean separation within columns by Duncan test at the 5% level (n = 50).

Both Mahogany and Spanish cedar responded differently to PPM™ treatments used as rinses for surface sterilization and added to culture media. PPM™ contains two isothiazolones, which are a broad-spectrum industrial biocides (Niedz 1998); however, the level of

contamination recorded for both species was similar to that reported by Flores (2001) for nodal explants from 5-year-old Mahogany plants when using Ca(OCl)₂ and NaOCl during surface sterilization. This author recorded 44% contamination of explants receiving dou-

ble surface sterilization treatments with $\text{Ca}(\text{OCl})_2$ (10% for 20 min then 8% for 10 min) and 45% contamination with NaOCl at 50% for 15 min. However, donor plants used in this research were 5 years older than those used by Flores (2001), and donor plant age is related to the incidence of endogenous contamination (Maruyama *et al.* 1989).

The results indicated that PPM™ can be added at 2 ml l⁻¹ to the culture medium during *in vitro* establishment of Mahogany to control contamination without impairing bud break or shoot elongation. For Spanish cedar, however, more experiments with this product are necessary to reduce contamination. Niedz (1998) indicated that PPM™ can be routinely added to tissue culture medium to control air- and waterborne bacterial and fungal contaminants effectively.

2. Response of Spanish cedar and Mahogany to *in vitro* culture

2.1 Effect of BAP on bud break

Various concentrations of BAP failed to affect bud break of Spanish cedar ($F_{4, 15} = 0.91$, $P = 0.5$) or Mahogany ($F_{5, 23} = 1.83$, $P = 0.1$) explants similarly. Nevertheless, both species responded differently to BAP treatments ($F_{5, 39} = 4.94$, $P = 0.001$), and in general bud break was almost twofold higher for Mahogany explants compared to those of Spanish cedar ($44.7\% \pm 3.7\%$ and $23.5\% \pm 2.1\%$, respectively). However, different species usually have different responses *in vitro* (Kane 2000), so the highest BAP concentration resulted in higher percentages of bud break for Mahogany than for Spanish cedar (table 6). This result could be explained because although both species were the same age, Mahogany tissues were woodier than Spanish cedar.

Rodríguez *et al.* (2003) found a better response for *in vitro* bud break of a *Swietenia* hybrid compared to Spanish cedar after BAP treatment. These authors stated that stem explants from both species responded to increasing concentrations of BAP by sprouting more buds; however, Collado *et al.* (2004) stated that *S. macrophylla* nodal explants sprouted better at low concentrations (1 or 2.5 μM) of BAP which agreed with this study since there was not statistical difference among treatments (table 6). For Spanish cedar and Mahogany, Pérez *et al.* (2006) and Flores (2001) respectively, reported that nodal explants responded to 2.2 μM or 6.5 μM better than to 20 μM BAP. The last authors used nodal explants from *in vitro* seedlings or 2-year-old plants, but here nodal explants from 10-year-old plants were used.

Table 6. Effect of BAP on mean percentage bud break of Spanish cedar (*C. odorata*) and Mahogany (*S. macrophylla*) nodal explants

| BAP (μM) | Bud break (%) | |
|-----------------------|---------------|----------|
| | Spanish cedar | Mahogany |
| 0 | 25 a | 41 a |
| 2.2 | 27 a | 49 a |
| 4.4 | 25 a | 29 b |
| 8.8 | 15 a | 37 ab |
| 17.7 | 25 a | 64 a |

Mean separation within columns by Duncan test at the 5% level ($n = 40$ and 50 for Spanish cedar and Mahogany explants, respectively).

For Mahogany, BAP at 17.7 μM seemed to favor the bud break, which differed with the results of Collado *et al.* (2004). These authors attained 63.9% bud break on nodal explants from Mahogany by using BAP at 1 μM . However, they did not state the age of their mature plants. Mroginski *et al.* (2003) also found that BAP at 22.2 μM impair bud break in nodal explants from 2-year-old *Toona ciliata* (Meliaceae) plants but improved it for explants from 10-year-old *T. ciliata* plants.

2.2 Improvement of the culture atmosphere

In all the previous experiments, some shoots grew ca. 1 to 1.5 cm and produced leaves. Leaves stayed on the explants until the 15th day after inoculation and then fell off. So, three experiments were conducted in an attempt to prevent leaf abscission by improving the culture vessel atmosphere.

Effect of silver nitrate and activated charcoal on Spanish cedar. Neither activated charcoal nor AgNO_3 alone or combined prevented abscission of new foliage from Spanish cedar nodal explants (data not shown, $F_{3, 16} = 1.12$, $P = 0.3$). Ozudogrl *et al.* (2005) demonstrated that AgNO_3 improved *in vitro* culture of some herbaceous species. In the present experiment, although AgNO_3 at 3 mg l⁻¹ lacked a statistical significant effect, it tended to decrease contamination while increasing leaf abscission.

The effect of AgNO_3 on explant contamination could have been due to Ag^+ , which has bactericidal activity (Kuvshinov *et al.* 1999). Kane (2000) suggested AgNO_3 at 1% as a surface disinfectant for explants; although we used 0.0003% AgNO_3 , this lower concentration

may have helped to prevent contamination of inoculated explants. The effect of AgNO₃ increasing leaf abscission could be explained by a phytotoxic effect of Ag⁺, which is a heavy metal (Pua 1999). For *Petunia* hybrid, AgNO₃ also inhibited shoot proliferation (Dimasi-Theriou *et al.* 1993).

Activated charcoal also helped to prevent leaf abscission, although treatments were statistically similar (data not shown). This positive effect could be explained because activated charcoal is typically an absorbent for gases such as ethylene (Pan and van Staden 1998). Therefore, activated charcoal could have improved the vessel atmosphere and the explant responses.

Use of vented caps for Mahogany. The types of caps used on cultures failed to affect contamination percentages ($F_{1,10} = 0.09$, $P = 0.77$) and bud break ($F_{1,10} = 4.04$, $P = 0.11$) of Mahogany nodal explants. Nevertheless, the culture atmosphere seemed to be improved since fewer contaminated nodal explants were in vessels covered with vented caps than in normal caps ($33.3 \pm 16.2\%$ and $43.2 \pm 20.8\%$, respectively). Also, bud break was over double for nodal explants established in vessels with vented caps compared to those with normal caps ($52.7 \pm 13.0\%$ and $25.3 \pm 4.4\%$, respectively). These results could be explained by a positive ventilation effect in the vessel atmosphere, specifically by the filter of vented caps that might have allowed the exchange of air without microbial contaminants entering the vessel.

During photoautotrophic micropropagation of *Eucalyptus*, ventilation improved growth and quality, as well as lowered the percentage of contamination due to the manipulation of environmental conditions inside the culture vessels (Zobayed *et al.* 2004). In this study, such conditions prevented leaf abscission from shoots about 2 cm in length at least up to day 20. Leaves remained on shoots up to day 35, and then they started to drop from the shoot.

Use of Micropore tape as sealant for Spanish cedar.

The results for the type of sealant preventing leaf abscission were similar to results for the type of caps. Contamination was similar for vessels sealed with either Micropore tape or plastic wrap (the normal sealant) ($F_{1,10} = 1.57$, $P = 0.24$), and bud break was also unaffected by the type of sealant ($F_{1,10} = 0.41$, $P = 0.54$). Nodal explants in vessels sealed with Micropore tape, however, grew better than those in vessels with the normal sealant since the contamination percentage was lower ($30.1\% \pm 11.0\%$ compared to $50.0\% \pm 11.4\%$) and the bud break percentage was higher ($52.8\% \pm 10.0\%$ compared to $43.0\% \pm 11.5\%$). The lower contamina-

tion yet higher bud break percentages could be due to better control of humidity inside the container allowed by the Micropore sealant.

Micropore tape is a breathable paper tape that allows gas exchange but keeps the culture atmosphere free from airborne bacteria and contaminants (Burne 2006). Gaseous substances such as CO₂ and ethylene in the headspace of cultured explants could affect their *in vitro* response (Demeester *et al.* 1995). Sealing the containers with closure materials that allow gas exchange may be essential for bud break. Therefore, Micropore tape could be combined with vented caps in order to get a better control of the *in vitro* atmosphere to improve responses by Spanish cedar and Mahogany nodal explants.

In conclusion, PPM™ at 2 ml l⁻¹ or NaOCl at 2% added to the medium and combined with NaOCl at 15% for 20 min as surface sterilization decreased contamination for single nodal explants from both Spanish cedar and Mahogany. These disinfection treatments allowed the establishment of nodal explants from mature *Meliaceae* plants as the first stage of *in vitro* propagation. For both species, the combination of vented caps and micropore tape as sealant might improve the *in vitro* response of nodal explants during the establishment phase of micropropagation.

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