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# DISINFECTION PROTOCOL AND *in vitro* GERMINATION OF SEEDS OF *Dalbergia nigra*

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## HIGHLIGHTS

NaOCl (14 min) is indicated for the disinfestation of seeds of bahia rosewood.

NaOCl exhibited toxicity in the lettuce seed model.

The WPM medium is more suitable than the MS.

In the medium WPM, the use of glutamine is suggested.

Keywords:
Bahia rosewood
*In vitro* cultivation
Disinfecting agents
Sodium hypochlorite toxicity
Culture media
Glutamine

## ABSTRACT

The Atlantic Forest is a biome that has suffered anthropic actions, such as the extraction of hardwood, which can lead to the extinction of endemic species of great economic and ecological value, such as *Dalbergia nigra*. In this perspective, large-scale multiplication studies are necessary to contribute to the conservation of this species. The objective of this work was to establish a protocol for the *in vitro* production of axenic seedlings of *D. nigra*. Four experiments were performed: I. Immersion times in NaOCl. II. Disinfecting agents in seed germination of *D. nigra*. III. NaOCl toxicity in seeds of the model species *Lactuca sativa* L. IV. Culture media and glutamine in the germination and initial growth of *D. nigra*. All experiments were arranged in a completely randomized design. For a better disinfestation and less harmful effects to the seedlings, it is recommended that the seeds be treated with 70% alcohol (1 min) and NaOCl (14 min). NaOCl has a high phytotoxic, cytotoxic, and genotoxic effect, and its mechanism of action in the *L. sativa* cell cycle is clastogenic and aneugenic, suggesting the formation of abnormal seedlings in *D. nigra*. The WPM medium and its supplementation with glutamine (0.75 mg·L$^{-1}$) are recommended.

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## INTRODUCTION

*Dalbergia nigra* (Vell.) Fr. All. Ex Benth. (Papilionoideae) is an endemic species to the Atlantic Forest, popularly known as Bahia rosewood (Lorenzi, 2016). Due to the intense exploitation of its timber resources, along with anthropogenic actions on the forest, attacks by pests (*Troezon championi* Lima) on seeds, and predators (*Sylvilagus brasiliensis* Linnaeus) on seedlings, its population has been disappearing and is now on the Red List of Threatened Species (Brasil, 2014; Leite et al., 2014).

*In vitro* germination is an alternative for the propagation of species with difficulty for seminiferous multiplication under natural conditions, allowing large-scale production in a shorter period of time and space, besides obtaining seedlings with high phytosanitary quality (Franceschi et al., 2019). One of the major problems faced in *in vitro* propagation is the explant disinfestation and its establishment free of contaminants, which may be either of exogenous (fungus) origin, limiting the tissue surface, or endogenous (bacteria), localized within the organism (Karmakar et al., 2019; Mendy et al., 2019), and making multiplication unfeasible.

Disinfestation protocols vary between the species and type of explant used, which are exposed to disinfecting agents with their respective exposure times (Santos et al., 2019). Woody species present greater difficulty for *in vitro* establishment due to the greater contamination of explants (Sarmast, 2018).

Given the great benefits of *in vitro* propagation and the need for more detailed studies on the behavior of bahia rosewood, in this condition, this work aimed to establish a protocol for the *in vitro* production of axenic seedlings of *Dalbergia nigra*.

## MATERIAL AND METHODS

Mature seeds of *Dalbergia nigra* harvested from matrices located in Viçosa, Minas Gerais (20°45'35.1"S, 42°52'06.1"W) and obtained with the Society of Forest Investigations (SIF) of the Federal University of Viçosa (UFV) were used in the experiment. The SIF is registered in the Ministry of Agriculture, Livestock, and Supply.

### Experiment I: Disinfestation and *in vitro* germination of *D. nigra* seeds with different NaOCl immersion times.

In a laminar flow cabinet, the seeds were immersed in alcohol (70%) for one minute, and then in a commercial solution (Candura®) of sodium hypochlorite (NaOCl) (2.0-2.5% active chlorine), in which the immersion times of 0; 5; 10; 15; 20, and 25 min constituted the treatments. Afterward, a triple wash with distilled and autoclaved water was performed after the use of each disinfecting agent. The experiment was conducted in a completely randomized design with four replications of 25 seeds.

One seed was placed per test tube containing 10 mL of the Sigma-Aldrich® Woody Plant Medium (WPM) culture medium (Lloyd and McCown, 1980), supplemented with sucrose (3%, Dinamic®), myo-inositol (100 $mg \cdot L^{-1}$, Sigma-Aldrich®), pH adjusted to 5.7±0.1, and solidified with agar (7 $g \cdot L^{-1}$, Kasvi®), being sterilized by autoclaving for 20 min at 121°C and 1 atm pressure. The *in vitro* germination experiments were kept in a growth room under a 50 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetic photon flux density obtained by white LED lamps, in a 16-hour photoperiod and temperature of 27±2 °C.

The following characteristics were evaluated for 30 days: contamination (%), germination (%), non-germinated seeds (%), germination speed index, mean germination time (days), normal seedlings (%), abnormal seedlings (%), total length (cm), number of leaves, collar diameter (mm), and total dry mass of seedlings (mg).

Germination (%) was conducted with four replications of 25 seeds for each treatment. The germinated seeds were counted daily for 30 days. Germination was characterized by the emission of the primary root with a length greater than or equal to two millimeters (Brasil, 2009). The results were expressed as the percentage of germination.

The germination speed index (GSI) was determined concomitantly with the germination test, daily computing the number of seeds that showed protrusion of the primary root equal to or greater than 2 mm (Maguire, 1962), being counted until the 30th day. The GSI was calculated by summing the number of seeds germinated at each day and dividing this value by the number of days elapsed between sowing and germination: GSI = (G1/N1) + (G2/N2) + (G3/N3) + ... + (Gn/Nn), where: GSI = germination speed index; G1, G2, G3, ..., Gn = number of germinated seeds computed at the first, second, third, and last count; N1, N2, N3, ..., Nn = number of days from sowing to the first, second, third, and last count.

The equation by Laboriau (1983) was used for the determination of the mean germination time: (MGT) $MGT = (\Sigma niti)/\Sigma ni$, in which, ni = number of seeds germinated per day; ti = incubation time (days).

The percentage of normal seedlings was calculated considering the seedlings with all formed structures (root, hypocotyl, and cotyledons) in relation to the germination percentage (seedlings with primary root protrusion) at 30 days after sowing, according to Brazil (2009). Were considered as abnormal seedlings all those that did not follow the above-mentioned normality pattern, such as the rosette shape of the shoot part.

The data were subjected to analysis of variance and the means were compared through regression analysis ($p<0.05$) (Cruz, 2016).

## Experiment II: Disinfectants in the establishment and *in vitro* germination of seeds of *D. nigra*.

The seeds were immersed in alcohol (70%) in a laminar flow cabinet for one minute, as well as in the disinfectant agents, according to the defined treatments.

In order to enhance the number of normal seedlings and reduce the high percentage of contamination, the disinfestation experiment II was performed with the inclusion of hydrogen peroxide ($H_2O_2$), Captan® fungicide, and Kasumin® bactericide. NaOCl (14 min) was selected as one of the treatments to be used, as it presented the highest percentage of normal seedlings in experiment I.

The treatments consisted of different combinations of disinfecting agents: Candura® commercial NaOCl (2.0-2.5% active chlorine); Alphatec® hydrogen peroxide ($H_2O_2$); Captan SC Adama® fungicide, and the Kasumin Arysta LifeScience® bactericide, as follows: T1 - NaOCl 2.5% (14 min); T2 - Captan 0.5% (5 min) + Kasumin 1 mL·L$^{-1}$ (10 min); T3 - Captan 0.5% (5 min) + Kasumin 1 mL·L$^{-1}$ (10 min) with residue; T4 - $H_2O_2$ 10% v·v$^{-1}$ (5 min); T5 - $H_2O_2$ 20% v·v$^{-1}$ (5 min); T6 - NaOCl 2.5% (14 min) + $H_2O_2$ 10% v·v$^{-1}$ (5 min); T7 - NaOCl 2.5% (14 min) + $H_2O_2$ 20% v·v$^{-1}$ (5 min). A triple wash was then performed with distilled and autoclaved water, and the experiment was performed in a completely randomized design, with seven treatments and four replications of 10 seeds.

One seed was inserted into each test tube, which contained 10 mL of the WPM culture medium (Lloyd and McCown, 1980) supplemented with sucrose (30 g·L$^{-1}$, Dinamic®), myo-inositol (100 mg·L$^{-1}$, Sigma-Aldrich®), pH adjusted to 5.7±0.1, and solidified with agar (5.5 g·L$^{-1}$, Kasvi®), autoclaved for 20 min at 121 °C and a pressure of 1 atm.

The following characteristics were evaluated for 30 days: contamination (%), germination (%), non-germinated seeds (%), germination speed index, mean germination time (days), normal seedlings (%), abnormal seedlings (%), total length (cm), number of leaves, collar diameter (mm), and total dry mass of seedlings (mg). The data were subjected to analysis of variance and the means were compared by the Scott-Knott clustering test ($p<0.05$) (Cruz, 2016).

## Experiment III: Evaluation of NaOCl toxicity in a *Lactuca sativa* L. seed bioassay as a model plant.

*L. sativa* seeds are used for bioassays because they are highly sensitive to the environment, presenting low reserve tissues that allow them to easily absorb the compounds to which they are subjected (Andrade et al., 2010; Rodrigues et al., 2013).

Seeds of the lettuce (*Lactuca sativa* L.) Manteiga (Feltrin Sementes®) were used for the toxicity test, being analyzed in a completely randomized design, with treatments corresponding to five concentrations of NaOCl (0.1; 0.5; 1.0; 1.5, and 2.0%), a glyphosate herbicide solution (positive control), and distilled water (negative control), with five replications of 25 seeds.

The seeds were placed in Petri dishes with Germitest® paper moistened with 2.5 mL of the solution corresponding to each treatment. These were then taken to a BOD (Biochemical Oxygen Demand) germination chamber with a 12-hour photoperiod and temperature of 27±2 °C. The germination and GSI were counted every eight hours over a period of 48 hours.

After 48 hours, the root length (cm) was measured with a digital caliper. Ten seedlings from each treatment were randomly selected for fixation in a solution of methanol and acetic acid (3:1), and placed in a freezer. After 120 hours, the shoot length (cm) of the seedlings that remained in the Petri dishes was measured with a digital caliper.

At room temperature, the fixed seedlings were subjected to a triple distilled water wash (10 min per wash), immersed in an HCl 5N solution for 18 min, and again in distilled water for 10 min. The mitotic index was then determined using the crush technique (Guerra and Souza, 2002).

In a microscope slide, the hood was removed to obtain the apical meristem, which was stained with 2% acetic orcein and covered by a coverslip. The slides were observed under an objective microscope lens (Nikon® BE Plan 40x/0.65) and were accounted for by the observation of 1000 cells per treatment in interphase, prophase, metaphase, anaphase, telophase, dividing cells, mitotic index (%), and chromosomal changes (%).

The mitotic index was obtained by dividing the number of cells in mitosis by the total number of cells observed, multiplying by 100. The data were subjected to analysis of variance and the means were compared by Dunnett's test ($p<0.05$) (Cruz, 2016).

## Experiment IV: Culture media and glutamine in the germination and initial growth of *D. nigra*.

In a laminar flow chamber, the seeds were disinfected in alcohol (70%) for one minute, followed by immersion in sodium hypochlorite, NaOCl (2.0-2.5% active chlorine) (SuperGlobo®) for 14 min. After the use of each of these disinfecting agents, the seeds were subjected to a triple wash in distilled and autoclaved water. The culture media used were the MS (Murashige and Skoog, 1962) and WPM (Lloyd and McCown, 1980), supplemented with sucrose (30 g·L$^{-1}$, Dinamic®), myo-inositol (100 mg·L$^{-1}$, Sigma-Aldrich®), glutamine (0; 0.25; 0.5, and 0.75 mg·L$^{-1}$), and solidified with

agar (5.5 g·L$^{-1}$, Kasvi®). The pH of the culture medium was adjusted to 5.7±0.1. The culture medium was distributed in plastic-capped vials and sterilized by autoclaving for 20 min at a temperature of 121 °C and a pressure of 1 atm.

The experiment was arranged in a completely randomized design, in a 2 x 4 factorial scheme (culture media: MS and WPM x Glutamine: 0; 0.25; 0.5, and 0.75 mg·L$^{-1}$), totaling eight treatments with four replications of 25 seeds each. The characteristics analyzed were: contamination (%); germination (%); non-germinated seed (%); germination speed index; mean germination time (days); normal seedlings; abnormal seedlings (%); non-developed seedling (%); number of leaves; collar diameter (mm); shoot length (mm); root length (mm); number of lateral roots; shoot dry mass of seedlings (mg), root dry mass of seedlings (mg), and total dry mass of seedlings (mg).

The data were subjected to analysis of variance and the F test at 5% significance for both media types and the culture media x glutamine concentrations interaction. The means were analyzed by regression (Cruz, 2016).

## RESULTS AND DISCUSSION

### Experiment I: Disinfestation and *in vitro* germination of seeds of D. nigra with different NaOCl immersion times

The different immersion times in NaOCl showed no significant difference for the variables of contamination, germination, non-germinated seeds, GSI, MGT, abnormal seedlings, total seedling length, leaf number, collar diameter, and total seedling dry mass (Figure 1).

The contamination (29.25%) of explants at different NaOCl concentrations (Figure 1) was 100% bacterial. The germination percentage, regardless of the time of exposure to NaOCl, was 52.41% (Figure 1), with a mean germination time of 9.47 days (Figure 1). The highest percentage of normal seedlings (41.95%) was obtained with 14 min of exposure to NaOCl (Figure 1). NaOCl reduced contamination and, consequently, favored a higher percentage of normal seedlings of *Melanoxylon brauna* up to a maximum exposure time of 25 min (Silva et al., 2019). In *D. nigra*, above 14 min of exposure of the seeds to NaOCl, there was a reduction in seedling normality (Figure 1). The determination of this exposure time to the NaOCl disinfecting agent is extremely important as it helps to reduce cytotoxicities and genotoxicities. Santos et al. (2019) used a lower concentration of NaOCl (1%), which was efficient for the *in vitro* establishment of nodal segments of *Olea europaea* L. 'Arbequina' and 'Koroneiki', not promoting seedling toxicity.

NaOCl adsorbs to the seed surface and, even after several washes with water, the residue is still capable of causing reactions with organic compounds. As the exposure time to the product increases, more residue is adsorbed by the seed, reacting with amino acids and generating a high concentration of ammonium chloride ($NH_4Cl$) and carbon dioxide ($CO_2$) in the test tube. Furthermore, NaOCl hydrolysis produces hypochlorous acid (HClO), a toxic compound that leads to cellular and photosynthetic changes, among others, negatively affecting seedling growth, which

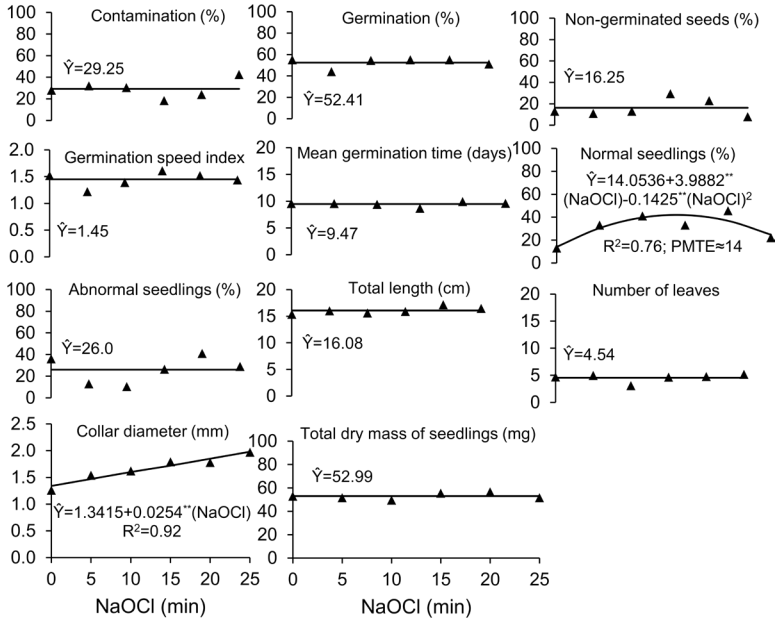


**FIGURE 1** Characteristics evaluated in seeds and seedlings of *D. nigra* after different seed immersion times in NaOCl. **Significant at a 5% level of probability. Abbreviation: PMTE. Point of maximum technical efficiency.

causes abnormal seedling formation (Abdul-Baki, 1974; Gamage et al., 2018).

With the increase in the exposure to NaOCl, there was an increase in the diameter of the seedling collar (Figure 1). This may be related to the response of the seedlings to the effect of NaOCl, promoting a thickening of the collar, a phenomenon characterized as rusticity, mediated by stress.

## Experiment II: Disinfecting agents in the establishment and *in vitro* germination of seeds of *D. nigra*

There was a significant difference for contamination, shoot length, shoot dry mass, root dry mass, and total dry mass of seedlings (Table 1).

Although no statistical difference was observed between the treatments that provided the lowest contamination rates (T1, T2, T6, and T7), it was decided to choose T1 for the disinfestation of seeds of *Dalbergia nigra*, aiming at a lower tissue exposure to stress and the reduction of production costs of axenic seedlings using economically viable disinfecting agents, such as alcohol and NaOCl. Treatments T3, T4, and T5 presented the highest percentages of contamination, having in common the absence of hypochlorite (Table 1). This is an important disinfecting agent since the chlorine present in the molecule combines with proteins in the cell membranes of the microorganism, forming toxic compounds that inhibit essential enzymes for its proliferation (Donini et al., 2005). For example, the antibacterial efficiency (93%) of the use of NaOCl (2.5%), for 30 min, was proven in seeds of *Calendula officinalis* L. (Bevilacqua et al., 2011).

In experiment II, the percentage of twinning and normal seedlings did not differ between treatments, with a mean of 77.86% (25.45% higher than experiment I) and 66.07% (24.12% higher than experiment I), respectively. It was also verified that the contamination was 3.9 times lower when compared to the experiment I, indicating that the origin of the batch has a great influence on these characteristics (Table 1).

Contamination can be a hindrance in *in vitro* establishment, as contaminants compromise seedling growth, either by competition for nutrients or the production of phytotoxic metabolites, such as lactic and acetic acids (Monfort et al., 2015).

The seedlings of T1, along with treatments T2, T3, and T7 presented one of the highest values of SDMS (8.04 mg). Its RDMS (31.45 mg) and TDMS (39.49 mg) did not differ statistically from most treatments, except for T4, which presented the lowest value (20.67 mg) (Table 1). The dry mass content incorporated by the seedlings is one of the main quality characteristics, which are important for acclimatization, as it indicates a greater rusticity and ability to overcome water loss, oscillations in relative humidity, temperature, and light intensity (George et al., 2008).

## Experiment III: Evaluation of NaOCl toxicity in a *Lactuca sativa* L. seed bioassay as a model plant

Only in the treatment with the lowest concentration of NaOCl (0.1%) there was germination (Table 2), which evidences the presence of phytotoxicity in higher concentrations.

**TABLE 1** Disinfestation treatments on germination, vigor, and *in vitro* growth of seedlings of *D. nigra*.

| TREAT | CONT (%) | GER (%) | NGS | GSI |
|---|---|---|---|---|
| T1 | 07.5±09.57b[(1)] | 87.5±18.93$^{ns}$ | 12.5±19.00$^{ns}$ | 1.095±0.23$^{ns}$ |
| T2 | 15.0±12.91b | 72.5±15.00 | 27.5±15.00 | 0.932±0.29 |
| T3 | 30.0±08.16a | 70.0±21.60 | 30.0±22.00 | 0.815±0.28 |
| T4 | 20.0±08.16a | 87.5±05.00 | 12.5±05.00 | 0.870±0.16 |
| T5 | 25.0±10.00a | 70.0±14.14 | 30.0±14.00 | 0.842±0.18 |
| T6 | 12.5±05.00b | 75.0±10.00 | 25.0±10.00 | 0.985±0.20 |
| T7 | 12.5±12.58b | 82.5±12.58 | 17.5±13.00 | 1.220±0.13 |
| TREAT | MGT (days) | NS (%) | AS (%) | SL (cm) |
| T1 | 10.3±0.93$^{ns}$ | 75.0±12.91$^{ns}$ | 12.5±12.58$^{ns}$ | 6.63±0.95b |
| T2 | 10.8±1.37 | 65.0±12.91 | 07.5±09.57 | 7.27±0.98a |
| T3 | 09.2±1.03 | 47.5±22.17 | 22.5±15.00 | 8.33±0.95a |
| T4 | 11.1±1.47 | 72.5±17.08 | 15.0±17.32 | 5.85±1.28b |
| T5 | 10.2±0.81 | 67.5±09.77 | 02.5±05.00 | 6.54±0.63b |
| T6 | 10.3±0.72 | 65.0±05.77 | 10.0±14.14 | 6.86±1.03b |
| T7 | 08.9±0.97 | 70.0±08.16 | 12.5±05.00 | 7.95±0.80a |
| TREAT | RL (cm) | SDMS (mg) | RDMS (mg) | TDMS (mg) |
| T1 | 2.93±0.57$^{ns}$ | 8.04±2.01a | 31.45±7.57a | 39.49±6.19a |
| T2 | 3.57±0.56 | 8.61±2.47a | 27.40±4.19a | 36.02±4.87a |
| T3 | 4.12±0.55 | 8.78±3.38a | 30.94±3.20a | 39.72±5.57a |
| T4 | 3.15±0.44 | 5.48±1.77b | 20.67±5.53b | 26.16±7.00b |
| T5 | 3.18±0.53 | 4.87±0.62b | 30.54±3.25a | 35.42±3.00a |
| T6 | 2.99±0.36 | 6.16±1.32b | 31.60±1.26a | 37.76±1.47a |
| T7 | 4.54±3.00 | 8.44±1.19a | 29.65±3.56a | 38.09±4.70a |

[(1)]Means followed by the same letter do not differ from each other by the Scott-Knott cluster test ($p<0.05$). nsNot significant. Note: The seeds in all treatments were initially immersed in 70% alcohol for 1 min. Abbreviations: TREAT. Treatments [T1. NaOCl (2.5%) 14 min; T2. Captan® (0.5%) 5 min + Kasumin® (1 mL·L$^{-1}$) 10 min without residue; T3. T2 with residue; T4. $H_2O_2$ (10%) 5 min; T5. $H_2O_2$ (20%) 5 min. T6. NaOCl (2.5%) 14 min + H2O2 (10%) 5 min; T7. NaOCl (2.5%) 14 min + $H_2O_2$ (20%) 5 min]. Abbreviations: CONT. Contamination; GER. Germination; NGS. Non-germinated seeds; GSI. Germination speed index; MGT. Mean germination time; NS. Normal seedlings; AS. Abnormal seedlings; SL. Shoot length; RL. Root length; SDMS. Shoot dry mass of seedlings; RDMS. Root dry mass of seedlings; TDMS. Total dry mass of seedlings.

**TABLE 2** Seed germination, seedling growth, cell division, mitotic index, and chromosomal changes of meristematic cells of *L. sativa* after immersion in different NaOCl concentrations

| TREAT | [ ] (%) | GER (%) | GSI | RL (cm) | SL (cm) |
|---|---|---|---|---|---|
| | 0.1 | 84.0±12.65 | 0.96±0.18 | 3.99±0.19b | .46±0.26b |
| | 0.5 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| NaOCl (%) | 1.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| | 1.5 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| | 2.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| Water | - | 98.4±2.19a[(1)] | 1.42±0.23a | 8.69±0.34a | 4.63±1.05a |
| Glyphosate | 0.01 | 97.6±2.19b | 1.38±0.09b | 4.25±0.39b | 1.37±0.23b |
| TREAT | [ ] (%) | IC | PC | MC | AC |
| | 0.1 | 954.0±12.59 | 21.2±3.90 | 14.2±6.38a | 9.4±3.91 |
| | 0.5 | 0.0±0.0 | 0.0±0.0b | 0.0±0.0b | 0.0±0.0b |
| NaOCl (%) | 1.0 | 0.0±0.0 | 0.0±0.0b | 0.0±0.0b | 0.0±0.0b |
| | 1.5 | 0.0±0.0 | 0.0±0.0b | 0.0±0.0b | 0.0±0.0b |
| | 2.0 | 0.0±0.0 | 0.0±0.0b | 0.0±0.0b | 0.0±0.0b |
| Water | - | 918.0±8.97a | 32.0±11.42a | 22.0±9.67a | 23.2±6.61a |
| Glyphosate | 0.01 | 996.0±1.22b | 2.4±1.14b | 0.6±0.90b | 1.0±0.71b |
| TREAT | [ ] (%) | TC | DC | MI (%) | CC (%) |
| | 0.1 | 1.2±0.84 | 46.0±12.59 | 4.6±1.26 | 7.2±4.09 |
| | 0.5 | 0.0±0.0b | 0.0±0.0b | 0.0±0.0b | 0.0±0.0ab |
| NaOCl (%) | 1.0 | 0.0±0.0b | 0.0±0.0b | 0.0±0.0b | 0.0±0.0ab |
| | 1.5 | 0.0±0.0b | 0.0±0.0b | 0.0±0.0b | 0.0±0.0ab |
| | 2.0 | 0.0±0.0b | 0.0±0.0b | 0.0±0.0b | 0.0±0.0ab |
| Water | - | 3.8±1.79a | 81.0±8.83a | 8.11±0.89a | 0.6±1.34a |
| Glyphosate | 0.01 | 0.0±0.0b | 4.0±1.22b | 0.40±0.12b | 0.4±0.55b |

[(1)]Means followed by the same letters indicate significant similarity by Dunnett's test ($p<0.05$). Abbreviations: TREAT. Treatments; [ ]. Concentration; NaOCl. sodium hypochlorite; GER. germination; GSI. germination speed index; RL. root length; SL. Shoot length; IC. Interphase cells; PC. Prophase cells; MC. Metaphase cells; AC. Anaphase cells; TC. Telophase cells; DC. Dividing cells; MI. Mitotic index; CC. Chromosomal changes.

Regarding root and shoot length, the NaOCl concentration of 0.1% was similar to that of the glyphosate control, reducing root length by about half and tripling the shoot length relative to water (Table 2).

Glyphosate is a systemic herbicide, used as a positive control in phytotoxicity and cytotoxicity assays as it acts directly on the roots in a negative and prolonged manner, also presenting genotoxicity (Andrade et al., 2010; Rodrigues et al., 2013; Loren et al., 2019). When in contact with plant tissue, glyphosate enters the extracellular matrix and, upon reaching the cytoplasm, it is transported via phloem and distributed to the cellular tissues, causing lethal effects to the plant and also inhibiting the shikimate pathway in plants, consequently interfering with the biosynthesis of lignins and aromatic amino acids, such as tryptophan (Bochkov et al., 2012). Toxicity tests of glyphosate formulations in *Lenna minor* evidence their inhibitory power on the synthesis of chlorophyll *a* and *b*, carotenoids, photosystem II activity, besides increasing the shikimic acid content by 15 to 21% with the application of glyphosate in low concentrations (Sikorski et al., 2019).

NaOCl delays the process of cell division, not being as harmful as glyphosate, whose mechanism of action in plants is related to the enzyme 5-enolpyruvalchiquimate-3-phosphate synthase (EPSPs), interfering with the synthesis of chorismate, a precursor of the tryptophan, tyrosine, and phenylalanine amino acids. In humans, it presents a growing tendency to correlate with various degenerative diseases, especially with increased autism in children (Nevison, 2014). Among these amino acids, the tryptophan produced in chloroplasts plays an important role, acting as an immediate precursor for the synthesis of IAA in plants. Auxin is the main regulator of organogenesis, acting on plant development and growth, regulating cell division, stretching, and differentiation, leading to the formation of shoot and root. Some tryptophan derivatives also act as precursors of IAA; however, they are considered dependent on tryptophan, such as indole pyruvic acid (AIP), indole acetaldoxime (IAOx), and indole acetamide (IAM) (Casanova-Sáez and Voß, 2019).

However, with NaOCl, there is a decrease in cell division and in the mitotic index, generating clastogenic (anaphasic bridge and chromosomal break) and aneugenic (loss and delay in chromosome formation) effects on cells (Figure 2), which explains the reduction in the germination, germination speed index, root and shoot length variables (Andrade et al., 2010).

This fact explains the high number of abnormal seedlings with the species *D. nigra* in treatments with longer exposure to NaOCl since this product is difficult to remove and, despite the treatments being exposed to the same concentration, the prolonged contact of seeds to the product triggered high cellular changes, which reinforces the need for a more effective disinfestation protocol, although with less harmful effects to the explant, as observed in this study when exposing seeds of *D. nigra* to NaOCl for 14 min.

## Experiment IV: Culture media and glutamine in the germination and initial growth of *D. nigra*.

Contamination, germination, GSI, MGT, normal and abnormal seedlings, non-developed seedlings, non-germinated seedlings, shoot length, number of lateral roots, shoot dry mass of seedlings, root dry mass of seedlings, and total dry mass of seedlings were not affected by the type of culture medium (Table 3). Rios et al. (2015) observed a lower MGT (5 days), and according to these authors, the higher glucose content and the presence of maltose in mature dry seeds and the imbibition phases I and II may be associated with the longer time required for the preparation of seed germination in *D. nigra*.

The WPM medium provided a larger number of leaves and collar diameter of seedlings than the MS medium (Table 3). This last characteristic is indicative of rusticity, which is of great importance for the acclimatization of *ex vitro* seedlings. The MS medium provided a longer seedling root length in relation to the WPM medium.

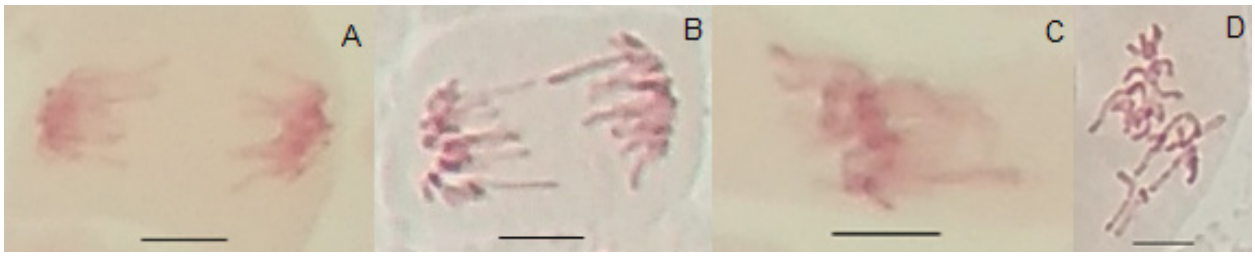


**FIGURE 2** Meristematic cells of *L. sativa* roots exposed to 0.1% NaOCl in (A) normal anaphase, with (B) anaphasic bridge, in (C) normal metaphase, and with (D) chromosome breakage in metaphase with isolated chromosomes. Scale: 10 $\mu$m.

**TABLE 3** Culture media on *in vitro* growth of *D. nigra*

| Culture media | CONT (%) | GER (%) | GSI | MGT (days) |
|---|---|---|---|---|
| MS | 8.7±7.2$^{ns}$ | 80.6±11.2$^{ns}$ | 1.0±0.2$^{ns}$ | 9.6±1.5$^{ns}$ |
| WPM | 5.0±7.3 | 86.2±10.9 | 0.9±0.2 | 10.3±1.3 |
| Culture media | NS (%) | AS (%) | NDS (%) | NGS (%) |
| MS | 2.5±13.4$^{ns}$ | 3.1±4.8$^{ns}$ | 14.3±1.3$^{ns}$ | 20.0±10.3$^{ns}$ |
| WPM | 68.7±13.1 | 7.5±10.0 | 13.0±0.9 | 14.3±12.1 |
| Culture media | NL | CD (mm) | SL (cm) | RL (cm) |
| MS | 3.9±0.6$^{*}$ | 1.5±0.1$^{*}$ | 10.9±1.0$^{ns}$ | 5.3±0.9$^{*}$ |
| WPM | 4.5±0.9 | 1.6±0.1 | 10.2±1.4 | 4.4±0.7 |
| Culture media | NLR | DSMS (mg) | RDMS (mg) | TDMS (mg) |
| MS | 22.1±6.7$^{ns}$ | 37.1±3.4$^{ns}$ | 20.4±5.4$^{ns}$ | 57.6±6.9$^{ns}$ |
| WPM | 20.6±5.2 | 34.9±3.5 | 21.2±5.0 | 56.2±7.1 |

*Significant ($p<0.05$) and nsNot significant by the F test. Abbreviations: MS. Murashige and Skoog; WPM. Lloyd and McCown; CONT. Contamination; GER. Germination; GSI. Germination speed index; MGT. Mean germination time; NS. Normal seedlings; AS. Abnormal seedlings; NDS. Non-developed seedling; NGS. Non-germinated seeds; NL. Number of leaves; CD. Collar diameter; SL. Shoot length; RL. Root length; NLR. Number of lateral roots; DSMS. Dry shoot mass of seedlings; RDMS. Root dry mass of seedlings; TDMS. Total dry mass of seedlings.

The WPM medium is formulated for woody species; however, these species can adapt to *in vitro* cultivation using the MS medium. The woody plant medium (WPM) was suitable for all *in vitro* stages in *Campomanesia xanthocarpa* O. Berg (Myrtaceae), during the multiplication and rooting process, compared to the MS medium (Machado et al., 2020), which, according to these authors, is a culture medium with reduced salts and more suitable for rooting. The superiority of the WPM medium in the meristem culture of *Ilex paraguariensis* St.-Hill. can be attributed to the lower salt concentrations (especially nitrogen and potassium) (Tomasi et al., 2018). For the *in vitro* establishment of sprouting apexes of *Dipteryx alata* Vog., the MS medium was considered to be more adequate compared to the WPM, providing seedlings with longer root length and higher leaf number, and an increase in dry mass was observed with the salt concentrations used (25; 50; 75, and 100%) for both *in vitro* culture media (Araruna et al., 2017).

The number of lateral roots produced in the MS medium decreases with increasing glutamine concentrations, and the opposite happens with the WPM medium (Figure 3). The gain in lateral roots in the WPM medium at the highest glutamine concentration (0.75 mg $L^{-1}$) was eight roots. Root length, root dry mass of seedlings, and total dry mass of seedlings showed a linear growing behavior with increasing glutamine concentrations, for both culture media (Figure 3). Transgenic plants of *Betula pubescens* Ehrh., which are carriers of the *GS1* gene that encodes the cytosolic form of glutamine synthetase, have a high content of glutamine, as well as glutamic and aspartic acids, and rooted more rapidly compared to the control plants (Lebedv et al., 2018). These authors observed a positive correlation between the increase in the levels of auxin in these transgenic plants and an increase in the rooting rate.

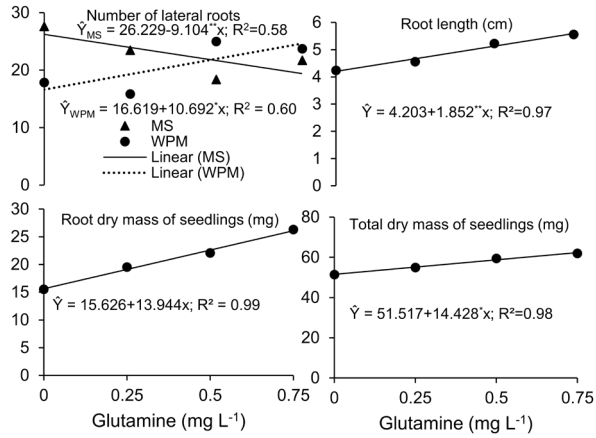


**FIGURE 3** Characteristics evaluated of seedlings of *D. nigra* grown in different culture media (MS and WPM) and glutamine concentrations. **Significant ($p<0.05$).

The WPM medium has only 45% of the total ionic strength of the MS medium, and lower concentrations of nitrate ($NH_3^-$) (MS 40 $\mu$M; WPM 9.7 $\mu$M) and ammonium ($NH_4^+$) (MS 20 $\mu$M; WPM 4.9 $\mu$M) (Phillips and Garda, 2019). Consequently, the WPM has a low total inorganic nitrogen concentration (14.7 $\mu$M) compared to the MS (60.0 $\mu$M) (Rocha et al., 2007). The growth of some plants may be impaired in media containing high concentrations of $NH_4^+$, reflecting toxicity induced by the accumulation of excess ammonium ions. Under normal circumstances, the toxic effect of ammonium is prevented by converting the ion into amino acids, in which L-glutamic acid is produced from glutamine by the action of the enzymes glutamine synthetase (GS) and glutamate synthetase (GOGAT) (George et al., 2008).

The use of amino acids in *in vitro* crops has a positive influence in stimulating SE maturation, reducing embryonic abnormalities, and increasing plant conversion (L-glutamine) and at rooting as it provides organic nitrogen for the explant, a readily available form that acts on seedling growth and root system development (George et al., 2008; Rathore et al., 2012; Daniel et al., 2018; Garcia et al., 2019). Asparagine, cysteine, citrulline, and L-glutamine were tested for the *in vitro* rooting of CAB-6P (*Prunus cerasus* L.) and Gisela 6 (*P. canescens* × *P. cerasus*) cherry rootstocks, using four concentrations (0; 0.5; 1 and 2 mg $L^{-1}$) combined with 2 mg $L^{-1}$ of indole-3-butyric acid. CAB-6P presented a higher number of roots compared to Gisela 6, highlighting the cysteine and L-glutamine amino acids at a concentration of 0.5 mg $L^{-1}$ (14.18 and 11.08 respectively) (Sarropoulou et al., 2016).

## CONCLUSIONS

I and II. NaOCl (2.5%) for 14 min is recommended for the disinfestation of seeds of *Dalbergia nigra*. III. NaOCl has a high phytotoxic, cytotoxic, and genotoxic effect, and its mechanism of action in the *Lactuca sativa* cell cycle is clastogenic and aneugenic, suggesting that it may influence the formation of abnormal seedlings of *Dalbergia nigra*. IV. The WPM medium and its supplementation with glutamine (0.75 mg·$L^{-1}$) is recommended.

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