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Optimization of jenipapo *in vitro* seed germination process

Otimização do processo de germinação *in vitro* de sementes de jenipapo

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ABSTRACT

The *in vitro* seed germination is an effective alternative for quickly obtaining explants with sanitary quality. However, jenipapo seeds present slow and uneven germination. Therefore, internal and external factors to seed which directly interfere in the process, they must be identified, in order to adapt better techniques to obtain seedlings. In this sense, this work aimed to optimize the *in vitro* germination of *Genipa americana* L. seeds by evaluating different factors (light quality, GA_3 treatment, pre-soaking in distilled water, growing media and stratification in the dark). It was found that the seed germination of *G. americana* was indifferent to light, however, the best results were obtained under conditions of continuous darkness; There was no effect of the application of exogenous GA_3; The pre-soaking in distilled water for 48 h contributes to obtaining better germination rates; And the reduction in MS medium salts, and laminating the pretreatment in the dark maximizes the germination potential of seeds.Therefore, the optimal conditions for *in vitro* germination of *G. americana* L. seeds requires pre-soaking in distilled water for 48 hours and inoculation into culture media consisting of ½ MS + 15 g L^{-1} sucrose, with stratification in the dark for 16 days, followed by the transfer to growth chambers with lighting provided by white fluorescent lamps.

Index terms: *Genipa americana* L.; *in vitro* culture; ligth quality; Rubiaceae; stratification in the dark.

RESUMO

A germinação *in vitro* de sementes é uma alternativa eficaz para obtenção de explantes com rapidez e qualidade sanitária. No entanto, sementes de jenipapeiro apresentam germinação lenta e desuniforme. Para tanto, fatores internos e externos às sementes que interferem diretamente no processo, devem ser identificados, visando à adaptação de melhores técnicas para obtenção de mudas. Neste sentido, objetivou-se otimizar a germinação *in vitro* de sementes de *Genipa americana* L. por meio da avaliação de diferentes fatores (qualidade de luz, tratamento com GA_3, pré-embebição em água destilada, diferentes composições no meio de cultivo e estratificação no escuro). Verificou-se que a germinação das sementes foi indiferente a luz, no entanto, os melhores resultadados foram obtidos sob condições de escuro contínuo; Não houve efeito da aplicação de GA_3 exógeno; A pré-embebição em água destilada durante 48h contribui para obtenção de melhores taxas de germinação; E a redução de sais do meio MS, bem como o pré-tratamento de estratificação no escuro maximizam o potencial germinativo das sementes. Portanto, as condições ótimas para germinação *in vitro* de sementes de *G. americana* L. requer a pré-embebição em água destilada por 48 horas e a inoculação em meio de cultivo constituído de ½ MS + 15 g L-1 de sacarose, com estratificação no escuro por 16 dias, seguida pela transferência a salas de crescimento com iluminação fornecida por lâmpadas fluorescentes branca.

Termos para indexação: *Genipa americana* L.; cultivo *in vitro*; qualidade de luz; Rubiaceae; estratificação no escuro.

INTRODUCTION

Jenipapo (*Genipa americana* L.) belongs to the Rubiaceae family and is native from Brazil. It is adapted to tropical forest conditions, widely distributed in South America, Mexico and the Caribbean. It has a great ecological importance, due to its characteristics of selective hygrophytes and phytoremediators, being recommended for the restoration of riparian forests and recovery of degraded areas. Moreover, jenipapo has an ornamental potential, and may be used in urban afforestation, since it has a dense pyramidal crown shape with short branches (Almeida et al., 2015; Santana et al., 2012). Its fruits are rich in a compound known as genipin with ability to develop blue color of low toxicity. These characteristics have attracted interest in its use as, a natural dye for food and beverages; developer in fingerprint forensic science and dyeing wool, cotton and leather (Lee et al., 2003; Levinton-Shamuilov et al., 2005; Ramos-de-la-Peña et al., 2014).

The socio-economic potential of *G. americana* is notorious. However, there are several limitations

that prevent its commercial exploitation as: lack of varieties and selected seedlings; seeds sensitivity to dehydration, with loss of viability in the short term; slow, low and uneven germination (Magistrali et al., 2013; Santos; Silva-Mann; Ferreira, 2011). Thus, *in vitro* seed germination is the most effective way for quickly obtaining explants with sanitary quality. However, for most species, speed, uniformity and germination rate depend on factors which are external and internal to the seeds. Internal factors are commonly associated with the presence of inhibitors or germination promoters, whereas external factors are related to ecophysiological characteristics of each species (Demotes-Mainard et al., 2016; Plue et al., 2010).

The *in vitro* culture is an excellent alternative to overcome the difficulties imposed by the traditional jenipapo propagation system, enabling large-scale multiplication and the conservation of this species. In addition, through *in vitro* cultivation techniques, it is possible to optimize the production of secondary metabolites with ethnopharmacological importance in several species (Bhuvaneshwari et al., 2016; Venugopalan; Srivastava, 2015). In this context, considering that there is little information regarding the *in vitro* culture of *G. americana*, this study aimed to optimize the *in vitro* germination of *G. americana* seeds by identifying internal and external factors affecting the germination process.

MATERIAL AND METHODS

Plant material

Ripe fruits of *G. americana*, were collected at latitude 21°13'40'' South and longitude 44°57'50'' West, at an altitude of 919 m. The fruits were pulped and the seeds were washed in running water on screen for complete mucilage removal. Subsequently, the seeds were placed on paper towel to dry at room temperature for 24 hours and then subjected to treatments.

Quality of light

For the assessment of the effects of light quality on *in vitro* germination, jenipapo seeds were sterilized in a 2.5% NaOCl solution (v/v) for 20 minutes in a laminar flow hood, and then inoculated into MS medium (Murashige; Skoog, 1962), with 3% sucrose and 0.7% agar. The pH was adjusted to 5.8 before autoclaving at 120 °C for 20 minutes. After inoculation, the material was maintained in a growth chamber with a temperature of 25 ± 2 °C, average relative humidity of 70% and 16-hour photoperiod. The different tested light conditions were: white fluorescent lamps (with a density of about 36 µmol photons m^{-2} s^{-1} and photosynthetically active radiation (PAR) ranging from 400 to 700 nm), Light Emitting Diode [(LED: 70% red + 30% blue) with a density of approximately 60 µmol photons m^{-2} s^{-1} and PAR from 460 to 660 nm)] and absence of light. To determine the quality of light (Figure 1), a spectroradiometer (Red TIDE USB 650 UV, LICOR, USA) was used.

The germinated seeds were counted daily and radicle protrusion (≥ 2.0 mm) was used as a criterion for germination. Assessments were concluded at 120 days and the final germination percentage, as well as germination speed index (GSI), was then obtained, according to Maguire (1962). The experimental design used was a randomized block with three treatments (LED, White light and dark). Seven replicates and tem tubes per plot were used (1 seed/tube), for a total of 210 seeds.

GA_3 treatment

Jenipapo seeds were sterilized and then inoculated into MS medium with 3% sucrose, 0.7% agar and six GA_3 concentrations (0; 0.2; 0.4; 0.8; 1.8 and 3.6 mg L^{-1}). The material was kept in a growth chamber under controlled conditions. Assessments were performed daily for 120 days and, at the end of this period, the final percentage of germination and GSI were calculated. The experimental design was completely randomized with six treatments (0; 0.2; 0.4; 0.8; 1.8 and 3.6 mg L^{-1} GA_3). Four replicates and tem tubes per plot were used (1 seed/tube), for a total of 240 seeds.

Pre-soaking in distilled water

The seeds were subjected to pre-soaking in distilled water under constant agitation (Shaker table; Orbital – MA 140/CFT) at different time intervals: 0 (without pre-soaking), 18 hours, 24 hours and 42 hours. After pre-soaking, the seeds were sterilized and inoculated into MS medium, added with 3% sucrose and 0.7% agar. The cultures were kept in a growth chamber under controlled conditions. The experimental design was completely randomized with four treatments (0, 18, 24 and 42 hours pre-soaking). Five replicates and ten tubes per plot were used (1 seed/tube), for a total of 200 seeds.

Different compositions in the culture medium

The seeds were pre-soaked in distilled water under constant agitation (Shaker table; Orbital – MA 140/CFT) for 48 hours; they were then sterilized and inoculated into

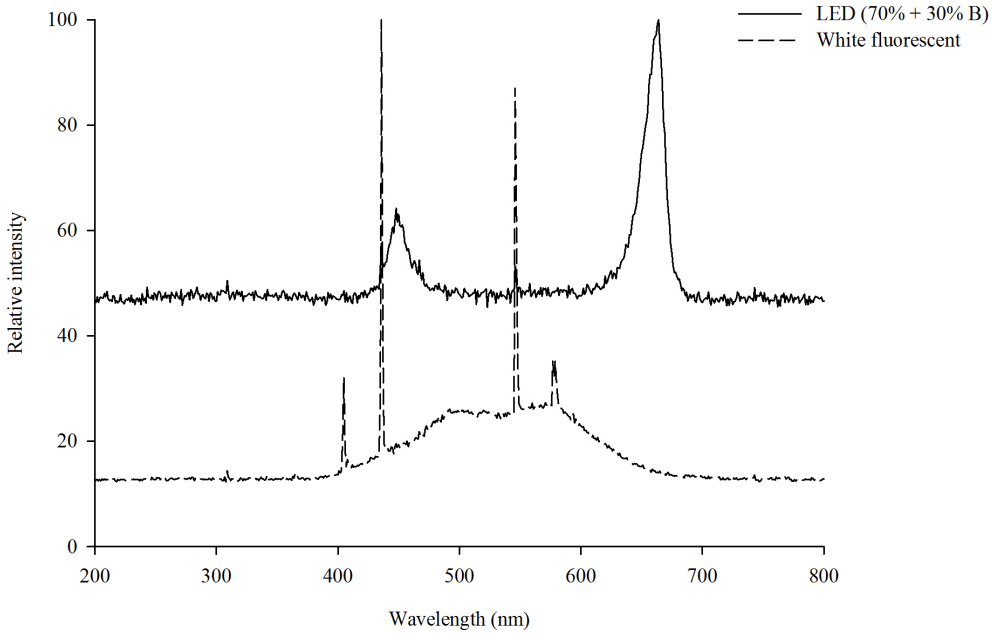

Figure 1: Spectral characteristics of lamps used as a light source for the germination of *Genipa americana* seeds stored in growth chambers.

test tubes containing 15 mL culture medium. The tested media consisted of: absence of MS salts and sucrose; ½ MS salts + 15 g L^{-1} sucrose; MS salts (total force) without sucrose. All media were solidified with 0.7% agar. After inoculation, the cultures were maintained in a growth chamber under controlled conditions. Assessments were performed daily for 45 days. The experimental design was completely randomized with three tratament and seven replicates of ten tubes per plot, for a total of 210 seeds.

Stratification pretreatment in the dark

The seeds were subjected to pre-soaking in distilled water under constant agitation for 48 hours and, subsequently, they were sterilized and inoculated into test tubes containing medium consisting of ½ MS salts, 15 g L^{-1} sucrose and 0.7% agar. After inoculation, the cultures were placed in the dark for various periods (0, 1, 2, 4, 8 and 16 days), and then transferred to a growth chamber under controlled with a temperature of 25 ± 2 °C, average relative humidity of 70% and 16-hour photoperiod. Assessments were performed daily for 30 days and, at the end of this period, the final percentage of germination and GSI were calculated. The experimental design was completely randomized with six treatments. Four replicates and ten tubes per plot were used, for a total of 240 seeds.

Statistical analysis

Data from different experiments were subjected to analysis of variance for diagnosing significant effects by the F test. The means of qualitative treatments were compared by the Skott-Knott test ($P>0.05$), whereas the means obtained in quantitative treatments were adjusted to regression and the equations were selected based on the highest coefficient of determination (R2). The analyses were performed using the software Sistema para Análise de Variância - SISVAR (Ferreira, 2014).

RESULTS AND DISCUSSION

Quality of light

The quality of light influenced the *in vitro* germination of jenipapo seeds ($p<0.05$). The highest germination rates obtained were 40% and 34%, respectively, for continuous dark conditions and white light (Figure 2). This result indicates that the germination of *G. americana* seeds is indifferent to light.

However, it was found that seeds kept under light provided by LED lamps had their germination potential reduced, indicating that the effect of light on seed germination is variable in many species, and these responses depend strolngly on the quality of light (Demotes-Mainard et al., 2016; Plue et al., 2010).

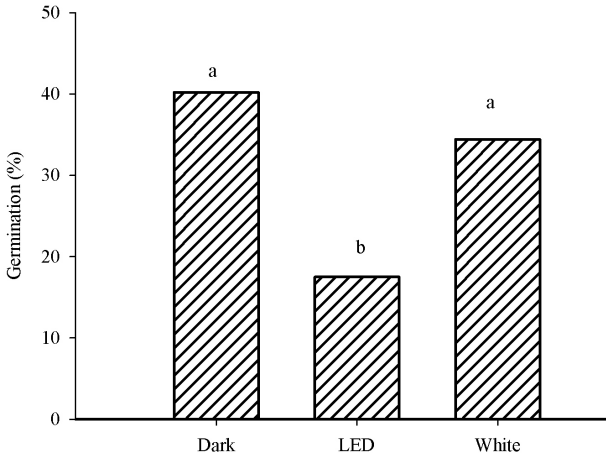

Figure 2: Percentage germination of *Genipa americana* L. seeds under various light qualities at 120 days of *in vitro* culture. Means followed by the same letter do not differ by the Scott-Knott test at $P<0.05$.

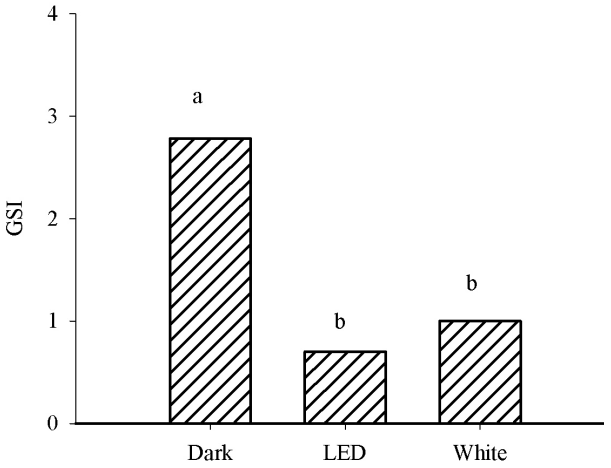

Figure 3: Germination speed index of *Genipa americana* L. seeds under various light qualities at 120 days of *in vitro* culture. Means followed by the same letter do not differ by the Scott-Knott test at $P<0.05$.

In this context, the negative effect of LED on the germination of *G. americana* seeds shows a possible adaptation of the species to understory environments, since species adapted to these conditions have higher germination rates in environments with low energy levels and plenty of far-red light (Liu et al., 2012; Plue et al., 2010). Therefore, the high level of energy provided by LED (peak at 460 nm, referring to blue light with a high energy level; Figure 1), associated with a higher intensity of red light, reduced germination performance of *G. americana* seeds. On the other hand, the light provided by white fluorescent lamps with lower red light intensities resulted in higher germination rates. The results obtained in this study also suggest that the light provided by LED lamps (70% R + 30% B) should be avoided during the germination of *G. americana* seeds.

Regarding GSI, the continuous dark condition favoring the increased in speed and of *G. americana* seeds germination uniformity (Figure 3). However, plantlets obtained under this condition showed blanching, due to light deficiency.

Treatments GA_3

The use of GA_3 at the different tested concentrations (0; 0.2; 0.4; 0.8; 1.8 and 3.6 mg L^{-1}) did not affect percentage and *in vitro* germination speed index. This result may be related to the endogenous availability of GA_3 at an ideal concentration, or close to that, in which both the potential and the germination speed of jenipapo seeds are stimulated. Therefore, the germination of *G. americana* seeds does not require the use of external GA_3.

According to Debeaujon e Koornef (2000), the GA_3 showed two mechanisms of action in the contol of seed germination. Initially, there is induction of expression of genes encoding enzymes related to hydrolysis of compounds in the endosperm, wich confer resistence of the radicle protusion. Thus, the use of techniques to reduce the concentration of inhibitors present within the seeds and/ or weakening the seed coat may be sufficient to promote germination in the absence of exogenous GA_3.

Pre-soaking in distilled water

Pre-soaking in distilled water affected the germination potential of jenipapo seeds. It was also found that the percentage of germination and GSI increased as a function of the length of stay, with a maximum estimated period of 48 hours (Figures 4 and 5).

This response may be related to a possible action in weakening the seed coat, facilitating breaking and radicle protrusion. Studies conducted by Queiroz et al. (2012), demonstrated that one of mechanisms contributing to slow and uneven germination of *G. Americana*, is related to strength imposed in the micropylar endosperm preventing the expansion and elongation radicle. Therefore, the main enzyme involved in the weakening of the micropylar endosperm (endo-β-mannanase) it has its activity increased when the seeds are soaked in water. This effect coincides with the reduction of the force necessary to pirece the micropylar endosperm of jenipapo.

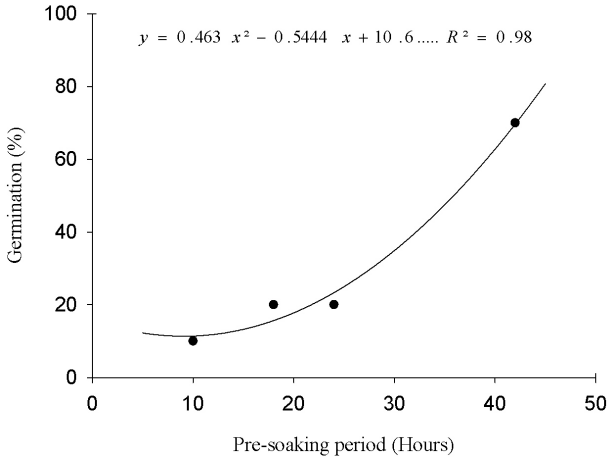

Figure 4: Percentage of germination of *Genipa americana* L. seeds as a function of various pre-soaking periods in distilled water at 120 days of *in vitro* culture.

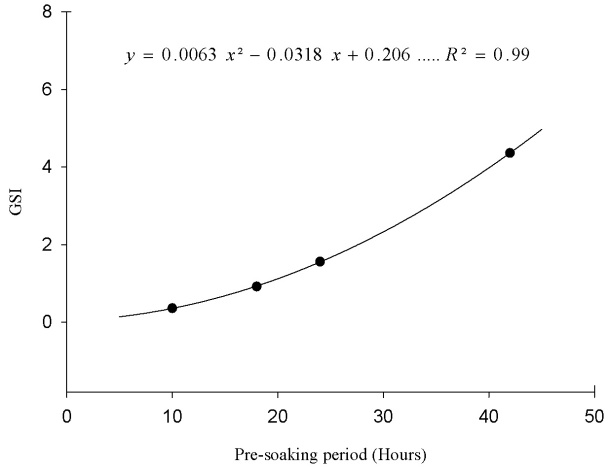

Figure 5: Germination speed index of *Genipa americana* L. seeds as a function of various pre-soaking periods in distilled water at 120 days of *in vitro* culture.

Water can also stimulate the respiratory activity, mobilization of reserves for growth resumption of the embryonic axis and reduction in the concentration of inhibitors present in the seed (Ribeiro et al., 2015; Weitbrecht; Muller; Leubner-Metzger, 2011). Therefore, increasing the germination percentage and GSI this study indicate that treatment of pre-soaking in distilled water, allows the weakening micropliar endosperm, giving less resistance to expansion and elongation of the radicle. And enable greater availability of metabolites, ready for use in resuming the growing embryo, providing a uniform and faster germination.

Different compositions in the culture medium

The different compositions tested in the cultivation medium affected the germination potential of jenipapo seeds, which was more efficient with the reduction in MS salts (Table 1).

The use of different combinations of MS salts and sucrose had been noted as factors affecting the *in vitro* germination of *G. americana* seeds (Almeida et al., 2013), and the absence of MS salts and sucrose was indicated to provide better germination rates. However, in this study, the combination which provided the best germination performance of *G. americana* seeds (81%) was ½ MS salts + 15 g L^{-1} sucrose, which indicates that the contents of reserves in the seed could affect the germination process, reflecting a greater or lower demand for mineral nutrients (Manzur; Penella; Rodriguez-Burruezo, 2013; Pêgo; Paiva; Paiva, 2013).

Sucrose added to the medium also has a strong effect on seed germination, since its requirement depends on the embryonic stage of development, being used as an energy source and/or to maintain an adequate osmotic equilibrium (Benmahioul et al., 2009; Li et al., 2012; Manzur; Penella; Rodriguez-Burruezo, 2013). Therefore, it is possible to state that the germination of *G. americana* seeds has low requirements with respect to the mineral nutrients added to the medium, and the addition of sucrose is essential for the maintenance of osmotic balance.

Table 1: Percentage and germination speed index (GSI) of *Genipa americana* L. seeds under different culture media at 45 days of *in vitro* culture.

Treatments	Germination (%)	GSI
0 MS + 0 Sucrose	67 b	3.87 a
½ MS + 15 g L^{-1} Sucrose	81 a	4.47 a
MS + 0 Sucrose	41 c	1.49 a
CV (%)	20.9	33.0

Means followed by the same letter in the column do not differ by the Scott-Knott test ($p<0.05$).

Stratification pretreatment in the dark

Stratification in the dark affected the percentage of *in vitro* germination and GSI of jenipapo seeds (Figures 6 and 7).

There is evidence that the germination of seeds of some species requires low radiation after being subjected to a stratification period in the dark (Goggin et al., 2010). During this period, fully hydrated seeds have a higher activity of α-amylase and β-endo-mannanase. These enzymes are essential, since they provide preparatory substrates for a rapid expansion of the embryonic axis, immediately after the perception of light stimuli (Goggin et al., 2011). Different studies on the *G. americana* germination have shown that the germination process is highly desuniform and can vary between 45 and 90 days (Magistrali et al., 2013; Magistrali et al., 2015). In contrast, the present study showed that the stratification pretreatment in the dark for a period of 16 days resulted in 100% germination, with an average time of 30 days, indicating that the use of this pretreatment is required, followed by a transfer to the 16 h photoperiod with light provided by white light, so that *G. americana* seeds express their maximum germination potential.

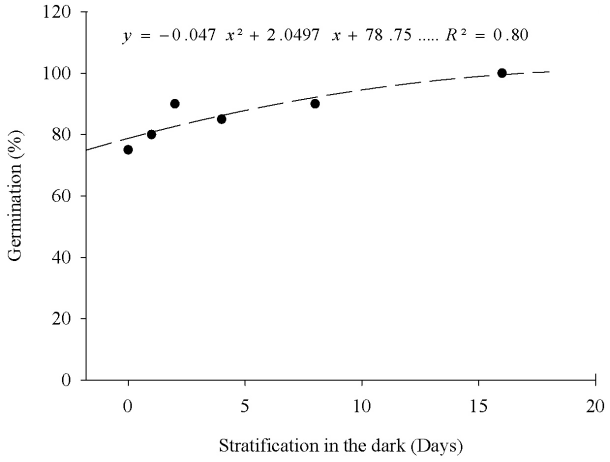

Figure 6: Percentage of germination of *Genipa americana* L. seeds as a function of various periods in the dark at 30 days of *in vitro* culture.

The results obtained in this study allowed the identification of several factors that contribute to accelerate and unever *in vitro* germination of *G. americana* seeds. These results provide obtaining seedlings in less time and more effectively, in order the demands generated in laboratories and in the field.

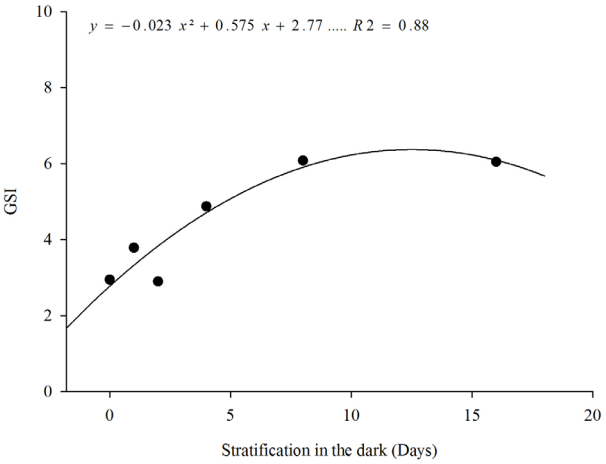

Figure 7: Germination speed index of *Genipa americana* L. seeds as a function of various periods in the dark at 30 days of *in vitro* culture.

CONCLUSION

The optimal conditions for the *in vitro* germination of *Genipa americana* L. seeds require pre-soaking in water for 48 hours and inoculation into a medium consisting of ½ MS + 15 g L^{-1} sucrose, with stratification in the dark for 16 days, followed by the transfer to growth chambers with lighting provided by white fluorescent lamps.

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