

Development of a protocol for the *in vitro* establishment of *Stevia rebaudiana* Bertoni Morita II variety

Desarrollo de un protocolo para el establecimiento *in vitro* de *Stevia rebaudiana* variedad Bertoni Morita II

Desenvolvimento de um protolo para o estabelecimento in vitro da variedade Morita II (Stevia rebaudiana Bertoni)

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Abstract

Due to the cross-pollinated condition of the plant, which causes variation in the sweetener's composition and in the morphological characteristics in terms of shape and color of the leaves, seed propagation is limiting for commercial purposes, so it is convenient to use asexual propagation systems. In this study, a protocol for *In Vitro* establishment of *Stevia rebaudiana* was developed, for which eight disinfection treatments were applied using 5% and 4% concentrations of sodium hypochlorite (NaClO) with different exposure times, 5, 10, 15 and 20 minutes. The best disinfection percentage (76.75%) and sprouting (58.91%) was obtained using a 5% concentration of NaClO with 20 minutes of exposure.

Key-words: concentration, culture media, disinfection, explant.

Resumen

Debido a la condición alógama de la planta, que ocasiona variación en la composición del edulcorante y en las características morfológicas en cuanto a forma y color de las hojas, la propagación por semilla es limitante para fines comerciales, por lo que es conveniente el uso de sistemas asexuales de propagación. En el presente estudio se desarrolló un protocolo para el establecimiento *In Vitro* de *Stevia rebaudiana*, para lo cual se aplicaron ocho tratamientos de desinfección utilizando concentraciones de hipoclorito de sodio (NaClO) al 5% y 4% con diferentes tiempos de exposición 5, 10, 15 y 20 minutos. El mejor porcentaje de desinfección (76.75%) y brotación (58.91 %) se obtuvo utilizando una concentración de NaClO al 5% durante 20 minutos de exposición.

Palabras clave: concentración, medio de cultivo, desinfección, explante

Resumo

Devido à condição de alogamia da planta, a qual causa variação na composição do adoçante e nas características morfologia de forma e color das folhas, a propagação de sementes é limitante para propósitos comerciais, fato pelo qual é conveniente usar sistemas assexuais de propagação. Neste estudo, um protocolo para o estabelecimento *in vitro* de (*Stevia rebaudiana*) foi desenvolvido. Para isso foram aplicados oito tratamentos de desinfecção

utilizando concentrações de 5% e 4% de hipoclorito de sódio (NaClO) em tempos de exposição de 5, 10, 15 e 20 minutos. O melhor percentual de desinfecção (76.75%) e brotação (58.91%) foi obtido na concentração de 5% de NaClO com 20 minutos de exposição.

Palavras-chave: concentração, meio de cultura, desinfecção, explante

Introduction

The demand for natural sweeteners is increasing in the world because of the side effects of synthetic monosaccharide-based sweeteners in people with sugar problems. Japan has already replaced half the consumption of sugar cane by Stevia and other first world countries are following the same trend. The stevia (*Stevia rebaudiana* Bertoni) is a subtropical rustic plant, native to northwestern province of Misiones in Paraguay. The sweetener obtained from this plant, has beneficial effects on the absorption of fats and in the regulation of blood pressure, in turn, it does not increase sugar levels in the blood; on the contrary, its hypoglycemic property has been demonstrated, by improving glucose tolerance (Guerrero, 2005).

As for the production of dried leaf (Morita variety) in Colombia, there is an estimate of 150 ha of crops with a production of nine tons per hectare. The Colombian product is grown mainly in the departments of Antioquia, Valle del Cauca and the eastern plains; much of the production is exported to Europe and Asia (Borda, *et al.*, 2009). In Colombia the 'Morita I' and 'Morita II' genotypes are grown; Morita II Variety, developed in Japan by

Toyosigue Morita, has a higher production of dry leaves and a better chemical content (Mitsubishi, *et al.*, 1975); therefore, it is the most commercially cultivated variety.

Regarding the Stevia crops, Tamura, *et al.*, (1984) state that it is difficult to maintain genetically stable conditions of crops with high stevioside content, due to variation caused by the segregation of genes. In turn, Miyazaki and Wanten (1974), claim that the self-incompatibility in pollination and the high heterozygosity are limiting factors in the sexual spread for large-scale cultivation. Therefore, the use of asexual propagation methods that allow obtaining genetically homogeneous populations with desirable and stable phenotypic characters is being considered; in this case, regarding the content of the sweetener (Tamura, *et al.*, 1984).

Another alternative method of vegetative propagation is *in vitro* propagation, which turns out to be efficient in the production of seedlings on a large scale in less time; As an example, Lyakhovkin, (1993), reports a multiplication rate from three to four cuttings in each sub-culture using apical meristems, obtaining an approximate total of 3200 seedlings per explant within a period of eight months.

In the *in vitro* culture, it is essential to find an efficient procedure to remove spores, fungal tissue, bacteria and other contaminants without damaging the tissue or reducing the regenerative capacity of the explant.

It is therefore important to assess biotechnological techniques to develop plant regeneration protocols for commercial alternatives, preserving the homogeneous characteristics for both the plant morphology and the content of sweetener.

Given the importance of the cultivation of *Stevia*, this research was conducted in order to 1) determine the origin of endogenous, exogenous, bacterial and / or fungal contamination of explants grown in Murashige & Skoog (MS) culture media; and 2) to determine the best disinfection protocol for the induction of *in vitro* shoots allowing a subsequent and complete development of an alternative methodology for efficient clonal reproduction.

Materials and methods

Location and plant material:

Phase 0, selection and preparation of stem plants was carried out in the Tesorito farm at the University of Caldas, Manizales, Colombia; with a height of 2,280 meters above the sea level, an average temperature of 17 ° C and a relative humidity of 78%. Phase I, *In Vitro* establishment of the culture, was developed in the Tissue Culture Laboratory of the Caldas University.

Stevia rebaudiana mother plants, Morita II variety, were provided by the company “Agro-steviant GP”, located in Puerto Tejada, Cauca, Colombia. They were kept in a seedbed under controlled irrigation conditions and agronomic management recommended by the supplier.

Experimental design and treatments

The experimental design was fully randomized blocks, by blocking the experiment with each planting conducted, for a total of four blocks comprised of eight treatments (Table 1) and 14 repetitions each. The selection of the planting for each explant was random, placing 5 of them in each container, and thus setting them as experimental units. For the *in*

Vitro establishment of *Stevia rebaudiana*, four disinfection assays were performed as a way to reduce and control experimental error variance for greater accuracy; in each assay eight treatments were applied for disinfecting explants, each treatment had 14 repetitions for a total of 448 crops.

Sprouting of the seeded explants and the percentage of contamination generated by fungi and bacteria were evaluated, the latter was evaluated every week for a month. After discarding the plants by contamination or oxidation of the explant, a percentage of feasibility that was assessed every eight days for a month was obtained.

During Phase 0, plants were treated weekly with Fungibiol (1.5 cc / L of water) in order to reduce *in vitro* fungal contamination, cuttings nodal segments were selected and entered into the laboratory to continue with Phase 1.

Explants were treated with a mixture of powder detergent and tween 80 for 30 minutes, rinsed with tap water and then immersed in a solution of sodium hypochlorite (v/v) at concentrations of 4% and 5% in combination with different exposure times: 5, 10, 15 and 20 minutes, then rinsed 3 times were with sterile water and immersed in a solution of 70% ethanol (Table 1). Within the laminar flow chamber, they were divided into nodal segments between 1.5 and 2 cm in length with 2 or 3 mm of thickness, containing at least one axillary bud. Subsequently, they were seeded vertically in Murashige and Skoogs (MS) medium supplemented with 3% sucrose, 1.5 mg / L of BA and 0.1 mg / L of NAA. The pH of the medium was adjusted to 5.8 and sterilized at 121 ° C and 15 psi for 20 minutes. 2.5 mg / ml of cephalexin (broad spectrum antibiotic) were added to the culture medium in order to reduce contamination by bacteria. Incubation was carried out at a temperature of 27 ± 2 ° C with a photoperiod of 16 hours light, using white light lamps. Incubation was carried out at a temperature of 27 ± 2 ° C with a photoperiod of 16 hours of light, using white light lamps.

Table 1. Treatments proposal (T) for surface disinfection of *Stevia rebaudiana* explants.

NaClO Concentration %	Exposure time (min)			
	5	10	15	20
4	T1	T2	T3	T4
5	T5	T6	T7	T8

Statistical analysis

The data obtained from the eight treatments evaluated were subject to ANOVA through the GENES statistical program. Significance was set at the level of 5% ($P \leq 0.05$). Afterwards, comparison tests were conducted for the averages of the treatments (Tukey).

Results and discussion

Concentrations of sodium hypochlorite showed no significant differences on contamination levels by fungi and bacteria and the survival of the nodal segments (Table 2).

Table 2. Comparative test of means of contamination and survival of nodal segments disinfected on eight different treatments

Treatment	Percentage estimates (%)				
	Fungus	Bacterium	With Contamination	without Contamination	Sprouting
1	32,13 a	14,28 b	46,44 a	53,55 e	58,91 a
2	14,28 f	21,42 a	35,73 c	64,27 c	58,91 a
3	17,87 de	14,28 b	32,17 d	67,83 b	58,91 a
4	16,06 ef	16,06 b	32,17 d	67,83 b	49,98 c
5	19,64 cd	16,07 b	35,73 c	64,26 c	42,84 d
6	21,42 bc	19,63 a	41,08 b	58,9 d	53,55 b
7	23,2 b	16,06 b	39,31 b	60,69 d	47,99 c
8	14,28 f	8,92 c	23,24 e	76,75 a	58,91 a

* Averages with different letters show statistical differences according to the comparative Tukey test at 5%

Establishment is a fundamental stage for the beginning of the *In Vitro* culture of any material. Teixeira (2004) and Villegas (2006), mentioned that the requirements of each selection, crop or variety are specific, so it is necessary to determine the ideal conditions for each genotype, especially in the disinfection process. Establishment's success depends on several factors, including health conditions of mother plants in the field or greenhouse and their

age: the older plants are, the greater the likelihood of *In Vitro* contamination because the time of exposure to environmental pollutants is increased (Villegas, 2006). Moreover, aseptic surface processes are important to maintain the viability and facilitate the recovery of growth and development of the explant (Rache & Pacheco, 2012). Roca & Mroginski (1997) ensure that avoiding contamination is a basic aspect for establishing *in vitro* cultures, because in the

best-case scenario, microorganisms do not destroy crops but they either compete with the explant for the nutrients of the culture medium or they modify it.

Although the histogram indicates that there are no significant differences among the eight treatments (T1 to T8), it can be demonstrated that the

explants subjected to sodium hypochlorite 5% for 20 minutes of exposure (T8) presented the lowest percentage of contamination (23.24%) and the highest percentage of sprouting (58.91%), these results show better a performance of the sterilization process with increasing concentration of NaClO (Figure 1).

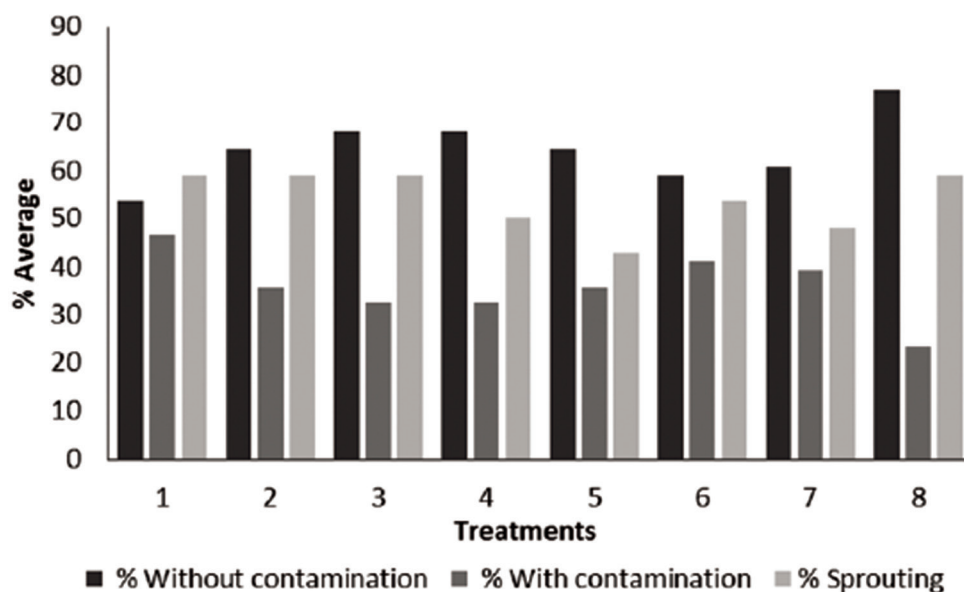


Figure 1. Averages of treatments without contamination, with contamination and sprouting of nodal segments

Likewise, it may be asserted that the *Stevia* explants of the Morita II variety subjected at concentrations of 4 and 5% NaClO between 5 and 20 minutes, are equally efficient for use in the In Vitro establishment; with contamination and survival rates ranging from 23.24% to 46.44%; 58.91% 42.84% and; respectively. This coincides with the results reported by Anbazhagan *et al.*, (2010), in which using a concentration of NaClO to 5% for 5 minutes of exposure, resulted in a (21.11%) of contamination and a (55.56%) of survival.; however, the explants that were subjected to 4% sodium hypochlorite for 5 minutes of exposure (T1), they showed higher contamination percentages, (46.44%). These results match the report by Vazquez, 2012, who, by using a low concentration of sodium hypochlorite 1.8% for 5 minutes, obtained a contamination percentage of (52.45%). However, the results differ from the results reported by Mohamed & Alhady, (2011) who, by using a concentration of sodium hypochlorite 1.5% for 20 minutes,

obtained better disinfection results with (30.39%) of contamination and with a (80%) of survival.

Moreover, the highest percentages of contamination by fungus occurred in the T1 with (32.13%), while the T8 treatment had the lowest percentage of contamination (14.28%). Regarding bacterial contamination, the T2 had the highest percentage of contamination (21.42%), while the T8 showed a clear decrease in contamination by these pathogens (8.92%) (Figure 2). Sanchez & Saenz (2005), says that prolonged exposure of nodal segments to higher concentrations of NaClO allow for better disinfection rates of fungi and bacteria; which occurs, according to the author, because the NaClO has more time to reach the plant tissue and execute the removal of endogenous microorganisms without harming tissue explant, since the disinfecting agent decreases the hydroxyl ions (OH), through the formation of water; lowers the pH, which stimulates the presence of hypochlorous acid

which, when in contact with organic matter, acts as a solvent; and releases chlorine which combines with the amino group of proteins, forming chloramines. Hypochlorous acid and hypochlorite

ions (ClO⁻) lead to degradation and hydrolysis of amino acids; therefore, it reduces the surface tension of the membrane and allows the lysis of the microorganisms.

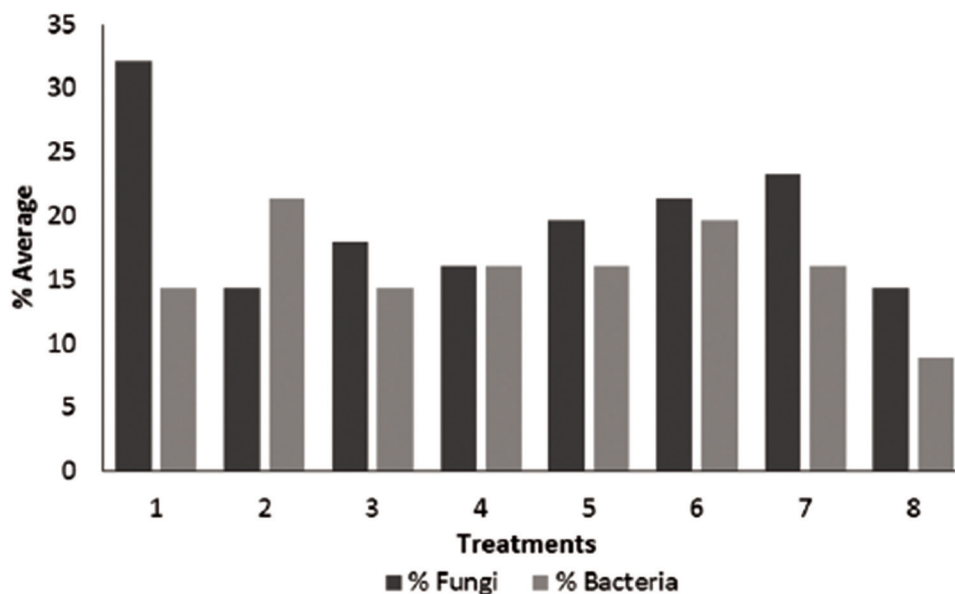


Figure 2. Averages of treatments for contamination by fungi and bacteria

Similarly Aamir *et al.* (2010), obtained decontamination and survival rates of 15.56% and 53.33% respectively, by using a high concentration of sodium hypochlorite 10% for 15 minutes for disinfection of *Stevia rebaudiana*. However, Shatnawi *et al.*, (2011) and Alhady (2011) reported percentages of contamination and survival of 25.56% and 32.22% respectively for a concentration of 1% sodium hypochlorite for 30 minutes of exposure and 68.89% and 63.33%, using a 1.5% concentration during 20 minutes of exposure.

This study evidences the results reported by Sánchez (2005) and Aamir *et al.* (2010) since the best percentages of decontamination and sprouting occurred in explants subjected to high concentrations of NaClO and longer exposure times.

The statistical analysis results showed that, for the sprouting variable, there were no statistically significant differences between treatments. According to Vasquez (2012) basal buds usually do not

emerge or are slow to emerge, and those used in this study were both apical and basal as intermediates. Contamination levels seem not to directly affect the emergence of nodal segments, since the cell differentiation occurred without changes (Figure 3). However, after the fourth week of planting, mainly fungal contamination affecting the emerged seedlings began, thus generating a strong competition for the nutrients of the medium, which was evidenced by the delayed emergence and development of explants. Meanwhile, Hernandez & Gonzales (2010) mention that many microorganisms require a period of adaptation to the new conditions before manifesting its presence and cause damage to tissues. Besides, they compete with the explant for the nutrients of the medium and cause them direct and indirect damage by colonization of their tissue or release into the environment of toxic metabolites. The authors themselves say that a frequent contaminant in the In Vitro culture causes a decrease in the reproduction coefficient, followed by rapid deterioration of crops.

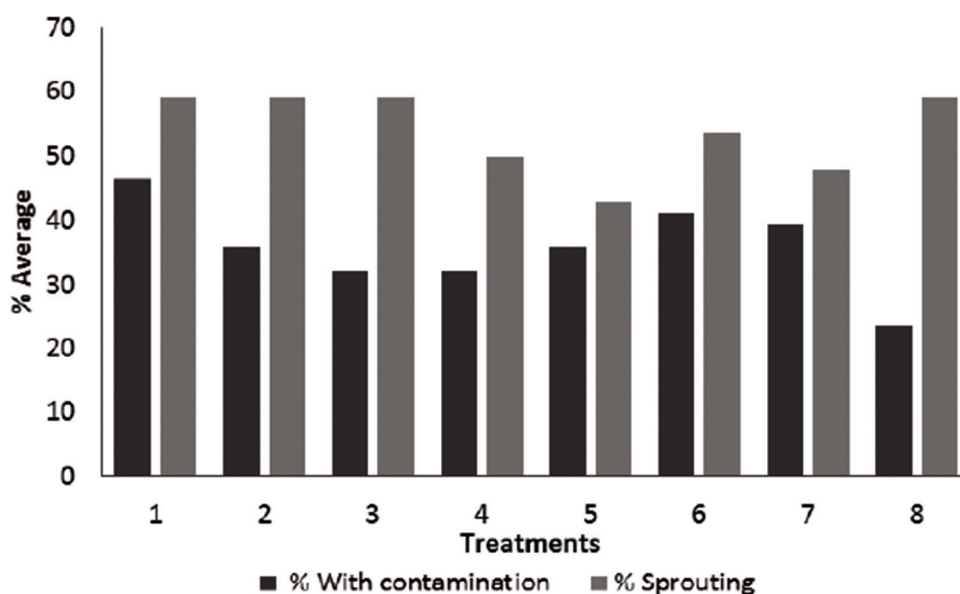


Figure 3. Averages of treatments for contamination and sprouting of nodal segments

Blanco *et al.* (2003), also states that contamination of explants with fungi or bacteria healthy morphogenic limits the capacity thereof. Methods have been developed to eradicate viruses and bacteria, these methods range from the removal of suspect tissues to the combined use of antibiotics alone or combined with *In Vitro* cultures. Similarly, in this study, 2.5mg/ml of Cephalexin were added to culture medium in the eight treatments evaluated for the same purpose. Meanwhile, Vásquez (2012) also reports the addition of fungicides and bactericides before sowing and within the culture medium, thus reducing contamination to 1.77% and achieving survival of 98.23%.

Nodal stevia explants can be considered as explants difficult to disinfect, due to pubescence of stems favoring the housing of microorganisms and small insects. On the other hand, according to Vasquez (2012), the high levels of contamination, mainly by fungi and bacteria are favored by the high concentration of sugars that the plant produces.

Conclusions

The results obtained in this work suggest that contamination of the explants by fungi and bacteria does not affect the emergence of nodal segments. Furthermore, although there is no significant difference between disinfection treatments for *In Vitro* establishment of *Stevia rebaudiana* Bertoni, Morita II variety, the treatments (T2 and T8) had the lowest percentage of fungal contamination (14.28%) and treatment (T8) had the lowest percentage of contamination by bacteria (8.92%). Similarly, treatments (T1, T2, T3 and T8) showed a higher germination percentage (58.91%). Similarly, treatments (T1, T2, T3 and T8) showed a higher germination percentage (58.91%). Although the statistical analysis clearly showed no significant difference between treatments, it is important to note that, for the nodal segments of the Morita II variety of *Stevia rebaudiana*, it was possible to obtain levels of decontamination by 75 and 85% by fungi and bacteria respectively, when exposed at concentrations of NaClO 4 and 5% during exposure times between 5 and 20 minutes, with the addition of 2.5mg / ml of antibiotic to the culture medium.

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Conflicto de Intereses

Los autores declaran no tener ningún conflicto de intereses

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