

Induction of shoot development of *Stevia rebaudiana* Morita II by vitamins and cysteine

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ABSTRACT

Objective: The aim of this study was to evaluate the influence of B vitamins and cysteine on the formation and development of shoots in *Stevia rebaudiana* (*S. rebaudiana*) Morita II, without the addition of plant growth regulators.

Design/methodology/approach: The starting material consisted of nodal segments, subjected to eight different vitamin treatments. The results were analyzed using mean comparisons and Tukey's test to identify statistical differences.

Results: The most effective treatment was treatment number two, which yielded an average of 7.5 shoots per explant, demonstrating its efficiency for multiplication. For explant elongation, treatment five proved to be the most effective.

Limitations of the study/implications: This study aims to establish a multiplication protocol with enhanced efficiency; however, a limitation is the transferability of the results to temporary immersion bioreactors.

Findings/conclusions: Thiamine and cysteine are two organic compounds that induce multiple sprouting, while nicotinic acid and pyridoxine promote the elongation of *Stevia* explants. This research represents a significant step towards transferring this knowledge to grassroots technological enterprises.

Keywords: *Stevia rebaudiana*, B vitamins, cysteine, Morita II.

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INTRODUCTION

Stevia rebaudiana Bertoni is known worldwide for its medicinal properties (González Hernández *et al.*, 2019). This plant belongs to the Compositae family (Ahmad, Blundell, & Mahomoodally, 2020) and is a perennial shrub native to Paraguay (Vázquez Hernández *et al.*, 2019). Its leaves contain steviol glycosides, which make them 100 to 300 times



sweeter than sucrose. This property has led to their consumption as sweeteners and their use as a sugar substitute in the food and pharmaceutical industries (Ahamad, Blundell, & Mahomoodally, 2020), as they are not metabolized by the human body (López Medina, López Zavaleta, & De la Cruz Carrillo, 2017). Stevia has been reported to possess anticancer, antimicrobial, anti-inflammatory, and antitumor properties without toxicity (Ruíz *et al.*, 2015). The genus *Stevia* comprises approximately 150 species (Abdullateef & Mohamad, 2011), among which *S. rebaudiana* variety Morita II and the creole variety are cultivated in the state of Yucatán.

However, the chemical composition of these varieties differs; Morita II has a higher daily biomass production per cm² of leaf area (Jarma, Rengifo, & Araméndiz Tatiz, 2006). Additionally, it synthesizes a greater concentration of rebaudioside A in relation to the concentration of steviosides, although its propagation by seed remains unsatisfactory (Ohta *et al.*, 2010). In the case of *S. rebaudiana* Bertoni, it has been demonstrated that in Murashige and Skoog culture medium supplemented with 0.37 mg/L of indole-3-butyric acid (IBA), an average of 14 shoots can be formed per nodal segment (Villamarín Gallegos *et al.*, 2020). This indicates that the type of response exhibited by the nodal segment of *S. rebaudiana* Bertoni will depend on the composition of the culture medium. Moreover, the vitamins used in the culture medium play an important role in the metabolism and development of plants, especially *in vitro*. According to Bhojwani and Razadan (1996), White reported the successful continuous growth of tomato root apices in 1934, initially using a culture medium composed of inorganic salts, yeast extract, and sucrose. However, in 1937, the substitution of yeast extract with pyridoxine, thiamine, and nicotinic acid allowed for the maintenance of tomato cultures for nearly 30 years, thus recognizing the importance of B vitamins. In *S. rebaudiana* Morita II, nodal segments form multiple shoots in the presence of B vitamins (4 mg/L of each), 6-benzylaminopurine (0.5 mg/L), kinetin (0.25 mg/L), and cysteine (4 mg/L) (Domínguez-May A. V., 2018). However, the latter, in the absence of plant growth regulators, increases shoot length in both *Vicia faba* (El-Awadi M. E. *et al.*, 2016) and EM26 apple (Sotiropoulos T. E. *et al.*, 2005). This could be advantageous, as it would reduce the *in vitro* production costs of these species and potentially enhance the production of other commercially valuable crops. Therefore, the objective of this work was to evaluate the influence of B vitamins and cysteine on the formation and development of shoots of *S. rebaudiana* Morita II, without the addition of plant growth regulators.

MATERIALS AND METHODS

Biological Material

The initial biological material for obtaining nodal segments originated from *S. rebaudiana* Morita II seedlings produced by the National Institute of Forestry, Agricultural and Livestock Research (INIFAP), located in the city of Mérida, Yucatán, Mexico.

Propagation of Shoots of *S. rebaudiana* Morita II for the Acquisition of Biological Material

In the preparation of the culture medium, 4.31 g/L of Murashige & Skoog (1962) salts from Sigma was used, supplemented with 100 mg/L of myo-inositol, 4 mg/L of glycine, 4

mg/L of pyridoxine, 4 mg/L of nicotinic acid, 4 mg/L of thiamine, 4 mg/L of cysteine, and 30 g/L of sucrose, along with 7 g/L of agar. The pH was adjusted to 5.8. The Murashige and Skoog culture medium was supplemented with 0.5 mg/L of 6-benzylaminopurine (BAP) and 0.25 mg/L of kinetin (KIN) as growth regulators. Finally, it was sterilized in an autoclave for 20 minutes at 121 °C and 1.032 kg/cm² of pressure.

To produce multiple shoots at 41 days of age, 200 nodal segments with a length of 1.5 cm were cultivated in the Murashige and Skoog culture medium in the presence of the previously mentioned growth regulators. The nodal segments were maintained for 41 days in a growth chamber at 25 ± 2 °C under photoperiod conditions according to Domínguez-May *et al.* (2018).

Culture Medium for the Induction of Shoots in Nodal Segments

Nodal segments of 1.5 cm in length were separated from 41-day-old shoots in culture medium. These were cultivated in eight different treatments, with 20 nodal segments inoculated in each treatment. All treatments contained 4.31 g/L of Murashige and Skoog (1962) medium, 100 mg/L of myo-inositol, 30 g/L of sucrose, and 7 g/L of agar, with the pH adjusted to 5.8. The addition of each vitamin (considering that 4 mg/L of thiamine is generally used) was maintained at 4 mg/L, along with cysteine at 4 mg/L. The combinations of these are shown in Table 1. It is important to note that in the evaluation of the effect of vitamins and cysteine, neither BAP nor KIN was added in any of the treatments.

All treatments were sterilized for 20 min at 121 °C with 1.032 kg cm² of pressure; afterwards, they were incubated in a growth chamber at 25 ± 2 °C under photoperiod conditions of 16 h of light and 8 h of darkness for a duration of 41 days.

Evaluated parameters

The number and length of shoots of Morita II were evaluated in each of the eight treatments.

Statistical Analysis

The results of the treatments were evaluated using IBM SPSS Statistics version 19, with a one-factor design (treatment) followed by mean analysis and Tukey's test.

Table 1. Treatments in Nodal Segments of *Stevia rebaudiana* Morita II for Shoot Induction.

Treatment	Vitamins, Cysteine o combination (4 mg L ⁻¹)
T1	Absence of vitamins and cysteine
T2	Thiamine
T3	Cysteine
T4	Nicotinic acid
T5	Pyridoxine
T6	Thiamine + Cysteine
T7	Thiamine + Cysteine + Nicotinic acid
T8	Thiamine + Cysteine + Pyridoxine

RESULTS AND DISCUSSION

Number of Shoots Generated

The application of the treatment in the absence of vitamins and cysteine (T1) to the nodal segments of *S. rebaudiana* Morita II did not allow for adequate shoot formation, exerting a negative effect on shoot development. After 41 days of cultivation, it produced a callus thickening at the base of the nodal segment with notable leaf deformation, resulting in an average of 0.55 ± 0.22 shoots generated.

In the presence of 4 mg/L of thiamine (T2), *S. rebaudiana* Morita II formed an average of 7.55 ± 0.88 shoots per nodal segment after 41 days of cultivation; however, the multiple shoots generated did not develop normal leaves. The addition of 4 mg/L of cysteine solely to the medium (T3) resulted in the formation of 4.30 ± 0.77 shoots per explant, with abnormal stem development and leaf rosettes forming over the callogenic thickening of the explant. The influence of only nicotinic acid in the medium (T4) allowed the development of shoots of *S. rebaudiana* Morita II with normal leaf formation, obtaining 3.55 ± 0.37 shoots per nodal segment. The medium supplemented solely with 4 mg/L of pyridoxine (T5) resulted in the formation of 2.50 ± 0.36 shoots per explant; however, the leaves formed were less expanded than those observed in the presence of nicotinic acid (T4) and exhibited greater internode elongation (Figure 1).

The combined effect of adding thiamine and cysteine to the medium (T6) showed the formation of 3.65 ± 0.39 shoots per nodal segment; however, there was also abnormal development of leaves directly attached to the explant without adequate stem development. The combination of thiamine, cysteine, and nicotinic acid (T7)

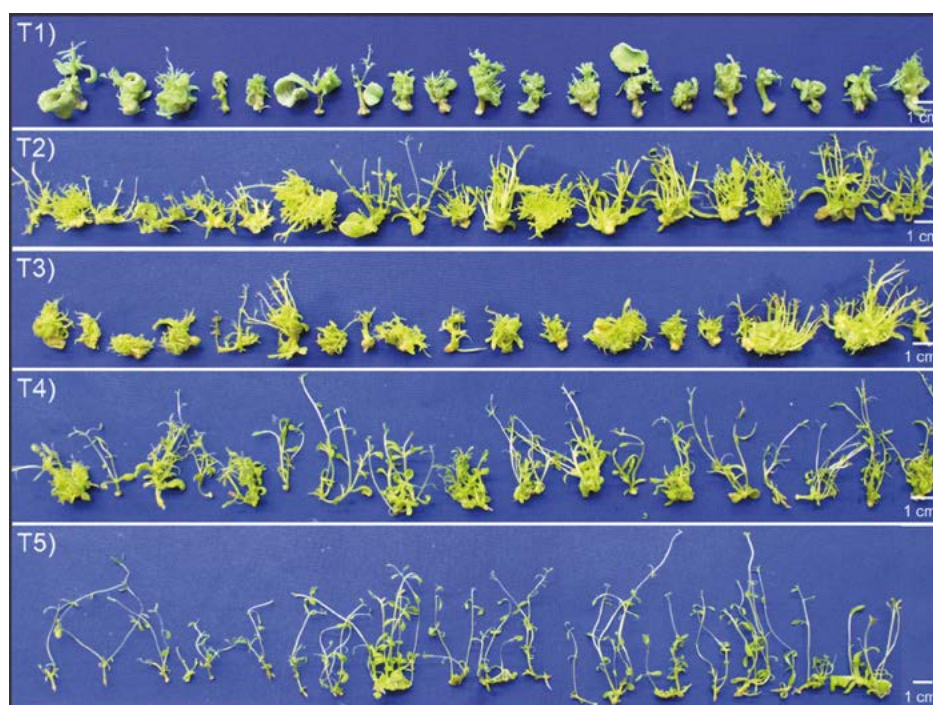


Figure 1. Development of nodal segments into shoots and their elongation. T1 Control, T2 Thiamine, T3 Cysteine, T4 Nicotinic Acid, T5 Pyridoxine.

stimulated the formation of 4.25 ± 0.38 shoots per explant, showing better development of multiple shoots, with normal leaf formation; however, there remained the presence of leaves emerging attached to the explant without stem elongation. Interestingly, the combination of thiamine, cysteine, and pyridoxine in the medium (T8) had a positive effect on the formation and development of shoots of *S. rebaudiana* Morita II, from the nodal segments, resulting in 4.55 ± 0.65 shoots per nodal segment, which exhibited better stem formation and leaf expansion; furthermore, abnormal formations, such as rosette leaves, were reduced (Figure 2).

The analysis of variance was applied to the eight treatments, finding a significant difference among them. Using Tukey's mean comparison test, three different groups were formed, with treatment T2 containing Thiamine showing the highest response in terms of the number of shoots generated, averaging 7.55 ± 0.88 per nodal segment (Table 2).

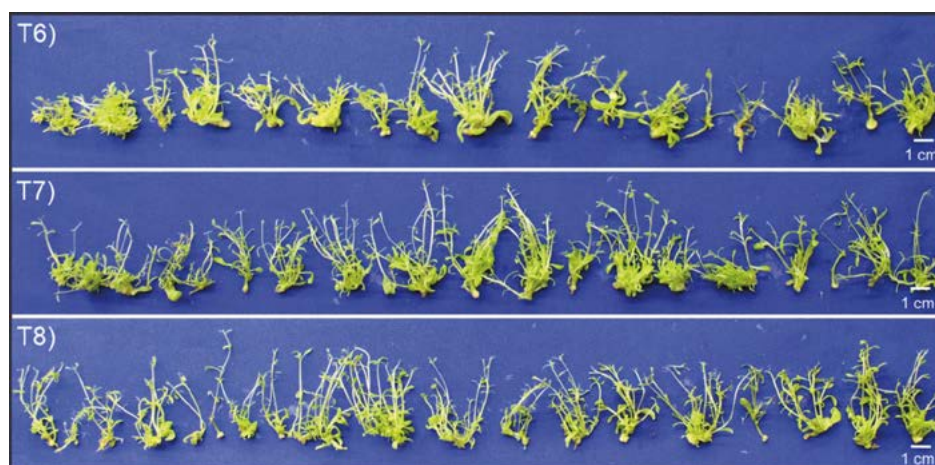


Figure 2. Development of nodal segments into shoots and their elongation. T6, Thiamine+Cysteine; T7, Thiamine+Cysteine+Nicotinic Acid; T8, Thiamine+Cysteine+Pyridoxine.

Table 2. Statistical analysis of number of shoots generated.

Treatment	Average number of shoots*	Typical error
T2	7.550 c	0.881
T8	4.550 b	0.650
T3	4.300 b	0.771
T7	4.250 b	0.383
T6	3.650 b	0.385
T4	3.550 b	0.366
T5	2.500 ab	0.359
T1	0.550 a	0.223

*Number of shoots generated

Tukey (HSD) $\alpha=0.05$; Means for groups in homogeneous subsets are shown. N=20 based on the harmonic mean of the sample size. Means for groups in homogeneous subsets are shown.

Although the analysis of variance shows that the highest effect on the number of shoots formation was with the addition of thiamine in the medium (T2), averaging 7.55 ± 0.88 shoots per explant, it exhibited abnormal development of the shoots and rosette leaves attached to the explant. On the other hand, treatment (T8), which consisted of the addition of thiamine, cysteine, and pyridoxine to the medium, demonstrated the development of shoots with better stem thickness and leaf expansion, averaging 4.55 ± 0.65 shoots per explant. This treatment is considered a better alternative for generating shoots of *S. rebaudiana* Morita II from nodal segments.

Shoot elongation

Upon evaluating the effect of the treatments on the shoot length of *S. rebaudiana* Morita II, it was found that the absence of vitamins and cysteine in treatment (T1) resulted in rosette-like shoot formations, without stem development or adequate shoot elongation, reaching an average length of 0.271 ± 0.08 cm. In the treatment with thiamine (T2), the elongation was 1.41 ± 0.05 cm. For the medium supplemented with cysteine (T3), the shoots formed with an elongation of 0.95 ± 0.06 cm, while in the medium supplemented with nicotinic acid (T4), the elongation reached 2.62 ± 0.14 cm. The treatment with pyridoxine (T5) promoted shoot elongation with an average of 4.02 ± 0.26 cm (Figure 2). The treatment with thiamine and cysteine (T6) allowed shoot elongation with an average of 2.61 ± 0.10 cm, showing a response like that of nicotinic acid. The treatment (T7) containing thiamine, cysteine, and nicotinic acid developed shoots with an elongation of 3.34 ± 0.11 cm. Likewise, the treatment (T8), supplemented with thiamine, cysteine, and pyridoxine, resulted in shoot elongation values of 3.24 ± 0.09 cm, with expanded stems and leaves (Figure 2).

For the case of shoot elongation, the analysis of variance shows a significant difference between treatments. Therefore, Tukey's test was applied, determining the presence of five different groups. It was found that the treatment with pyridoxine (T5) had the highest response, with a shoot elongation of 4.02 ± 0.26 cm. Even though the numerical value for elongation in treatment T5 was the highest, the treatment (T8) in the presence of thiamine, cysteine, and pyridoxine resulted in shoots with well-developed stems, internodes, and expanded leaves, reaching a length of 3.24 ± 0.09 cm (Table 3).

It is interesting to note that explants from nodal segments of *S. rebaudiana* Morita II have been used in biotechnological tissue culture processes to obtain shoots, but generally with the application of plant growth hormones. According to Macías Hernández (2019), who tested various growth regulators with explants from nodal segments, it was the Murashige and Skoog culture medium with 1 mg/L of BAP that allowed the production of shoots with average values of 4.4 cm in height and 5.8 shoots per explant after 4 weeks of incubation.

In this study, a phytohormone-like effect was observed in the generation and elongation of shoots, exerted by the presence of the B-type vitamins evaluated in the organogenic response of the explant, as shown in Figure 1, treatments T2, T4, and T5 in the induction of shoot formation. According to the results, thiamine increases the number of shoots in *S. rebaudiana* Morita II, but does not promote their elongation. Thiamine in *Calendula officinalis* L. not only increases the number of floral stems but also

Table 3. Statistical Analysis of Shoot Elongation Generated.

Treatment	Average length*	Typical error
T5	4.024 e	0.25939
T7	3.336 d	0.10489
T8	3.239 d	0.07506
T4	2.616 c	0.13998
T6	2.610 c	0.10304
T2	1.413 b	0.05481
T3	0.953 b	0.06241
T1	0.271 a	0.07506

*Length in cm; Tukey (HSD) $\alpha=0.05$; Means for groups in homogeneous subsets are displayed; N=60 based on the harmonic mean of the sample size; Group sizes are unequal. The harmonic mean of the group sizes will be used. Type I error levels are not guaranteed.

enhances their length (Soltani, Saffari, & Maghsoudi Moud, 2014); in *Phoenix dactylifera* L., this vitamin boosts the formation of somatic embryos depending on its concentration (Al Khayri, 2001). In *Triticum aestivum*, thiamine increases seedling length when present at low concentrations; however, at high concentrations, it reduces their length (Misratia & Mansur, 2018).

Cysteine also promotes the increase in the number of shoots of *S. rebaudiana* Morita II, with little stimulation of their elongation. In *Saccharum officinarum* L., cysteine at low concentrations increases the number of shoots, while a contrary effect is observed at higher concentrations (Asad, Arshad, Mansoor, & Zafar, 2009). On the other hand, in *Malus domestica* Borkh., the number of shoots increases; however, it does not stimulate elongation (Sotiropoulos, Dimassi, & Therios, 2005), which aligns with what was observed in Morita II.

Nicotinic acid in the medium (T4) produced a lower number of shoots compared to thiamine and cysteine but allowed for their elongation. In *Solanum tuberosum*, nicotinic acid does not increase the number of shoots in the Mozart variety; however, it promotes the elongation of shoots (Yaseen, Toma, & Carbonera, 2017), as seen in the case of Morita II. Similarly, the elongation effect of nicotinic acid was observed in *Triticum aestivum*, where seedlings increase in length at low concentrations, but their length may decrease if used at high concentrations (Misratia & Mansur, 2018). On the other hand, the medium supplemented with pyridoxine (T5) decreased the formation of shoots per explant of Morita II compared to thiamine; however, it exhibited the highest elongation value. This is contrary to the findings by Yaseen F. K. *et al.* (2017), where *Solanum tuberosum*, variety Mozart, did not see an increase in either the number or length of shoots per nodal segment. It is similar to the response found by Soltani Y. *et al.* (2012), where the foliar application of pyridoxine in *Calendula officinalis* L. plants increased the length of their stems. This suggests that both B vitamins and cysteine can generate a different physiological effect depending on the concentration used and the plant species they act upon.

The combination of B vitamins in *Hypericum perforatum*, when thiamine is at a low concentration (0.1 mg/L) compared to nicotinic acid (50 mg/L) and pyridoxine (50 mg/L), results in a lower number of shoots. However, when the concentration of thiamine is increased (10 mg/L), the number of shoots per explant increases. Conversely, when the thiamine concentration is 0.1 mg/L and the nicotinic acid concentration is 0.5 mg/L, without using pyridoxine, the shoots increase in length; but when thiamine is at 10 mg/L, the length of the shoots decreases. This is contrary to the case where the concentration of nicotinic acid is increased to 50 mg/L while maintaining thiamine at 0.1 mg/L, resulting in a significant increase in shoot length (Khakpour, Motallebi Azar, Bahman, Alizadeh Salte, & Hasani, 2015). In *Solanum tuberosum*, the number of shoots also increases when the thiamine concentration increases from 0.1 to 10 mg/L in the presence of pyridoxine (50 mg/L) and nicotinic acid (50 mg/L). The length of the shoots decreases when the thiamine concentration increases from 0.1 to 10 mg/L with pyridoxine (0.5 mg/L); however, when the concentration of the latter increases from 0.5 to 50 mg/L, the shoots elongate significantly (Kazemiani & Motallebi Azar, 2015). This indicates that thiamine induces the formation of multiple shoots without promoting elongation. In contrast, nicotinic acid and pyridoxine induce elongation in the shoots. A similar combined effect is observed for *S. rebaudiana* Morita II, where thiamine and cysteine induce the formation of multiple shoots, while nicotinic acid and pyridoxine promote shoot elongation.

Even though the numerical value for shoot elongation in treatment T5 is the highest, treatment T8, which included thiamine, cysteine, and pyridoxine, resulted in an average of 4.5 shoots per explant, with well-defined stem development, internodes, and expanded leaves, reaching a length of 3.24 ± 0.09 cm (Table 7). This is like the results obtained by Macías Hernández (2019) using the Murashige and Skoog medium supplemented with 1 mg/L of BAP. Regarding KIN and BAP, it has been documented that these synthetic cytokinins are involved in cell division, callus formation, organ development, and the induction of somatic embryogenesis from plant tissues (Thilaga *et al.*, 2013; Pasternak *et al.*, 2024). Currently, researchers have documented part of the reprogramming mechanism for the induction of somatic embryogenesis in coffee, indicating that a preconditioning period of 14 days in seedlings in the presence of the synthetic cytokinin KIN is necessary to increase the production of endogenous growth regulators such as indole-3-acetic acid (IAA) and conjugated auxins IAA-alanine and IAA-glutamic acid (IAA-Ala and IAA-Glu). Subsequently, during the induction of somatic embryogenesis, when KIN is removed from the culture medium, both free and conjugated endogenous auxins decrease drastically, leading to a reprogramming of transcripts *CcTAA1*, *CcYUC1*, and *CcYUC3* involved in the biosynthesis of IAA, which facilitates the induction of the embryogenic response in the presence of BA (6-benzylaminopurine). Two possible mechanisms of action are proposed: a) de novo synthesis for the formation of IAA, and b) the formation of IAA through the hydrolysis of conjugated auxins. It is suggested that the accumulation of conjugated auxins, which increased during the pretreatment in the presence of KIN, enabled the formation of new IAA during the induction of somatic embryogenesis in the presence of BA (Ayil *et al.*, 2013).

In our study on *S. rebaudiana* Morita II, the nodal segments were pretreated for 41 days with KIN, which was subsequently removed. We could suggest that the endogenous levels of conjugated auxins increased in the presence of KIN, preparing or reprogramming the nodal segments for bud formation through either the de novo synthesis pathway or the hydrolysis of conjugated auxins to form new IAA. Together with the vitamins, these mechanisms would facilitate physiological effects such as elongation and the complete development of the bud in *S. rebaudiana* Morita II.

CONCLUSIONS

The Murashige and Skoog culture medium, in the absence of B vitamins and cysteine, does not promote the formation or elongation of buds from nodal segments of *S. rebaudiana* Morita II. The Murashige and Skoog medium must contain any of these organic compounds for bud development per nodal segment; however, not all generate the same response. Thiamine and/or cysteine induce the formation of multiple buds but do not efficiently promote elongation, while nicotinic acid and/or pyridoxine stimulate elongation. Nevertheless, for the buds from nodal segments to develop adequately in stem and leaf expansion, a combination of thiamine, cysteine, and pyridoxine is required. The absence of plant growth regulators in the culture media allowed for the observation of the inductive effect on bud development, where the combination of B vitamins and cysteine appears to act as inducers in the development and formation of buds.

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