

IN VITRO GERMINATION AND VEGETATIVE PROPAGATION THROUGH BUD DEVELOPMENT OF SACHA INCHI (*Plukenetia volubilis*)

Germinación y propagación vegetativa *in vitro* a través del desarrollo de yemas de Sacha Inchi (*Plukenetia volubilis*)

Ana María HENAO RAMÍREZ¹ , Aura Inés URREA TRUJILLO² , Lucía ATEHORTÚA GARCÉS³ 

¹ Centro de Desarrollo Agrobiotecnológico y de Innovación – CEDAIT, Universidad de Antioquia, Km. 1,7 vía San Antonio de Pereira - Carmen de Viboral, A.A 054048, Carmen de Viboral, Colombia.

² Instituto de Biología, Facultad de Ciencias Exactas y Naturales, Universidad de Antioquia, Calle 70 No. 52-21, Medellín, Colombia.

³ Instituto de Biología, Facultad de Ciencias Exactas y Naturales, Universidad de Antioquia, Calle 70 No. 52-21, Medellín, Colombia.

** **For correspondence:** amaria.henao@udea.edu.co

Received: 30th June 2020. **Returned for revision:** 20th October 2020. **Accepted:** 06th January 2021.

Associate Editor: Hernán Mauricio Romero

Citation/ citar este artículo como: Heno Ramírez, A. M., Urrea Trujillo, A. I., and Atehortúa Garcés, L. (2022). *In vitro* germination and vegetative propagation through bud development of sacha inchi (*Plukenetia volubilis*). *Acta Biológica Colombiana*, 27(1), 70-78. <https://doi.org/10.15446/abc.v27n1.88727>

ABSTRACT

This study describes the *in vitro* seed germination and micropropagation of *Plukenetia volubilis* (sacha inchi), an oilseed crop rich in omega-3 fatty acids, with health benefits and several industrial applications. Seed germination was evaluated in different culture media (MS and 1/2 MS), seed coat presence/absence and culture temperature (18 °C and 28 °C). Micropropagation was performed using axillary bud development (ABD) on nodal segments from *in vitro* seedlings. KIN, BAP and 2-ip were evaluated for ABD, and the effect of modified MS in 453 mg L⁻¹ CaCl₂ and 351.62 mg L⁻¹ MgSO₄ on ABD and shoot survival was assessed to improve the process. Finally, six treatments were evaluated to optimize ABD and shoot leaf formation. Seed germination of 91.6 % was achieved in MS at 28 °C when the seed coat was removed. ABD was obtained in 45 % and 40 % with 0.4 mg L⁻¹ KIN and 0.6 mg L⁻¹ 2-ip, respectively, with the least CAL. The modification in 453 mg L⁻¹ CaCl₂ then allowed 76 % ABD and 82 % explant survival. ABD response was optimized to 95 % and 2.45 leaves with MS medium + CaCl₂ modification + 10 % coconut water + 0.4 mg L⁻¹ KIN. The same results were obtained by replacing the latter with 0.6 mg L⁻¹ 2-ip. Rooting was achieved in MS without PGR, and acclimatization was successful. The results indicate that plant production via germination and vegetative propagation is effective for commercial purposes.

Keywords: Axillary buds, Calcium, Kinetin, Micropropagation, 2-ip.

RESUMEN

Este estudio describe la germinación *in vitro* y micropropagación de *Plukenetia volubilis*, un cultivo oleaginoso rico en omega-3 beneficioso para la salud y con múltiples aplicaciones industriales. Se evaluó en la germinación diferentes medios de cultivo (MS y 1/2 MS), presencia-ausencia de testa y temperatura de cultivo (18 °C y 28 °C). La micropropagación se realizó vía yemas axilares (ABD) de plántulas *in vitro*. Se evaluó el efecto de KIN, BAP y 2-ip sobre ABD, seguidamente, para mejorar el proceso se evaluó el efecto de MS modificado en 453 mg L⁻¹ CaCl₂ y 351.62 mg L⁻¹ MgSO₄ sobre ABD y supervivencia del brote. Finalmente, se evaluaron seis tratamientos para optimizar ABD y la formación de hojas. Se logró una germinación 91,6 % en MS a 28 °C cuando se retiró la testa. Se obtuvo 45 % y 40 % de ABD con 0,4 mg L⁻¹ KIN y 0,6 mg L⁻¹ 2-ip respectivamente, ambos con la menor CAL. Posteriormente, la modificación de CaCl₂ permitió 76 % ABD y 82 % de supervivencia. Se optimizó ABD al 95 % con 2,45 hojas por brote con el medio: MS + modificación de CaCl₂ + 10 % de agua de coco + 0.4 mg L⁻¹ KIN, los mismos resultados se obtuvieron cambiando este último con 0,6 mg L⁻¹ 2-ip, se logró enraizamiento en MS sin PGR, y la aclimatización fue exitosa. Los resultados indican que la producción de plantas vía germinación y propagación vegetativa es efectiva con fines comerciales.

Palabras Clave: Calcio, Inducción de brotes, Kinetina, Micropropagación, 2-ip.

INTRODUCTION

Plukenetia L. (Euphorbiaceae) is a pantropical genus with 37 species of lianas and scrambling vines. Several species of *Plukenetia* have traditionally been cultivated in their native range, e.g., *Plukenetia volubilis* L. (Sacha Inchi), *Plukenetia huayllabambana* R. W. and *Plukenetia conophora* Müll. Arg. (Tropicos.org, 2020). It is found in the Lesser Antilles and South America, primarily in the northern and western regions and margins of the Amazon Basin in Suriname, Venezuela, Colombia, Ecuador, Peru, Bolivia and Brazil (Wang et al., 2018). It is known as the Inca peanut because the Inca Civilization used it as early as 3000 years ago. In the past decade, *P. volubilis* has become increasingly popular in the horticultural, pharmaceutical, cosmetic and food industries (Kodahl, 2020).

P. volubilis seeds are an excellent source of lipids; 77.5–84.4 % are polyunsaturated fatty acids (ω 3, ω 6 and ω 9), 8.4–13.2 % are monounsaturated fatty acids, and 6.8–9.1 % are saturated fatty acids (Maurer et al., 2012). In addition, its seeds have essential amino acids and are used as dietary supplements (Sathe et al., 2012). Food enriched with omega polyunsaturated fatty acids can play an important role in preventing and treating coronary heart and neurodegenerative diseases, diabetes, rheumatoid arthritis, pulmonary diseases and other inflammatory autoimmune diseases, making *P. volubilis* oil highly attractive to the food and pharmaceutical industries (Kerdiles et al., 2017; Watanabe and Tatsuno, 2017; Tenenbaum and Fisman, 2018). Furthermore, its seeds contain antioxidants, including vitamins A and E, which may explain the oil's increasing popularity in the cosmetic industry (Chirinos et al., 2016). All the potential uses of *P. volubilis* oil in different industries make this species a promising crop for farmers involved in oilseed production for food and non-food markets (Wang et al., 2018).

P. volubilis has a considerable degree of morphological variation and high genetic diversity between different populations. It is an allogamous species usually propagated by seeds. Therefore, its offspring is heterogeneous (Cachique et al., 2018). Moreover, its seeds have poor seed viability, a lower germination rate, lower disease resistance and delayed seedling rooting (Solis et al., 2016). Vegetative propagation allows maintaining 100 % of superior genotypes' identity, ensures germplasm conservation and increases genetic gain in short periods by using both additive and non-additive components of genetic variance (Solis et al., 2019).

The method of asexual propagation through cuttings has limited establishing commercial plantations because genotypes with desired traits cannot be propagated in masse. One strategy to overcome these limitations is *in vitro* tissue culture, which allows regeneration via organogenesis and/or somatic embryogenesis. *In vitro* regeneration offers a significant opportunity for mass propagation and genetic enhancement of plants. It can be performed in reduced spaces and makes it possible to keep a stock of disease-free plants.

Some studies have been developed for the *in vitro* propagation of *P. volubilis*. Seed germination times have been shorter in *in vitro* germination than in nurseries. However, some are still long (de la Rosa and Quijada, 2013; Cardoso et al., 2018; Kodahl et al., 2018). Some studies used apical meristems obtained from *in vitro* plantlets. Viegas Rodrigues et al. (2014) achieved shoot formation but did not report the rooting phase and Solis et al. (2018) also achieved shoot formation and rooting but did not reach the *ex vitro* phase.

Restrepo-Osorio et al. (2020) and Bordignon et al. (2012) used different hypocotyl and epicotyl segments of seeds germinated *in vitro* to obtain plants from induced shoots. Millones and Vásquez (2008) and Thuy and Nhung (2019) used hypocotyl segments and obtained shoots and rooting. However, Patthanajuck and Bunnag (2017) used the same explant they did not attend to, which was root formation in shoot regenerates. Dong et al. (2016) used cotyledon leaves to obtain shoot regeneration, but lacked rooting. Viegas Rodrigues et al. (2014) performed a field evaluation of *in vitro* regenerated plantlets, which showed an increase in productivity and no genetic variability compared to plantlets propagated by seeds.

Considering the results above, this study had two objectives: to evaluate plant production via germination and vegetative propagation by ABD. The following were evaluated in terms of seed germination: two culture media (MS and $1/2$ MS), two seed coat presence/absence conditions and two culture temperatures (18 °C and 28 °C). ABD was evaluated at different times, first using different types and concentrations of cytokines: KIN, 6-BAP and 2-ip between 0.1 to 0.8 mg L⁻¹. CAL was subsequently evaluated as an unwanted response. Second, the effect of modified MS in 453 mg L⁻¹ CaCl₂ and 351.62 mg L⁻¹ MgSO₄ supplemented 0.4 mg L⁻¹ KIN on ABD and shoot survival was assessed. Third, different treatments with 10 % coconut water, 0.6 mg L⁻¹ 2-ip and 0.4 mg L⁻¹ KIN were evaluated to optimize ABD and shoot leaf formation. Therefore, this investigation made it possible to develop an efficient protocol for *in vitro* plant production via seed germination and vegetative propagation through nodal explants. Acclimatization was also addressed in a preliminary manner. The results contribute to standardizing the *P. volubilis* micropropagation protocol for commercial purposes, since it includes both the rooting shoots and acclimatization stage.

MATERIALS AND METHODS

Plant Material, Seed Disinfection and Culture Conditions

The research was performed in the Plant Tissue Culture Laboratory at Universidad de Antioquia (Antioquia, Colombia). The fruits were obtained from Colombiana de Biocombustibles S.A. (Santa Fe de Antioquia, Antioquia, Colombia) and seeds were stored at 24 °C for a maximum

of 5 days prior to their introduction to the laboratory. Fruits in an advanced stage of physiological maturation were used (Fig. 1a-d) and manually processed to remove the pericarp and isolate the seeds for further disinfection (Fig. 1b-c). Seeds were disinfected with a 70 % v/v ethanol solution for 15 min followed by a 10 % v/v iodine solution (Merck) for 5 min in a laminar flow cabinet. They were subsequently immersed in a 3 % v/v sodium chloride and 10 % v/v acetic acid solution for 30 min and then rinsed with sterile distilled water. Seeds were manipulated and inoculated in the culture media (establishment phase) using sterilized tweezers and scalpels. Glass flasks (109.3 mm x 43.7 mm, 68.3 mm x 57.5 mm and 117 mm x 100 mm), each containing 20 -50 mL of culture medium, were used in all phases. Uniform culture conditions were maintained in all experiments. All culture media were supplemented with 20 g L⁻¹ sucrose and 2.7 g L⁻¹ gelling agent (Phytigel®), and medium's pH was adjusted to 5.8 using 1M NaOH or HCl with a pH meter (Metrohm) prior to autoclaving at 121 °C and 15 psi for 15 min.

In Vitro Germination

The establishment phase basically considered three factors' effect on seed germination: culture media evaluated considering a Murashige and Skoog basal medium (MS) (Murashige and Skoog, 1962) and a half basal medium MS (½ MS), seed coat presence or absence determined by manually removing coats with a scalpel under sterile conditions, and the effect of temperature under two conditions: 18 °C and 26 °C. Combinations of these factors contributed to a total of eight

treatments as follows: MS + coat 18 °C, MS + coat 28 °C; MS - coat 18 °C; MS - coat 28 °C; ½ MS + coat 18 °C; ½ MS + coat 28 °C; ½ MS - coat 18 °C; ½ MS + coat 28 °C.

One seed was cultured in a single glass flask (109.3 mm x 43.7 mm) and, after eight days, the number of seeds with radicle development in each treatment mentioned in the previous paragraph were recorded. Each treatment was replicated twice. In order to calculate the percentage of germination, the count performed in groups of 20 seeds was taken as a unit of measurement.

In vitro seeds were kept in complete darkness until radicle development occurred (Fig. 2c). After epicotyl extension, seedlings were transferred to glass flasks with MS medium and gelling agent (Phytigel®) (117 mm x 100 mm), where plantlet development was completed (Fig. 2f-g). Plantlets were kept at 23 ± 2 °C and with continuous light at an intensity of 2000 lux from fluorescent light tubes. The culture medium was renewed every three weeks.

In Vitro Vegetative Propagation by Axillary Bud Development (ABD)

The Effect of KIN, BAP and 2-ip concentrations on ABD

In the first experiment, the effects of different KIN, 2-ip and BAP concentrations (0.8 mg L⁻¹, 0.6 mg L⁻¹, 0.4 mg L⁻¹, 0.2 mg L⁻¹, 0.1 mg L⁻¹) on ABD and callogenesis (CAL) were evaluated in the multiplication phase as an unwanted response. In previous experiments with MS basal medium and without growth regulators, explants did not establish or develop. Therefore, these features were not considered for this experiment.

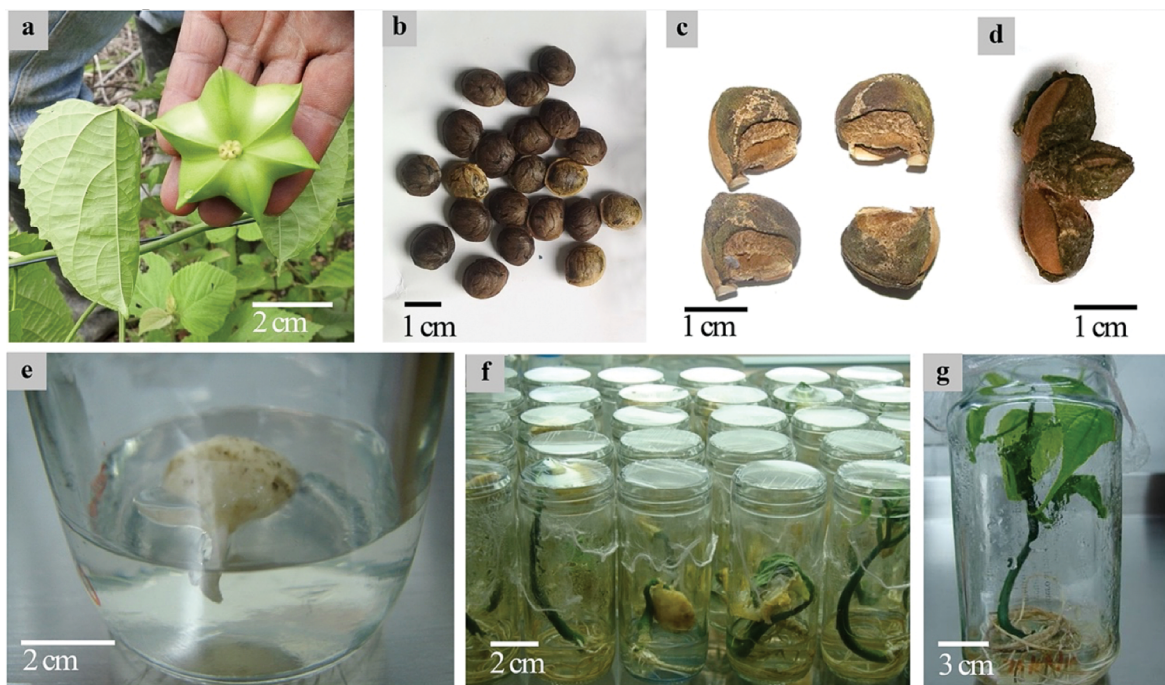


Figure 1. Sacha inchi (*Plukenetia volubilis*) fructification and seedling development: a) unripe fruit, b) seed, c) mature locules, d) mature fruit, e) radicle extension, f) seedlings after 15 days, g) plants after two months of culture.

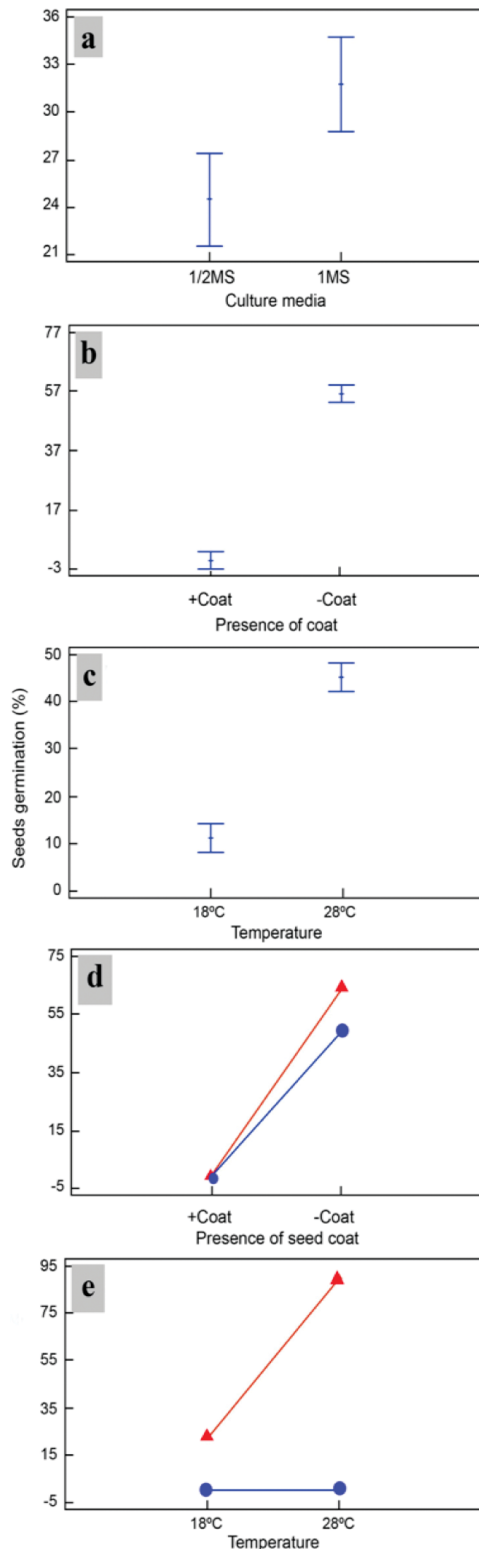


Figure 2. The independent effect of culture medium, a) seed coat presence b) and temperature c) on *in vitro* seed germination, as well as interactions between the effect of basal culture medium concentration [MS (triangle), $\frac{1}{2}$ MS (circle)] and seed coat presence or absence d) and seed coat [presence (circle) or absence (triangle)] and temperature e) on the germination of *Plukenetia volubilis* seeds. Differences were significant at $p \leq 0.05$ using multiple Fisher LSD rank comparisons.

Nodal segments (2.0 – 2.3 cm long) were collected from two- to three-month-old seedlings (Fig. 2c). The nodal segments were planted vertically with almost 30 % of their surface in contact with the MS basal medium and gelling agent (Phytigel®), using two nodal segments for glass flasks (68.3 mm x 57.5 mm). The count was performed in groups of 12 nodal segments, which were taken as experimental units to estimate ABD percentage. The size of the formed callus (CAL) was measured with millimeter paper under sterile conditions. Callus formation was an unwanted response and was avoided. Fifteen treatments were performed with six replications per treatment. Culture conditions were the same as plantlet maintenance for *in vitro* germination.

Effect of Calcium and Magnesium Modifications to the MS Basal Medium on ABD and Explant Survival

In the second experiment, modifications were made to calcium and magnesium concentrations to counteract the chlorosis and necrosis of young leaves after some weeks of culture. These modifications were performed to analyze the soil of a commercial plot of *P. volubilis* provided by Colombiana de Biocombustibles S.A. It was observed that Ca and Mg macronutrients and Cu and Fe micronutrients were lower in the MS medium's soil (Appendix 1). Consequently, Ca, Mg, Fe and Cu were the nutrient concentrations that should have been changed, but only macronutrient concentration was considered in this study. The effects of the following treatments on ABD and survival percentage were evaluated: (a) control full MS (332.2 mg L⁻¹ CaCl₂ and 180.7 mg L⁻¹ MgSO₄); (b) MS with 453 mg L⁻¹ CaCl₂ (Kao and Michayluk, 1975); (c) MS with 351.62 mg L⁻¹ MgSO₄ (White, 1964). Three treatments were performed, with three replications per treatment. The count was performed in groups of 25 nodal segments, which were taken as experimental units.

The Effect of Different PGR's and Coconut Water on ABD and Leaf Formation

In the third experiment, the effects of different treatments on ABD percentage and number of leaves were tested in order to optimize axillary bud growth in MS, modified according to the results above: (a) MS Control; (b) 0.4 mg L⁻¹ 2-ip; (c) 0.4 mg L⁻¹ KIN; (d) 10 % coconut water; (e) 0.4 mg L⁻¹ 2-ip supplemented with 10 % coconut water, (f) 0.4 mg L⁻¹ KIN supplemented with 10 % coconut water. In this case, six treatments were performed to estimate ABD percentage, with three replications per treatment. The count was performed in groups of 20 explants, which were taken as experimental units. Finally, the leaves formed in each explant were counted.

Statistical Analysis

The data was analysed using parametric analyses of variance (ANOVA) to test significance with One-way and Multi-way analyses. The Shapiro-Wilk Test was used for

normality and Bartlett's test for homoscedasticity. Fisher's Least Significant Difference (LSD) comparison was used to determine significant differences in averages. All tests were performed at a 5% level of significance ($p \leq 0.05$). The data was analysed using Statgraphics Centurion XVII software.

RESULTS

In Vitro Germination

Culture media, seed coat presence and culture temperature had a significant effect on the percentage of germinated seeds during seed germination ($p \leq 0.05$). Germination was higher in the full MS medium (31.75 %) than the $1/2$ MS medium (24.5 %) (Fig. 2a). It was 56.25 % when the seed coat was removed and 0 % when keeping the coat (Fig. 2b). When the seeds were kept at 28 °C, germination was 45 %, and it was 11.25 % when kept at 18 °C (Fig. 2c).

Interactions between the different factors corresponding to culture medium-seed coat presence (Fig. 2d) and culture temperature-seed coat presence (Fig. 2e) were found to be statistically significant ($p \leq 0.05$). These results indicated that all factors (culture media – seed coat and temperature – seed coat) are related and, therefore, the best culture conditions for seed germination are MS medium without coats (66.5 %) and 28 °C without coats (91.6 %) (Fig. 2d-e). It was observed that radicles appeared within two days after *in vitro* introduction.

In Vitro Vegetative Propagation by Axillary Bud Development (ABD)

Effect of KIN, BAP and 2-ip Concentrations on ABD and Callogenesis

The independent effect of KIN, BAP and 2-ip concentrations on the percentage of axillary bud development was significant ($p \leq 0.05$). The highest percentage of ABD was obtained with 0.6 mg L⁻¹ 2-ip (43.33 %). Nevertheless, there was no difference with 0.4 mg L⁻¹ KIN (42 %) (Fig. 3b). None of the BAP concentrations induced ABD, but they reached the highest values of CAL (4.84 cm²) with 0.8 mg L⁻¹ BAP (Fig. 3a). The desired result was the lowest CAL, which was obtained at 0.1 mg L⁻¹ (0.52 cm²), 0.2 mg L⁻¹ (0.62 cm²) and 0.4 mg L⁻¹ (0.47 cm²) 2-ip following 0.4 mg L⁻¹ (0.86 cm²) and 0.2 mg L⁻¹ (1.07 cm²) KIN (Table 1). The lower values of CAL, at 0.47 cm², matched the better ABD results with 0.4 mg L⁻¹ KIN. However, axillary bud development after 20 days of cultivation presented chlorosis and necrosis in young leaves (Fig. 3c).

Effect of Calcium and Magnesium Modifications in the Basal MS Medium on ABD and Explant Survival

As mentioned earlier, various modifications were made to Ca and Mg concentrations in MS basal medium to counteract chlorosis and necrosis during ABD. It was observed that macronutrient modifications in the MS basal

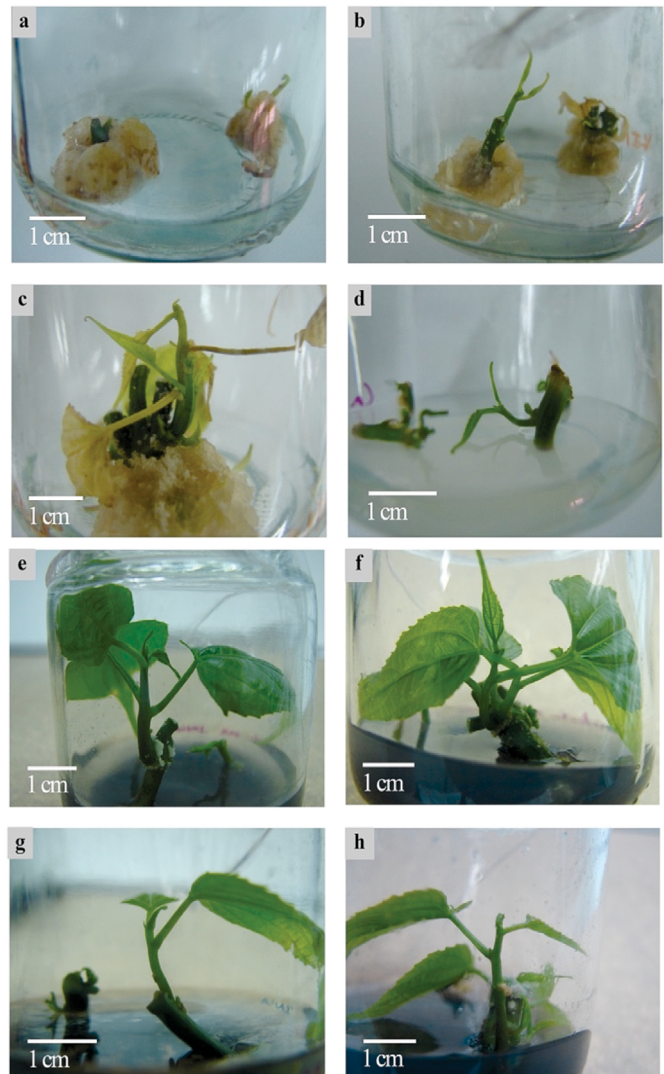


Fig. 3. *Plukenetia volubilis*. callogenesis with 0.1 – 0.8 mg L⁻¹ BAP (a), axillary bud development (ABD) in 0.8 mg L⁻¹ KIN (b), explant chlorosis and necrosis after 20 days of culture (c), ABD in 0.4 mg L⁻¹ KIN and MS modified in 453 mg L⁻¹ CaCl₂ in (d), ABD after 15 days of culture in MS modified with 453 mg L⁻¹ CaCl₂ and supplemented with 0.6 mg L⁻¹ 2-ip and 10% coconut water (e-f), ABD after 15 days of culture in MS modified with 453 mg L⁻¹ CaCl₂ and supplemented 0.4 mg L⁻¹ KIN and 10% coconut water (g-h).

medium significantly improved ABD and explant survival ($p \leq 0.05$). The rise in CaCl₂ concentration to 453 mg L⁻¹ increased ABD up to 76 % and explant survival was 82 %, with significant differences with respect to MgSO₄ (21 % for ABD and 39 % for explant survival) (Appendix 2). The buds developed in the medium modified for Ca concentration demonstrated more vigor, which means health and strength. Leaves showed normal development and leaf-fall and necrosis events decreased (Fig. 3d). Nevertheless, the bottom of the explants in contact with culture media presented oxidation. Activated carbon was used in culture media to avoid this oxidation (1 g L⁻¹).

Table 1. The effect of different plant growth regulators (PGR) (KIN, BAP and 2-ip) on axillary bud development (ABD) and callogenesis (CAL) in *Plukenetia volubilis*.

	Concentration (mg L ⁻¹)	ABD (%)	CAL (cm ²)
KIN	0.1	9.0e	2.53c
	0.2	28.33c	1.07fg
	0.4	42.0a	0.86gh
	0.6	8.33e	2.72c
	0.8	20.0d	1.49ef
2-ip	0.1	8.67e	0.52gh
	0.2	21.0d	0.62gh
	0.4	37.67b	0.47h
	0.6	43.33a	1.53ef
	0.8	18.0d	1.94de
BAP	0.1	0	4.24b
	0.2	0	2.23cd
	0.4	0	381b
	0.6	0	4.04b
	0.8	0	4.84a

Values followed by the same letter and in the same column are not significantly different ($p < 0.05$) using an LSD multiple rank analysis.

Effect of Different PGR's and Coconut Water on ABD and Leaf Formation

The effect of different treatments with different PGR's and coconut water on ABD and leaf formation was significant ($p \leq 0.05$) (Table 2). Ninety-five percent ABD was the best result obtained from culture media supplemented with 0.6 mg L⁻¹ 2-ip and 10 % coconut water (Fig. 3e-f) and 0.4 mg L⁻¹ KIN and 10 % coconut water (Fig. 3g-h), without a significant difference between them. Activated carbon (g L⁻¹) was added to media to counteract oxidation from using coconut water. Moreover, the highest number of leaves was 2.45 and 2.75 leaves, respectively, for the same treatments mentioned above, without a significant difference between them (Table 2). ABD only required 15 days in both treatments.

Table 2. The effect of different culture media supplemented with coconut water on axillary bud development (ABD) and leaf formation in *Plukenetia volubilis*.

Culture Media	ABD (%)	Number of leaves per explant
Control	70b	1.85 b
0.4 mg L ⁻¹ 2-ip	35c	1.15 b
0.4 mg L ⁻¹ KIN	45c	1.7 b
10 % coconut water	75b	1.5 b
10% coconut water + 0.6 mg L ⁻¹ 2-ip	95a	2.45 a
10% coconut water + 0.4 mg L ⁻¹ KIN	95a	2.75 a

The culture media in all treatments were MS modified with 453 mg L⁻¹ CaCl₂ and supplemented with 1 g L⁻¹ activated carbon.

^a Values followed by the same letter and in the same column are not significantly different ($p < 0.05$) using an LSD multiple rank analysis.

Root formation was achieved by removing PGR from the basal culture medium (KIN and 2-ip) for 8 - 15 days. Plantlets were subsequently transferred to *ex vitro* conditions using a fertilized soil substrate. Regenerated plants with at least five internodes and a 5 - 10 cm stem length were transferred into nursery conditions. The plants were washed with tap water to remove excess culture media from roots and transferred to a plot (5L) with sterilized soil. Each plant was completely covered with a bag to avoid dehydration and the bags were punctured two days later to favor gas exchange. Bags were removed on day 5, and a ½ MS culture medium was used for irrigation (data not shown).

DISCUSSION

The highest seed germination results were obtained in full MS basal medium, without seed coats and at 28 °C. Some studies have hypothesized that low nutrient concentrations facilitate the germination of mature zygotic embryos (Hesami et al., 2018). However, this cannot be generalized because each species has specific physiological requirements. Cardoso et al. (2018) only obtained 70 % seed germination in a full MS medium with *P. volubilis*, but did not remove seed coats. Germination took 20 - 25 days. The results obtained in the study are consistent with Kodahl et al. (2018), who attained 100 % germination when the testa was removed. They observed the radicle emerge 7 days after culture, while Restrepo-Osorio et al. (2020) reported 30 days. Nevertheless, we observed radicle extension two days after *in vitro* introduction, not only considering the MS culture medium and the absence of testa, but also keeping seeds at 28 °C in darkness. This result is most likely due to the resemblance to natural environmental conditions. *P. volubilis* grows in dry tropical forests, where an increase in temperature can trigger biochemical responses required for germination. Likewise, according to Azcón and Talón (2008), the type of seed coat can be responsible for controlling impermeability and the movement or imbibition of water. In addition, temperature, soil moisture and light conditions are the most important factors that determine the end of seed dormancy. Also, the absence of a seed coat greatly favors germination, and metabolic activity is immediately reactivated. These culture conditions (MS, without testa, 28 °C), which allowed obtaining 91.6 % germination, are recommended for improving the propagation process of *P. volubilis* through seeds.

The positive results obtained for ABD with 2-ip (0.8 mg L⁻¹) and KIN (0.4 mg L⁻¹) show that cytokinins must be added to media to stimulate axillary bud development in *P. volubilis*. It has been widely reported that axillary bud proliferation in *in vitro* cultures is achieved by supplementing media with cytokinin to avoid apical dominance and stimulate bud sprouting (Gupta et al., 2020). Furthermore, it was observed that low concentrations of cytokinins promote sprouting and decrease the formation of calli. These results

may be attributed to interactions between exogenous plant growth regulators and the endogenous contents of other phytohormones in plant tissue (Almeida et al., 2012).

Several researchers have reported using BAP and IBA for leading shoots from stem segments in *P. volubilis*. Bordignon et al., (2012) used explants hypocotyl segments. After nine weeks of *in vitro* culture with 0.5 mg L⁻¹ BAP or 1.0 mg L⁻¹ BAP + 0.1 mg L⁻¹ IBA, they obtained shoot induction. In addition, Patthanajuck and Bunnag (2017) obtained shoot induction with hypocotyl segments using 1 mg L⁻¹ BAP after 4 weeks. Furthermore, Thuy and Nhung (2019) regenerated shoots with 1.0 mg L⁻¹ BAP + 0.1 mg L⁻¹ IBA after 4 weeks using segments between the first and second leaves considered mature leaves. On the other hand, ABD was obtained in the study without any of the BAP concentrations, but there was callus formation with BAP. This is like the mentioned authors' results. ABD induction from nodal segments using 2-ip (0.8 mg L⁻¹) and KIN (0.4 mg L⁻¹) without callus formation was addressed for the first time in this study. Conversely, other works have used the apical meristem as an explant for shoot induction. Viegas Rodrigues et al. (2014) used 1.0 mg L⁻¹ BAP for six weeks of culture and Solis et al. (2018) used 0.1 mg L⁻¹ BAP and 0.05 mg L⁻¹ NAA for sprouting 14 days afterwards. This last 14-day response time was similar for this study, where axillary development was observed at 15 days.

The increase in Ca concentration, which changed from 332.2 mg L⁻¹ to 453 mg L⁻¹ CaCl₂, favored axillary bud development and prevented chlorosis in young leaves and subsequent leaf-fall. Several studies reported that high calcium concentrations prevented sprout tip necrosis without affecting growth and regeneration (Bairu et al., 2009). Tuteja and Mahajan (2007) propose two possible hypotheses for avoiding chlorosis and young leaf abscission: the first is that a deficiency in calcium affects cell division, and the mitotic index is high in these tissues. Secondly, the middle lamella is formed between two daughter cells, which is primarily calcium pectate, and a deficiency can alter its formation. Furthermore, it has been demonstrated that calcium is required as a second messenger for some hormones and environmental responses to function (Xu et al., 2009).

In this work, ABD response was optimized by adding coconut water (10 %). This result indicates *P. volubilis* may require additional complementary components to successfully develop axillary buds. Adding organic compounds to basal culture media is an alternative used routinely for the micropropagation of various species (Rai 2019; Agustini et al., 2020; Sembiring et al., 2020). The effect of coconut water on tissue culture mainly occurs due to the high complexity and content of sugars, minerals, vitamins, amino acids, enzymes, volatile aromatic compounds and other biochemical compounds (Burns et al., 2020).

The effect of coconut water as a growth factor on the micropropagation process occurs due to the singular or synergetic effect of its components, such as auxins and

cytokinins. Furthermore, some studies reported that medium supplementation with organic compounds increases *ex vitro* survival, which is one of the most challenging steps after *in vitro* culture (Moreno Martínez and Menchaca García, 2007). However, oxidation at the base of explants was a recurring problem when using coconut water in culture media. It was overcome using activated carbon (data not shown). Activated carbon is mainly used to control oxidation problems associated with tissue culture, such as the release of phenolic compounds that induce oxidation and tissue death. Therefore, activated carbon absorbs chemical substances and prevents them from interacting with tissue (Thomas, 2008).

CONCLUSIONS

In conclusion, the protocol developed in this study was successful and facilitated the *in vitro* germination of *P. volubilis* seeds. High percentages of *in vitro* seed germination were achieved by removing their coats, using MS culture media and keeping them at 28 °C for 2-3 days. The response in germination time improved compared to other works, shortening the seedlings' production times. These results could help overcome seed dormancy and expedite the germination process when rapid multiplication is desired. Furthermore, the protocol developed for the micropropagation of *P. volubilis* plantlets is feasible through axillary bud induction of nodal explants and represents a significant improvement in differentiating axillary buds, developing shoots and forming leaves on the plantlets, with respect to the previously reported works. The best axillary bud development was obtained when the culture medium was supplemented with 453 mg L⁻¹ CaCl₂, 0.6 mg L⁻¹ 2-ip or 0.4 mg L⁻¹ KIN, 10 % coconut water for 15 days of culture. This work contributes to improving the efficiency of the propagation of *P. volubilis* to improve its propagation and complement traditional vegetative propagation methods in nurseries. With a perspective towards the future, studying the genetic stability of micropropagated plantlets and examining the role parameters, such as age, cultivar genotype, liquid media and the concentrations of plant growth regulators play in regeneration efficiency in detail is proposed.

ACKNOWLEDGEMENTS

This work was supported by the Committee for Research Development of Universidad de Antioquia (CODI). A special thanks to Esther Julia Naranjo from the Plant Tissue Culture Laboratory of Universidad de Antioquia for her scientific advice and Colombiana de Biocombustibles S.A. and Carlos Andrés Palacio Lopera, who generously supplied the seeds for these experiments.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

- Agustini, V., Rahayu, I., Numberi, L., and Mah, Z. (2020). The role of chitosan as a growth driver for orchid culture *Dendrobium lasianthera*. J.J.Sm. *in vitro. Jurnal Biologi Papua*, 12(1), 43–49. <https://doi.org/10.31957/jbp.1096>
- Almeida, M., Almeida, C. V., Graner, E., Brondani, G. E., and Abreu-Tarazi, M. F. (2012). Pre-procambial cells are niches for pluripotent and totipotent stem-like cells for organogenesis and somatic embryogenesis in the peach palm: A histological study. *Plant Cell Reports*, 31(8), 1495–1515. <https://doi.org/10.1007/s00299-012-1264-6>
- Azcón, J., and Talón, M. (2008). *Plant Physiology Fundamentals*. Editorial McGraw-Hill Interamericana.
- Bairu, M. W., Jain, N., Stirk, W. A., Doležal, K., and Van Staden, J. (2009). Solving the problem of shoot-tip necrosis in *Harpagophytum procumbens* by changing the cytokinin types, calcium and boron concentrations in the medium. *South African Journal of Botany*, 75(1), 122–127. <https://doi.org/10.1016/j.sajb.2008.08.006>
- Bordignon, S. R., Bovi Ambrosano, M. G., and Viegas Rodrigues, P. H. (2012). *In vitro* propagation of Sacha inchi. *Ciência Rural*, 42(7), 1168–1172. <https://doi.org/10.1590/S0103-84782012005000049>
- Burns, D. T., Johnston, E. -L., and Walker, M. J. (2020). Authenticity and the Potability of Coconut Water - a Critical Review. *Journal of AOAC International*, 103(3), 1-7. <https://doi.org/10.1093/jaoacint/qs008>
- Cachique, D. H., Solsol, H. R., García Sanchez, M. A., Arévalo López, L. A., and Kodahl, N. (2018). Vegetative propagation of the underutilized oilseed crop sachá inchi (*Plukenetia volubilis* L.). *Genetic Resources and Crop Evolution*, 65(7), 2027–2036. <https://doi.org/10.1007/s10722-018-0659-9>
- Cardoso, A. A. S., Lopes, M. T. G., Valente, M. S. F., Quisen, R. C., and Chaves, F. C. M. (2018). Seed morphometry, *in vitro* germination and vegetative propagation in sachá inchi (*Plukenetia volubilis* L.). *Revista Brasileira de Ciências Agrárias*, 13(3), 1–7. <https://doi.org/10.5039/agraria.v13i3a5561>
- Chirinos, R., Necochea, O., Pedreschi, R., and Campos, D. (2016). Sachá inchi (*Plukenetia volubilis* L.) shell: An alternative source of phenolic compounds and antioxidants. *International Journal of Food Science & Technology*, 51(4), 986–993. <https://doi.org/10.1111/ijfs.13049>
- Dong, Y., Chen, M., Wang, X., Niu, L., Fu, Q., and Xu, Z. (2016). Establishment of *in vitro* regeneration system of woody oil crop *Plukenetia volubilis*. *Molecular Plant Breed*, 14(2), 462–470.
- Gupta, S., Kachhwaha, S., Lal Kothari, S., and Jain, R. (2020). Synergistic effect of cytokinins and auxins enables mass clonal multiplication of drumstick tree (*Moringa oleifera* Lam.): a wonder. *In Vitro Cellular & Developmental Biology - Plant*, 56, 458–469. <https://doi.org/10.1007/s11627-020-10065-0>
- Hesami, M., Daneshvar, M. H., and Yoosefzadeh-Najafabadi, M. (2018). Establishment of a protocol for *in vitro* seed germination and callus formation of *Ficus religiosa* L., an important medicinal plant. *Jundishapur Journal of Natural Pharmaceutical Products*, 13(4), 1–8. <https://doi.org/10.5812/jjnpp.62682>
- Kao, K., and Michayluk, M. (1975). Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. *Planta*, 126(2), 105–110. <https://doi.org/10.1007/BF00380613>
- Kerdiles, O., Layé, S., and Calon, F. (2017). Omega-3 polyunsaturated fatty acids and brain health: Preclinical evidence for the prevention of neurodegenerative diseases. *Trends in Food Science & Technology*, 69(Part B), 203–213. <https://doi.org/10.1016/j.tifs.2017.09.003>
- Kodahl, N. (2020). Sachá inchi (*Plukenetia volubilis* L.) from lost crop of the Incas to part of the solution to global challenges. *Planta*, 251(4), 1–22. <https://doi.org/10.1007/s00425-020-03377-3>
- Kodahl, N., García-Dávila, C. R., Cachique, D., Sørensen, M., and Lütken, H. (2018). An *in vitro* seed germination protocol for *Plukenetia volubilis* L. *ISHS Acta Horticulturae*, 1201, 549–554. <https://doi.org/10.17660/ActaHortic.2018.1201.73>
- Maurer, N. E., Hatta-Sakoda, B., Pascual-Chagman, G., and Rodriguez-Saona, L. E. (2012). Characterization and authentication of a novel vegetable source of omega-3 fatty acids, sachá inchi (*Plukenetia volubilis* L.) oil. *Food Chemistry*, 134(2), 1173–1180. <https://doi.org/10.1016/j.foodchem.2012.02.143>
- Millones, C. E., and Vásquez, E. R. (2008). Micropropagación de plantas de sachá inchi (*Plukenetia volubilis* L.) provenientes de la provincia de Rodríguez de Mendoza, Región Amazonas. *Investigaciones Amazonenses*, 2, 7–11.
- Moreno Martínez, D., and Menchaca García, R. A. (2007). Efecto de los compuestos orgánicos en la propagación *in vitro* de *Stanhopea Tigrina* Bateman (Orchidaceae). *Foresta Veracruzana*, 9(2), 27–32.
- Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, 15(3), 473–497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
- Patthanajuck, V., and Bunnag, S. (2017). Effect of plant growth regulators on shoot induction of sachá inchi (*Plukenetia volubilis* L.) *in vitro* (pp. 494–500). NIGRC KKU-2017.
- Rai, I. N., Sudana, I. M., Astawa, I. N. G., Dwiyani, R., and Fitriani, Y. (2019). Direct organogenesis *in vitro* propagation of local balinese banana with thidiazuron. *International Journal of Life Sciences*, 3(3), 32–40. <https://doi.org/10.29332/ijls.v3n3.360>
- Restrepo-Osorio, C., Gil-Correal, A., Chamorro-Gutiérrez, L., Ramírez-Ríos, V., Álvarez, J. C., and Villanueva-Mejía, D. (2020). Efficient direct shoot organogenesis and genetic stability in micropropagated sachá inchi (*Plukenetia volubilis* L.). *BMC Research Notes*, 13(1), 1–7. <https://doi.org/10.1186/s13104-020-05257-1>

- de la Rosa, R., and Quijada, J. (2013). Germination of Sacha Inchi, *Plukenetia Volubilis* L. (Mcbride, 1951) (Malpighiales, Euphorbiaceae) Under Four Different Conditions. *The Biologist*, 11(1), 9–14.
- Sathe, S. K, Kshirsagar, H. H, and Sharma, G. M. (2012). Solubilization, Fractionation, and electrophoretic characterization of inca peanut (*Plukenetia volubilis* L.) Proteins. *Plant Foods for Human Nutrition*, 67(3), 247–255. <https://doi.org/10.1007/s11130-012-0301-5>
- Sembiring, R., Hayati, M., and Kesumawati, E. (2020). Formation of potato micro tubers (*Solanum tuberosum* L.) by using BAP and coconut water in the *in vitro* culture. *IOP Conference Series: Earth and Environmental Science*, 425(1), 012072. <https://doi.org/10.1088/1755-1315/425/1/012072>
- Solis, R., Cachique, D., Guerrero-Abab, J., Ruiz Sánchez, M. E, and Tapiay Figueroa, L. (2018). *In vitro* propagation of sachá inchi through organogenesis. *Pesquisa Agropecuária Brasileira*, 53(3), 1285–1288. <https://doi.org/10.1590/s0100-204x2018001100012>
- Solis, R., Gonzales, N., Pezo, M., Arévalo, L., and Vallejos-Torres, G. (2019). Rooting of sachá inchi (*Plukenetia volubilis*) juvenile cuttings in microtunnels. *Acta Agronómica*, 68(11), 35–40. <https://doi.org/10.15446/acag.v68n1.72101>
- Solis, R., Pezo, M., Diaz, G., Arévalo, L., and Cachique, D. (2016). Vegetative propagation of *Plukenetia polyadenia* by cuttings: effects of leaf area and indole-3-butyric acid concentration. *Brazilian Journal of Biology*, 77(1), 580–584. <https://doi.org/10.1590/1519-6984.20415>
- Tenenbaum, A., and Fisman, E. Z. (2018). Omega-3 polyunsaturated fatty acids supplementation in patients with diabetes and cardiovascular disease risk: Does dose really matter? *Cardiovascular Diabetology*, 17(1), 1–3. <https://doi.org/10.1186/s12933-018-0766-0>
- Thomas, T. D. (2008). The role of activated charcoal in plant tissue culture. *Biotechnology Advances*, 26(6), 618–631. <https://doi.org/10.1016/j.biotechadv.2008.08.003>
- Thuy, L., and Nhung, N. (2019). Study on the suitable mediums for *in vitro* culture the stem segment of sachá inchi (*Plukenetia volubilis* L.) plant. *Naresuan University Journal: Science and Technology*, 207(14), 121–128.
- Tropicos.org. (4 de marzo de 2020). *Legacy Names. Missouri Bot. Gard. 2020.* <http://legacy.tropicos.org/Name/40012207?tab=subordinatetaxa>
- Tuteja, N., and Mahajan, S. (2007). Calcium Signaling Network in Plants. *Plant Signaling & Behavior*, 2(2), 79–85. <https://doi.org/10.4161/psb.2.2.4176>
- Viegas Rodrigues, P., Bordignon, S. B., and Bovi Ambrosano, G. (2014). Horticultural performance of *in vitro* propagated plants of Sachá inchi. *Ciência Rural, Santa Maria*, 44(6), 1050–1053. <https://doi.org/10.1590/S0103-84782014000600016>.
- Wang, S., Zhu, F., and Kakuda, Y. (2018). Sachá inchi (*Plukenetia volubilis* L.): Nutritional composition, biological activity, and uses. *Food Chemistry*, 265, 316–328. <https://doi.org/10.1016/j.foodchem.2018.05.055>
- Watanabe, Y., and Tatsuno, I. (2017). Omega-3 polyunsaturated fatty acids for cardiovascular diseases: present, past and future. *Expert Review of Clinical Pharmacology*, 10(8), 865–873. <https://doi.org/10.1080/17512433.2017.1333902>
- White, P, R. (1964). The cultivation of animal and plant cells. *Soil Science*, 97(1), 74. <https://doi.org/10.1097/00010694-196401000-00013>
- Xu, T., Li, T., and Qi, M. (2009). Analysis of calcium content, hormones, and degrading enzymes in tomato pedicel explants during calcium-inhibited abscission. *Agricultural Sciences in China*, 8(5), 556–563. [https://doi.org/10.1016/S1671-2927\(08\)60246-1](https://doi.org/10.1016/S1671-2927(08)60246-1)