

## IN VITRO INDUCTION OF MICRORRHIZOMES AND MICROTUBERS AS A PROPAGATION STRATEGY FOR *COSMOS ATROSANGUINEUS* (HOOK.) VOSS

MIGUEL ÁNGEL VERÁSTEGUI-VIDAL<sup>1,2,6</sup>, ROBERT BYE BOETTLER<sup>3</sup>, CÉSAR MATEO FLORES-ORTIZ<sup>4</sup>, YOLANDA POZOS-RUIZ<sup>2</sup>,  
 MARÍA TERESA ORTIZ-MELO<sup>5</sup>, JUAN GERARDO ORTIZ-MONTIEL<sup>2\*</sup>, AND VÍCTOR MANUEL CHÁVEZ-ÁVILA<sup>1\*</sup>

<sup>1</sup> Laboratorio de Cultivo de Tejidos Vegetales, Jardín Botánico del Instituto de Biología, Universidad Nacional Autónoma de México, Ciudad Universitaria, Mexico City, Mexico.

<sup>2</sup> Laboratorio de Cultivo de Tejidos Vegetales, Unidad de Morfología y Función, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Tlalnepantla, Estado de México, Mexico.

<sup>3</sup> Laboratorio de Etnobotánica, Jardín Botánico del Instituto de Biología, Universidad Nacional Autónoma de México, Ciudad Universitaria Coyoacán, Mexico City, Mexico.

<sup>4</sup> Laboratorio de Biogeoquímica, Unidad de Biología, Tecnología y Prototipos, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Tlalnepantla, Estado de México, Mexico.

<sup>5</sup> Unidad de Investigación en Biomedicina, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Tlalnepantla, Estado de México, Mexico.

<sup>6</sup> Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Ciudad Universitaria, Mexico City, Mexico.

\*Corresponding authors: [jgerardo@unam.mx](mailto:jgerardo@unam.mx); [victorm.chavez@ib.unam.mx](mailto:victorm.chavez@ib.unam.mx)

### Abstract

**Background:** *Cosmos atrosanguineus* (Asteraceae) is a perennial herbaceous plant with tuberous rhizomes endemic to Mexico. It is sterile and does not produce seeds, requiring alternative propagation methods.

**Questions:** Can *in vitro* techniques be used to develop microrrhizomes and microtubers as propagules for *Cosmos atrosanguineus*?

**Studied species:** The study focused on *C. atrosanguineus* explants cultured under controlled *in vitro* conditions, with emphasis on rhizome and tuber development.

**Study site:** Experiments were conducted *in vitro* under laboratory conditions.

**Methods:** Culture media were supplemented with auxins, particularly 1-Naphthaleneacetic Acid (NAA), and with varying concentrations of sucrose. Morphological, anatomical, and biochemical analyses were performed to confirm tuberization. Additionally, transpiration rates were compared between acclimatized plantlets and those regenerated from *in vitro* microrrhizomes.

**Results:** Supplementation with 1 mg/L NAA promoted microrrhizome development, while 12 % sucrose stimulated microtuber induction. Anatomical characterization of tuberized microrrhizomes confirmed their similarity to the naturally tuberizing species *Cosmos pringlei*. Plantlets derived from microrrhizomes successfully acclimatized *ex vitro*, showing functional transpiration.

**Conclusions:** The *in vitro* generation of microrrhizomes and microtubers represents a viable propagation strategy for *C. atrosanguineus*. These propagules are suitable for *ex vitro* establishment, offering an effective alternative for conserving and propagating this endemic sterile species.

**Keywords:** *In vitro* propagation, microrrhizomes, microtubers, 1-Naphthaleneacetic acid (NAA), endemic species conservation.

### Resumen:

**Antecedentes:** *Cosmos atrosanguineus* (Asteraceae) es una planta herbácea perenne con rizomas tuberosos endémica de México. Es estéril y no produce semillas, por lo que requiere métodos alternativos de propagación.

**Pregunta:** ¿Pueden emplearse técnicas *in vitro* para desarrollar microrrizomas y microtubérculos como propágulos de *C. atrosanguineus*?

**Especie de estudio:** El estudio se centró en explantes de *C. atrosanguineus* cultivados en condiciones *in vitro* controladas, con énfasis en el desarrollo de rizomas y tubérculos.

**Sitio de estudio:** Los experimentos se llevaron a cabo en condiciones de laboratorio *in vitro*.

**Métodos:** Los medios de cultivo se suplementaron con auxinas, en particular con ácido 1-naftalenacético (ANA), y con diferentes concentraciones de sacarosa. Se realizaron análisis morfológicos, anatómicos y bioquímicos para confirmar la tuberización. Además, se compararon las tasas de transpiración entre plántulas aclimatizadas y aquellas regeneradas a partir de microrrizomas *in vitro*.

**Resultados:** La adición de 1 mg/L de ANA promovió el desarrollo de microrrizomas, mientras que el 12 % de sacarosa estimuló la inducción de microtubérculos. La caracterización anatómica de los microrrizomas tuberizados confirmó su similitud con la especie naturalmente tuberizante *Cosmos pringlei*. Las plántulas derivadas de microrrizomas se aclimatizaron exitosamente *ex vitro*, mostrando transpiración funcional.

**Conclusiones:** La generación *in vitro* de microrrizomas y microtubérculos constituye una estrategia viable de propagación para *C. atrosanguineus*. Estos propágulos son adecuados para el establecimiento *ex vitro*, ofreciendo una alternativa efectiva para la conservación y multiplicación de esta especie endémica y estéril.

**Palabras clave:** Propagación *in vitro*, microrrizomas, microtubérculos, ácido 1-Naftalenacético (ANA), conservación de especies endémicas.

This is an open access article distributed under the terms of the Creative Commons Attribution License CCBY-NC (4.0) international.

<https://creativecommons.org/licenses/by-nc/4.0/>



**C***osmos atrosanguineus* (Hook.) Voss (Asteraceae) is a perennial subscapose herb with simple and pinnatifid leaves, a peduncle 10 to 45 cm long and a capitulum measuring 4 to 4.5 cm in diameter and consisting of 8 ray florets dark purple-violet to reddish-brown in color and 30 to 50 tubular disc florets of similar color (Castro-Castro *et al.* 2014). A notable characteristic is the chocolate-like aroma emitted by inflorescences. The rhizomes form tubers approximately 3 cm long and 1.5 to 3 cm in diameter, generally narrowing until they develop into a napiform taproot (Hind & Fay 2003, Castro-Castro *et al.* 2014).

This species is endemic to Mexico and was first described in 1861 by William Jackson Hooker as variety of *Cosmos diversifolius* Otto ex Knowles & Westc, based on plants cultivated from seeds sent earlier from Mexico (Hooker 1861), possibly from the state of Hidalgo (Ortgies 1861). It was subsequently circulated internationally as an ornamental plant. Herbarium collections from wild populations were obtained later from the states of Guanajuato, Hidalgo, Querétaro, and San Luis Potosí (Castro-Castro *et al.* 2014). The plants grown in gardens throughout the world are sterile and fail to produce viable achenes. Consequently, it must be propagated *in vitro* using tissue culture techniques (Wilkinson *et al.* 2003) or by *in vivo* division of tuberous roots, a very slow propagation method (Rice 2017). A clone preserved *in vitro* at the Royal Botanic Gardens, Kew has been distributed (Oku *et al.* 2008; Rice 2017) and was obtained by the Laboratorio de Cultivos de Tejidos Vegetales, Jardín Botánico, Instituto de Biología, UNAM (Ortega-Larrocea *et al.* 1997; Hind & Fay 2003). However, in Mexico, there are no reports from wild living populations of this plant, and the only data available on this species are from floristic lists based on herbarium specimens (Vargas-Amado *et al.* 2013). Meanwhile, the international market offers hybrid varieties that were developed in the 1990s. It is unknown whether those varieties are genetically related to the original material held at the Royal Botanic Garden of Kew (Rice 2017). *Cosmos atrosanguineus* has been micropropagated (Kozak 1991, Ortega-Larrocea *et al.* 1997, Wilkinson *et al.* 1998, Kozak *et al.* 2013), and the success of this practice depends significantly on the type of explant chosen, by subculture of stem sections with nodes or by dividing axillary shoots (Hosoki *et al.* 2003). The placement of explants in culture medium appears to be important (Orlikowska *et al.* 2000, Debnath 2005, Rajeswari & Palival 2008). Their horizontal or vertical placement in an inverted position, with the tip of the shoot facing down, increased the number of axillary shoots (Ziv *et al.* 1970, Seabrook *et al.* 1976, Kozak 1991, Orlikowska *et al.* 2000).

Numerous plant species have demonstrated that micropropagation is achievable through the propagation of not only entire plants but also structures that facilitate propagule production, such as the induction of microrhizome tubers (Nayak 2000, Labrooy *et al.* 2020, Shuangying *et al.* 2024). Tuberized microrhizomes offer several advantages, as these structures can be sprouted, stored, transported, or utilized in germplasm conservation efforts (Brijesh & Ajjappala 2023).

*In vitro*-formed tuberous microrhizomes and microtubers are modified stems or storage organs analogous to rhizomes and tubers produced *in vivo*, capable of sprouting and developing into a complete plant (Zahid *et al.* 2021). In the *in vitro* culture of *Alstroemeria*, the induction, development, and multiplication of rhizomes are enhanced following exposure to moderate abiotic stresses, including heat, cold, drought, anaerobiosis, or salinity (Pumisutapon *et al.* 2012). In the formation of both organs, a high concentration of sucrose has been identified as the primary metabolic signal for the induction of microrhizomes and tubers *in vitro* (Pelacho & Mingo-Castel 1991, Zahid *et al.* 2021).

Strategies for the induction of tuberized microrhizomes *in vitro* have been associated with the effect of sucrose at concentrations ranging from 6.0 to 9.0 % (Nayak 2000), as well as the addition of plant growth regulators (PGRs) to the culture medium, such as 6-benzylaminopurine (BAP), 1-naphthaleneacetic acid (NAA), or indole acetic acid (IAA) (Rout *et al.* 2001), and photoperiod (Shirgurkar *et al.* 2001).

The objective of this study was to evaluate the synergistic effect of different auxins and sucrose concentrations on the induction and development of tuberized microrhizomes in *C. atrosanguineus*. This was aimed at establishing a highly efficient *in vitro* propagation system and providing a viable structure for the long-term conservation of this sterile endemic species.

## Materials and methods

**Biological material source.** For this research, plants of *C. atrosanguineus* from the in vitro collection of the Jardín Botánico, Instituto de Biología, UNAM, were used. This material was transferred to the Laboratorio de Cultivo de Tejidos Vegetales, Unidad de Morfología y Función, Facultad de Estudios Superiores Iztacala, UNAM, to carry out the respective assays.

**In vitro microrhizome induction.** Binodal cuttings were obtained from *C. atrosanguineus* plants previously propagated *in vitro* and used as explants; they were cultured in MS medium (Murashige & Skoog 1962) containing 30 g/L sucrose, 0.1 mg/L thiamine, and 100 mg/L myo-inositol. The culture media were independently supplemented with the following PGRs: 1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), and indole-3-acetic acid (IAA), at concentrations of 0.0, 1.0, and 2.0 mg/L. Culture medium without PGRs was used in the control group. All culture media were adjusted to pH 5.7 prior to adding 3.0 g/L Phytigel™ (3.0 g/L) and were sterilized in an autoclave for 15 minutes at a pressure of 1.05 kg/cm<sup>2</sup>. Cultivation conditions consisted of a photoperiod of 16 h/8 h (light/dark), light of 135 μmol m<sup>-2</sup> s<sup>-1</sup>, and temperature of 26 ± 2 °C. After the explants were incubated for 25 days under these conditions and harvested the relative rhizome production index (RRPI) was calculated using the following equation (Medina *et al.* 2007):

$$RRPI = \frac{\left( \frac{\% \text{ Explants}}{w/\text{microrhizomes}} \right) \left( \frac{\text{Microrhizomes dry weight}}{\text{explant}} \right) \left( \frac{\text{Microrhizomes number}}{\text{explant}} \right)}{100}$$

**In vitro tuberization of microrhizomes.** After 25 days of growing in the microrhizome inducer media, the plantlets from the group supplemented with 1 mg/L NAA were selected based on their morphology. These plantlets were transplanted to two different media: 1) MS media without PGR and with the sucrose concentrations modified to 0.0, 3.0, 6.0, and 12.0 %. 2) MS media without sucrose but with equivalent molar concentrations (0.087, 0.175, 0.263, 0.350 M) of mannitol to evaluate morphogenesis in culture. To assess a possible osmolar effect. The gelling agent procedures and environmental conditions remained consistent with those previously described.

The evaluations of these treatments were conducted after 20 days, and the total soluble sugar content in leaves, stems, and microrhizomes was quantified using the phenol-sulfuric acid method (Dubois *et al.* 1951). To perform this quantification, the fresh weights of the plantlets' leaves, stems, and microrhizomes were individually measured. These samples were then macerated with 1 ml of distilled water, and 250 μl of the sample were mixed with 250 μl of 5 % phenol. Finally, 1.25 mL of 96 % sulfuric acid was added. Following a 30 min incubation period at room temperature, the samples were read in a Genesys 10S UV/V (Thermo Scientific, CA, USA) spectrophotometer at 485 nm.

**Acclimatization.** Plantlets that developed microrhizomes (obtained from the group added with 1 mg/L of NAA for 25 days) were transferred to containers with sterile agrolite and covered with a transparent plastic lid. Irrigation was carried out using MS inorganic salts (pH 5.7) and under the cultivation regime with 100 % relative humidity (RH), a photoperiod of 16 h/8 h (light/dark), light of 135 μmol m<sup>-2</sup> s<sup>-1</sup>, and temperature of 26 ± 2 °C. Subsequently, the plastic lid was removed after 30 days, causing the RH to decrease to 26 %, a condition maintained for an additional period of 60 days. Hydric conductance was measured with a porometer (leaf porometer, Decagon Devices, Inc., Model SC-1).

Tuberized microrhizomes (obtained from the group added with 1 mg/L of NAA for 25 days, followed by culture on MS medium without PGR and with 12 % sucrose for 20 days) were transferred to containers with sterile agrolite, under the previously specified conditions, with RH maintained at 26 % and without a transparent plastic cover. The hydric conductance measurement was carried out 30 days after new plantlets sprouted from the tuberized microrhizomes (approximately 7.5 cm in length).

**Anatomy technique.** The anatomical description of the tubers was conducted through the observation of fixed and embedded transverse sections. Fragments of microrrhizomes 1 cm long were placed in FAA (5 ml formaldehyde + 5 ml glacial acetic acid + 90 ml ethyl alcohol) according to Sandoval *et al.* (2005), for two hours and then gradually dehydrated with ethyl alcohol (30, 50, 70, 90, 96 %, and three changes in 100 %); each change was made at three-hour intervals. The embedding consisted of immersing the tissues in solutions of ethyl alcohol/xylol (75/25 %, 50/50 %, 25/75 %, 0/100 %), with changes every 3 h, and then heated in an oven at 56 °C (Sandoval *et al.* 2005). Liquid paraplast was added, doubling the volume. The cross-sections of 35 µm were obtained in a rotary microtome. Sections were attached to slides with Haupt solution (Haupt 1930) and then subjected to a safranin and fast green staining (Johansen 1941).

Wild tubers of *Cosmos pringlei* B.L. Rob. & Fernald from Chihuahua, Mexico, were used as a reference for anatomical study. It belongs to the same generic section *Discopoda* (DC.) Sherff of *Cosmos* as *C. atrosanguineus*. In this case, as the tissues were dry (this condition is how they are stored locally), it was necessary to rehydrate them with a solution of soap and water for two hours. Afterwards they were treated as described above with following changes in the times of the fixation procedure until their inclusion in paraplast: F A A: 48 h, ethyl alcohol changes every 24 h, ethyl alcohol/xylol changes every 24 h, and 20 days in liquid paraplast at 56 °C. Sections were obtained and stained as described previously.

**Statistical analysis.** A descriptive and inferential statistical analysis was performed using SigmaPlot v. 12.0 software; the count data had previously undergone a square root transformation when performing such analysis. A two-way ANOVA was performed for mean comparisons, and Tukey's post hoc tests were conducted when necessary. Statistical significance was accepted when  $P < 0.05$ .

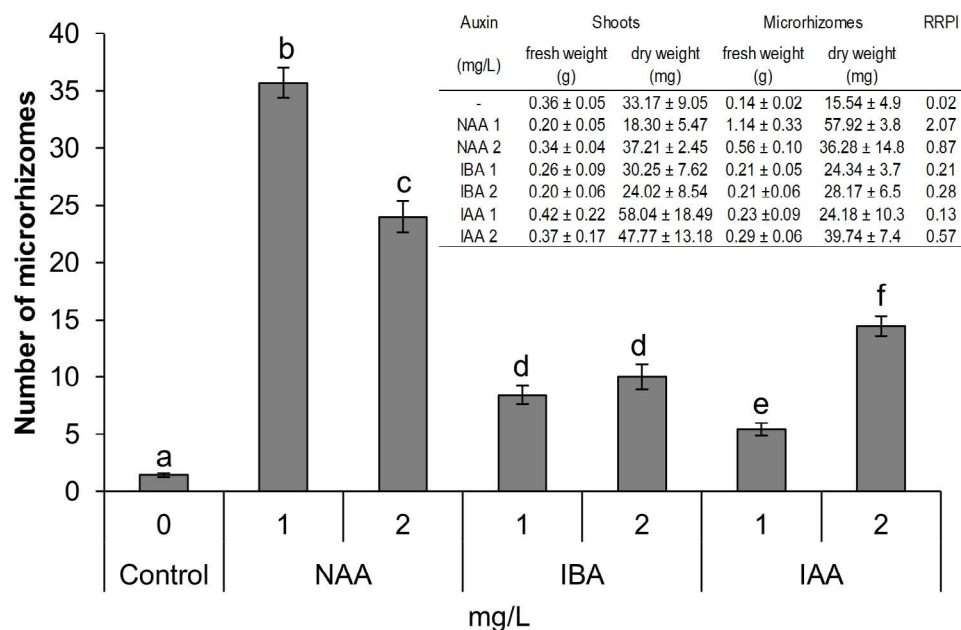
## Results

**In vitro microrrhizome induction.** In *C. atrosanguineus* binodal cuttings, the control group (no auxins added) produced  $1.4 \pm 0.2$  microrrhizomes per explant. In contrast, all the cuttings treated with the auxins presented statistically significant different number of microrrhizomes per explant compared to the control (Figure 1). IAA (1.0 mg/L or 2.0 mg/L) produced  $5.4 \pm 0.5$  and  $14.4 \pm 0.86$  microrrhizomes; IBA (1.0 or 2.0 mg/L) generated  $8.4 \pm 0.8$  and  $10 \pm 1.1$ . The greatest number of microrrhizomes per explant was produced by NAA treatments at 1.0 and 2.0 mg/L yielded  $35.7 \pm 1.3$  and  $24 \pm 1.3$ , respectively. Plantlets treated with 1 mg/L NAA had a lower aerial dry weight ( $18.30 \pm 5.47$  mg) compared to those with 2 mg/L NAA ( $37.21 \pm 2.45$  mg) (Figure 1).

The Rhizome Propagation Response Index (RRPI) for the 1 mg/L NAA treatment was 2.07, whereas all other treatments remained below 0.87 (Figure 1). Furthermore, treatments with the auxins IBA or IAA, and control explants (no auxin added) showed neither thickening in the meristematic tissues nor sprout formation (Figure 2A, B), while explants treated with 1 mg/L of NAA showed more regenerative tissues located in the area that divides the aerial from the underground part, generating multiple sprouts (Figure 2C, D). Therefore, in addition to the presence of several microrrhizomes and the highest RRPI, this treatment was selected for the subsequent tests.

**In vitro tuberization of microrrhizomes.** Tuberized microrrhizomes in media with 12 % sucrose showed a rise in fresh weight (Figure 3) and a significant increase in soluble sugar content in leaves, stems, and microrrhizomes after 20 days. These microrrhizomes increased from  $4.83 \pm 1.19$  mg/g to  $35.42 \pm 6.68$  mg/g of soluble sugar per fresh tissue (Figure 3, upper right chart).

Concentrations of 0, 3, and 6 % sucrose did not significantly affect soluble sugar levels in leaves, stems, or microrrhizomes over a 20-day period, as 12 % sucrose concentration did (Figure 4). The osmotic potentials corresponding to each sucrose concentration were calculated for equivalent treatment with mannitol: 0 g/l = 0 MPa, 30 g/l = -0.21 MPa, 60 g/l = -0.431 g/l and 120 g/l = -0.86 MPa. Mannitol treatments resulted in morphological deterioration (necrosis) and very low sugar concentrations in microrrhizomes with no significant differences between the distinct concentrations (Figure 4).



**Figure 1.** Explants with *in vitro* microrhizomes of *Cosmos atrosanguineus* after 25 days of treatment with auxins (mg/L). Naphthaleneacetic acid (NAA), indolebutyric acid (IBA), and indoleacetic acid (IAA) or control (no auxin addition). The upper right chart shows the Relative Rhizome Production Index. Average of microrhizome and shoot production per fresh and dry weight ( $n = 7$ )  $\pm$  standard error. The bars in the graph represent the average of 7 replicas. Different letters above the  $\pm$  standard error represent significant differences. Data transformed with square root (Tukey  $P < 0.05$ ).

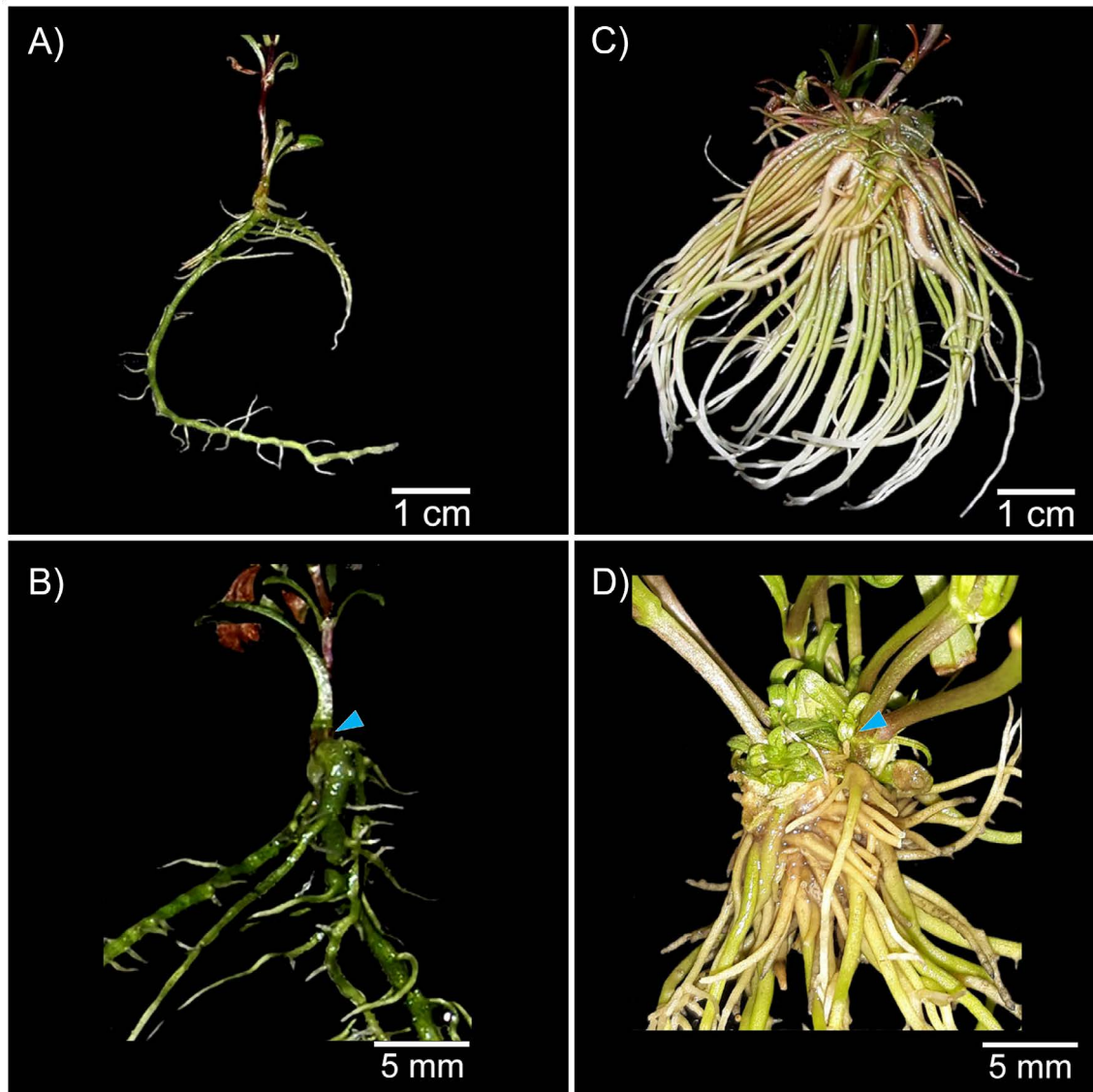
It was observed that 75 % of tuberized microrhizomes developed new shoots, while only 25 % of non-tuberized microrhizomes sprouted new shoots (data not shown).

**Acclimatization.** After thirty days, the plantlets with microrhizomes transpired an average of  $155 \pm 16 \text{ mmol m}^{-2} \text{ s}^{-1} \text{ H}_2\text{O}$ . As the days passed, these values decreased to a mean of  $98 \pm 14 \text{ mmol m}^{-2} \text{ s}^{-1} \text{ H}_2\text{O}$ . While the plantlets that sprouted *ex vitro* from the microtubers diminished their transpiration rate from the beginning until the end of the experiment (90 days), showing an average of  $46 \pm 8 \text{ mmol m}^{-2} \text{ s}^{-1} \text{ H}_2\text{O}$  and  $22 \pm 12 \text{ mmol m}^{-2} \text{ s}^{-1} \text{ H}_2\text{O}$ , respectively (Figure 5).

**Anatomical analysis.** To verify that the structures that we call tuberized microrhizomes were this kind of structure, an anatomical comparison was made with a wild tuber of the same genus, *C. pringlei*, and its description was carried out before comparison (Figure 6A). The central pith with irregular sinuous parenchyma cells and in its periphery the xylem with sparse tracheary elements and abundant parenchyma (Figure 6B). The vessels became more abundant and larger as they moved away from the pith. This xylem was limited by a band of dividing cells corresponding to the vascular cambium, which gave rise to both xylem and phloem (Figure 6C). In the parenchyma, a considerable reduction in the size of the cells was observed close to the epidermis. The parenchyma was interrupted by the presence of radial parenchyma and by xylem-forming rays with very few vessels in the area near the pith; these could be found solitary or in uni- or bi-serial groups. Finally, a very dense area of cortical cells was observed in expansion and division (Figure 6D). Immersed in the cortex, secretory canals of schizogenic origin with varying sizes were observed. In the periphery, a periderm was found, with its respective layers, phellogen, giving rise to phelloderm towards the interior and the phellem towards the outside of the tuber (Figure 6E).

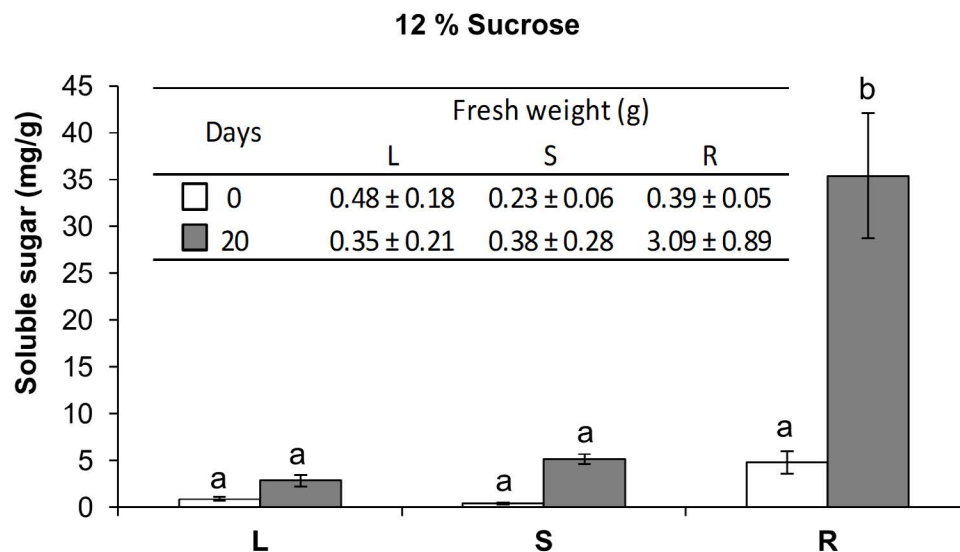
After 40 days in a medium supplemented with 12 % sucrose, histological preparations were made for the anatomical comparison of *C. atrosanguineus* tuberized microrhizomes *in vitro*. The histological preparations



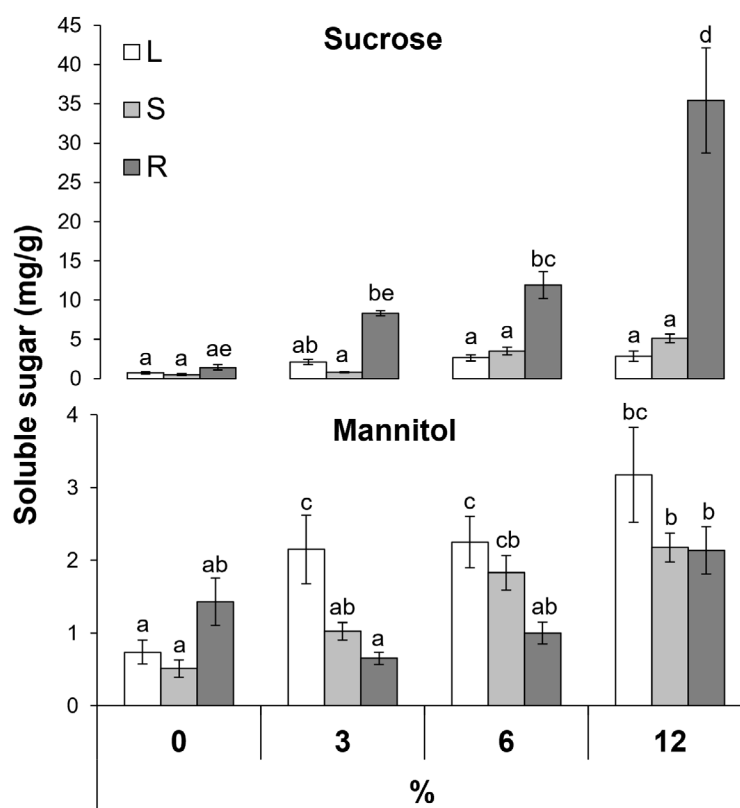


**Figure 2.** Comparison of *Cosmos atrosanguineus* microrhizomes developed in vitro in MS medium, A) and B) control (without added auxins), C) and D) media supplemented with 1 mg/L NAA. The blue triangle shows the meristematic region.

show three different stages of microtuber thickening (Figure 7A, E, I). In the initial stage, as in the wild tuber, the *in vitro* microtubers presented a similar anatomy in the development of small secretory canals of schizogenetic origin. At this stage, the pericycle is not clearly defined, the cells of the cortex remained in division, with only parenchyma cells. The vascular cylinder was narrow with few tracheary elements arranged in radial rows separated by parenchyma; the pith is wide and with thick parenchyma cells (Figure 7A, B, C, D). In the next stage of thickening (Figure 7E), the cortex and the pith continue with active divisions, the vascular cylinder also increases radially and in width with more abundant radial and axial parenchyma beginning to divide. A well-defined cell layer is observed that gives rise to the pericycle; the secretory canals are embedded within this layer (Figure 7E, F, G, H). In the last collected stage of microtuber thickening, few xylem vessels, a pith and a cortex with sinuous cells, elongating and dividing, were observed. Also, more cells adjacent to the secretory canals forming the pericycle could be observed. The periderm was present and the phellogen showed mitotic activity (Figure 7I, J, K, L).



**Figure 3.** Soluble sugar content of *Cosmos atrosanguineus* subjected to treatment with 12 % sucrose. Comparison of the concentration of soluble sugars of leaves (L), stems (S) and microrhizomes (R) on the start (0) and end (20) days of the experiment. Different letters above the  $\pm$  standard error ( $n = 6$ ) represent significant differences (Tukey  $P < 0.05$ ). The upper right chart shows the fresh weight of leaves (L), stems (S), and microrhizomes (R) ( $n = 6$ )  $\pm$  standard error.



**Figure 4.** Comparison of soluble sugar concentrations of *Cosmos atrosanguineus* in leaves (L), stems (S), and microrhizomes (R) after 20 days in the different sucrose and mannitol treatments. Different letters above the  $\pm$  standard error ( $n = 6$ ) represent significant differences (Tukey  $P < 0.05$ ).

## Discussion

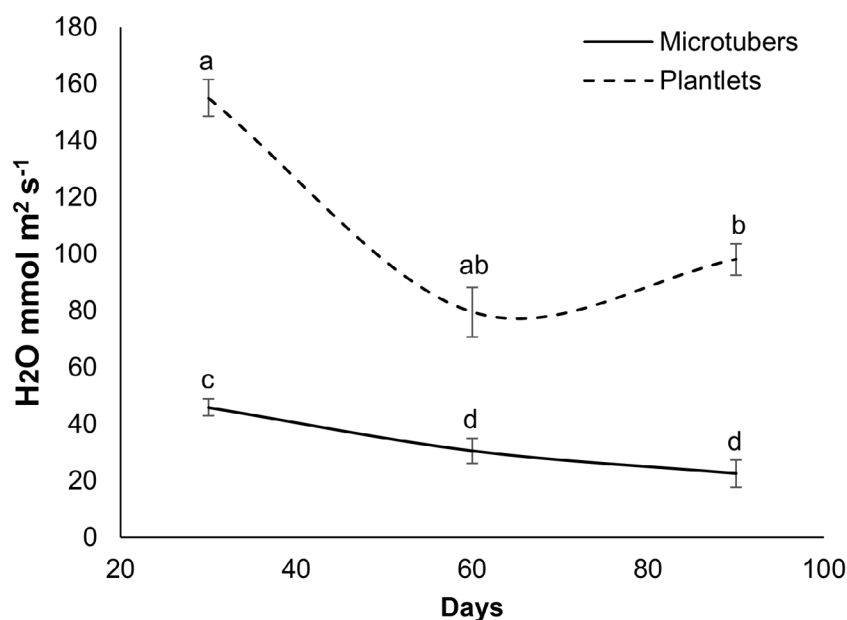
*In vitro* microrhizome induction. Auxin treatments are commonly used to develop supporting organs, such as roots and rhizomes *in vitro*, allowing for even longitudinal growth and thickening, a crucial factor for forming homogeneous tubers (Duan *et al.* 2015, Kondhare *et al.* 2021).

It has been suggested that in tuberous plants such as dahlia, potato or cassava, the investment of metabolic resources in developing the aerial part is higher than in the storage organs which receive less photoassimilates to thicken. Hence, the formation of tubers depends on how the plant distributes its energy and nutrients; therefore, any factor, such as hormones, light, nutrients, or stress, that increases aerial growth can reduce the formation of reserve organs (Medina *et al.* 2007). In this study, *C. atrosanguineus* plantlets treatment with 1 mg/L NAA invested less metabolic resources in the aerial part and more in the formation of microrhizomes.

Moreover, the interaction between NAA and sucrose concentration can be explained by the overexpression of genes related to the synthesis, signaling, and transport of auxins in response to sugar (Ljung *et al.* 2015). High levels of sugar increase the concentration of endogenous auxins in the plant (Sairanen *et al.* 2012). Treatments with higher auxin concentrations accumulate more auxin in the plant, which can negatively impact its development (Kolachevskaya *et al.* 2017). This could also be the reason for the reduction in the number of microrhizomes in the presence of 2 mg/L of NAA. On the other hand, our data do not align with the findings in *Alstroemeria*, where the formation of microrhizomes was observed in a medium with only 0.25 mg/L of NAA (Khaleghi *et al.* 2008).

Furthermore, the RRPI enabled the integration and visualization of variables to select the treatment with 1 mg/L of NAA as the one that generated the most microrhizomes per explant per dry weight, exhibiting an RRPI of 2.07, unlike the other treatments that remained at or below 0.87 (Figure 1). Given that treatment with 1 mg/L NAA produced the greatest number of microrhizomes and highest RRPI, this condition was selected as the optimal microrhizome inducer.

The previous idea aligns with the development of microrhizomes *in vitro* from various rhizomatous plants, where common elements include supplementing the media with phytohormones, such as NAA. For instance, in *Decalepis hamiltonii* Wight & Arn., 1.44 mg/L NAA produced an average of 5 microrhizomes per explant (Thangavel *et al.*

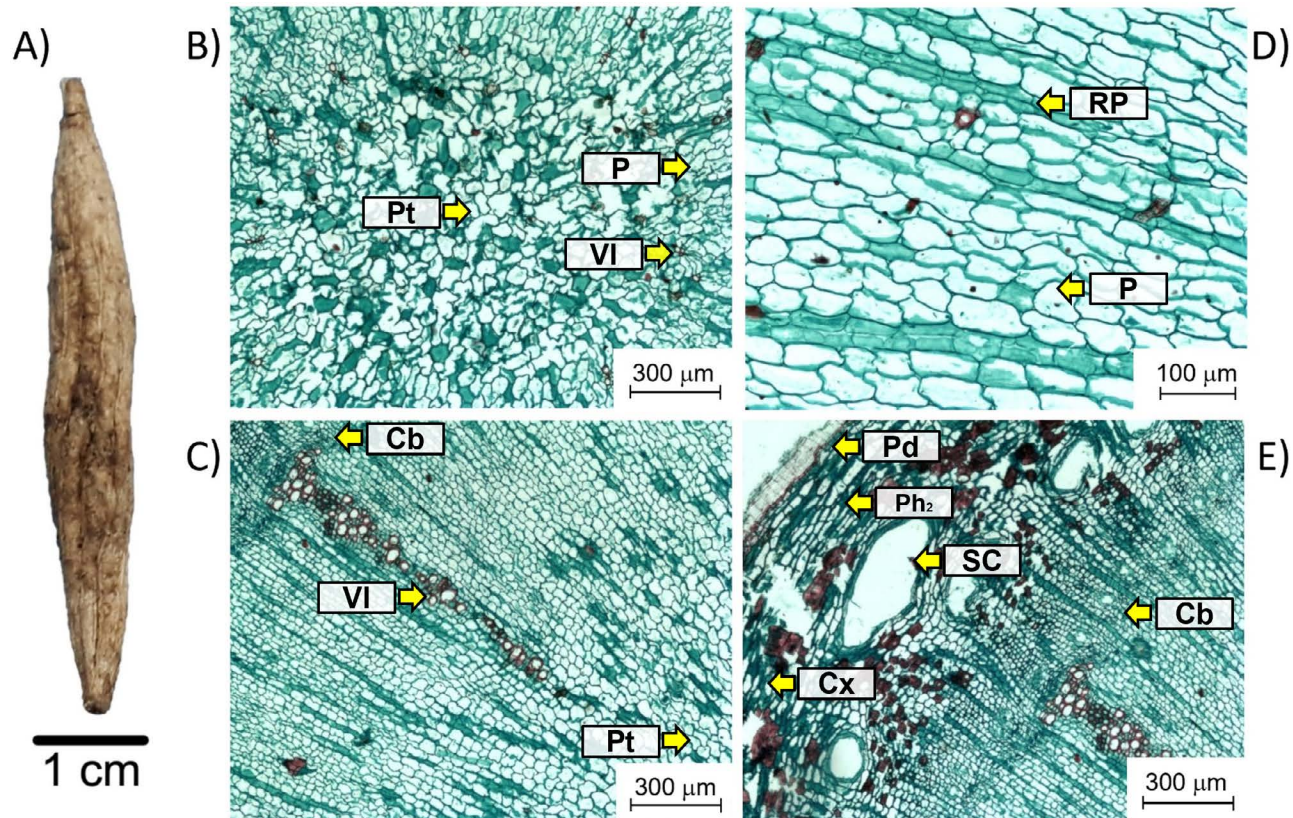


**Figure 5.** Transpiration of plantlets developed *in vitro* (dashed line) and plantlets that sprouted from *Cosmos atrosanguineus* tubers (solid line) during acclimatization (30, 60, and 90 days). Different letters above  $\pm$  standard error ( $n = 6$ ) represent significant differences (Tukey  $P < 0.01$ ).



2014). In other reports, 0.1 mg/L NAA and 0.5 mg/L IBA were used to induce rhizome development in *Zingiber officinale* Roscoe (Sharma & Singh 1995), *Curcuma aromatica* Salisb (Nayak 2000), and *Curcuma longa* L. (Sunitibala *et al.* 2001). Although the species cited belong to evolutionarily distant lineages from *Cosmos*, the comparison is not based on phylogenetic affinities, but on shared principles of developmental regulation. The formation of storage organs and rhizomatous structures in angiosperms is controlled by highly conserved regulatory networks associated with meristematic activity, including genes from the *WOX*, *CLE*, and *KNOX class I* families, as well as regulators of vascular development (Du & Groover 2010). These networks can be activated in a convergent manner during *in vitro*-induced tissue reprogramming, regardless of the phylogenetic origin of the species.

Additionally, the species that generate underground storage organs share physiological and anatomical processes in thickening or filling, having a very similar secondary growth, regulated mainly by cytokinins, auxins and gibberellic acid, as well as by vernalization and photoperiod (Natarajan *et al.* 2019, Kondhare *et al.* 2021), even though the organs have different origins and can be formed from leaves as in the case of garlic (*Allium sativum* L.) (Hong-jiu *et al.* 2020), from taproots such as beets (*Beta vulgaris* L.) (Kerk & Sussex 2012), from lateral roots such as cassava (*Manihot esculenta* Crantz) (Suárez & Mederos 2011), from adventitious roots such as sweet potato (*Ipomoea batatas* L.) (Yang *et al.* 2023), from stems that form stolons such as potatoes (*Solanum tuberosum* L.) (Jing *et al.* 2023), or from rhizomes such as ginger (*Zingiber officinale*) (Chittaragi *et al.* 2022).



**Figure 6.** Cross-sections of wild tuber of *Cosmos pringlei*. A) Wild tuber, B) Pith surrounded by a few vessel elements and parenchyma; C) General view of the secondary xylem, the conductive elements, vessels and few fibers are arranged in fascicles, the vessel elements form radii from the pith to the peridermis, in the upper left it is possible to see the cellular strip that makes up the cambium; D) Approach to secondary growth where parenchymal cells are observed with some solitary vessels immersed and radial parenchyma; E) Section with a large number of secretory canals of schizogenic origin of various sizes, in the upper left part the secondary phloem and the different layers that form the peridermis can be seen. Pt: pith; P: parenchyma; VI: vessels; Cb: cambium; RP: radial parenchyma; Cx: cortex; Ph<sub>2</sub>: secondary phloem; Pd: periderm; SC: secretory canals.

However, one of the essential characteristics that must be considered when using one of these organs as a propagule is the location of the meristem and dormancy capacity. While there are underground storage organs that present meristems throughout the body, others can be located at the base of each tuber, as is the case with dahlias, which have the ability to keep their buds in a latent state (Jiménez 2015). Some Asteraceae, such as *Smallanthus* (yacon), have a meristematic zone located at the base that joins the aerial part of the plant to the underground part, and forms structures known as rhizophores that may quickly lose their viability (Seminario *et al.* 2003). Nonetheless, they are used as propagules since they form new sprouts continuously (Ferreira *et al.* 2020a, b, Quaresma *et al.* 2020). In the case of *C. atrosanguineus*, explants treated with 1 mg/L of NAA showed more regenerative tissues located in the area that divides the aerial from the underground part, generating multiple sprouts (Figure 2C, D), in contrast, treatments with the auxins IBA or IAA, and control explants (no auxin added) showed neither thickening in the meristematic tissues nor sprout formation (Figure 2A, B).

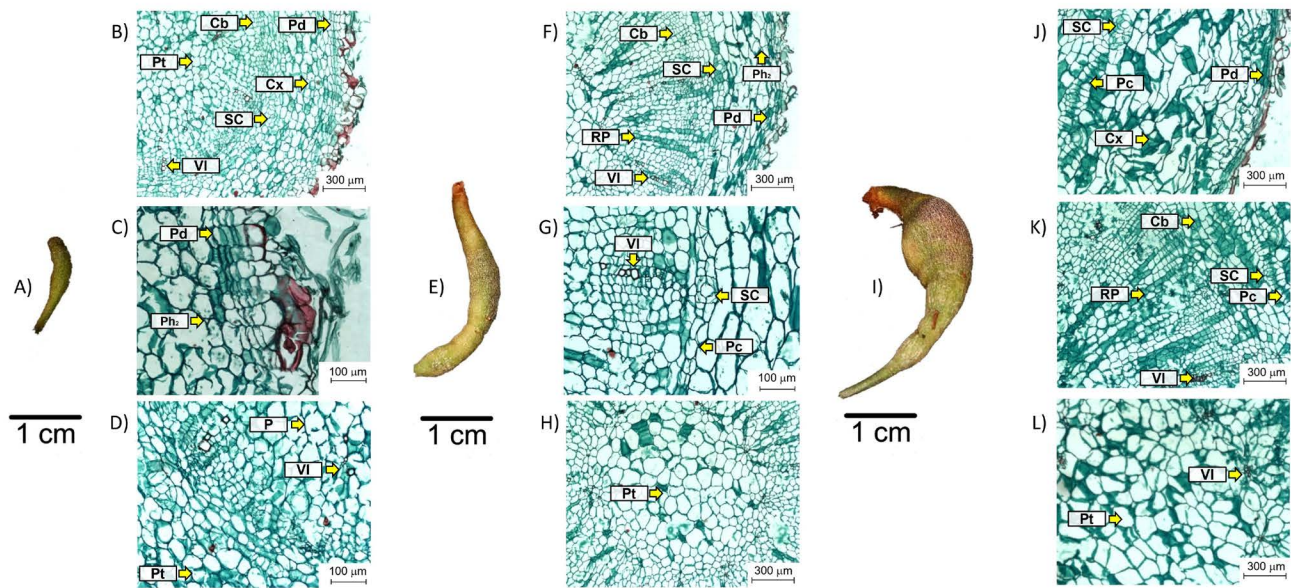
Sucrose concentration was expected to influence soluble sugar content, as carbohydrates play a central role in energy metabolism, signaling, and plant development. Sucrose is the primary product of photosynthesis, and its transport from source leaves to organs, such as stems and rhizomes, is finely regulated, essentially determining plant growth and productivity. In addition to their function as carbon storage, sugars actively participate in signaling pathways that modulate differentiation and response to the environment (Hennion *et al.* 2019). Therefore, modifications in the external availability of sucrose could alter its internal distribution and accumulation in the different organs, which happens in *in vitro* cultured plants, since they absorb the sucrose available in the culture medium. Accordingly, in plantlets of *C. atrosanguineus*, tuberized microrhizomes significantly increased their soluble sugar content in the treatment with 12 % sucrose (Figure 3), indicating that the consumption of sucrose by the tissues would incorporate it into the demanding organs, such as microrhizomes or tubers. Nonetheless, the addition of lower concentrations of sucrose (0, 3, and 6 %) for 20 days did not affect the content of soluble sugars in the leaves, stems, or microrhizomes. On the other hand, an osmotic effect on the tuber formation can be discarded in mannitol-treated plantlets that showed an evident morphological deterioration in the aerial part (necrosis), moreover the concentration of soluble sugars in these microrhizomes were very low and did not show significant differences between them (Figure 4).

The differential effect between sucrose and mannitol is explained by their different physiological functions. Sucrose acts as a source of carbon and a signaling molecule, influencing metabolism and the allocation of photo-assimilates, while mannitol, in many species, is not efficiently metabolized or absorbed and is mainly used as an osmotic agent (Lipavská & Vreugdenhil 1996, de Paiva Neto & Otoni 2003). Although increased mannitol would be expected to induce osmotic adjustment through the accumulation of soluble sugars, previous studies have shown that this effect does not always occur (de Paiva Neto & Otoni 2003, Ghaheri *et al.* 2017). The absence of significant differences in soluble sugar content, together with the deterioration observed in the aerial part, suggests that mannitol mainly affected the osmotic potential of the medium without substantially altering carbohydrate metabolism, unlike sucrose whose effect is associated with metabolic and signaling processes (Lipavská & Vreugdenhil 1996).

The application of mannitol in the medium is recommended to induce a certain proportion of osmotic stress assuming that plants do not absorb this compound and with the aim that the plant decreases its growth speed for conservation purposes (Chappell *et al.* 2020). Unlike this, sucrose even when it initially presents an osmotic potential according to its concentration, is reduced by its degradation through the activity of the enzyme invertase in the plant (de Paiva Neto & Otoni 2003). The goal of mannitol addition is related to the observation that stress can increase the number and size of tuberized microrhizomes (Pumisutapon *et al.* 2012).

Whereas the treatments with rising concentrations of sucrose in the culture media showed that the higher the concentration of sucrose, the greater the increase in the sugars stored in the microrhizomes (Figure 4). Such pattern occurs in *in vitro* micropropagation cultures that develop similar underground structures, such as in the case of *Zingiber cassumunar* Roxb. where  $5.83 \pm 0.48$  rhizomes were obtained per tube of with 7 % sucrose (Chirangini & Sharma 2005), and in *Bambusa bambos* (L.) Voss var. *gigantea* Bennet & R.C. Gaur, a 5 % sucrose concentration was used to obtain 2-6 rhizomes (Kapoor & Rao 2006). Both researchers recognized the importance of raising the sucrose concentrations in the culture media to form these storage organs.





**Figure 7.** Cross-sections of tubers of *Cosmos atrosanguineus* in vitro of different thicknesses. (A-L). The arrows point to Cb: cambium; Pd: periderm; Pt: pith; Cx: cortex; SC: secretory canals; VI: vassels; Ph<sub>2</sub>: secondary phloem; P: parenchyma; RP: radial parenchyma and Pc: pericycle.

Tubers are underground storage organs that primarily depend on the import of sugars from the aerial parts of the plant for thickening or filling, where photosynthates are transported from the source leaves to the forming organ for metabolism or storage (Sunitibala *et al.* 2001, Hennion *et al.* 2019). Our results indicate a significant sucrose incorporation into the tissues of the microrhizomes, contributing to the thickening or filling of the microtubers.

Additionally, tuberized and non-tuberized microrhizomes were subcultured *in vitro* to test sprouting percentage. From tuberized microrhizomes, 75 % developed new shoots, while from non-tuberized microrhizomes, only 25 % sprouted new shoots (data not shown). This demonstrates that in *C. atrosanguineus*, a critical step in implementing a successful *in vitro* propagation process is the tuberization of microrhizomes.

Nevertheless, it has been considered that propagation through tubers is very slow (Kozak *et al.* 2013). The proposal we present in this research suggests that tuberized microrhizomes are viable structures for use as propagules, thereby improving their propagation and *ex vitro* establishment.

**Acclimatization.** *In vitro* plantlets survival generally may be subjected to different factors, such as environmental gas alteration, high relative humidity, low luminosity, as well as environments rich in sugars and nutrients that encourage their development. Nonetheless, plants grown under these conditions may exhibit abnormal morphology as well as altered anatomy and physiology, which limit their resistance to *ex vitro* environmental conditions adaptation (Mohammed *et al.* 2023). Consequently, these plantlets must undergo an acclimatization process that gradually changes the environment in which they grow, as *in vitro* plantlets exhibit excessive water loss during soil adaptation. The mechanisms that disrupt the maintenance of water content and interfere with the regulation of leaf transpiration are probably related to malfunctions of the stomata (Misalová *et al.* 2009).

In the case of *C. atrosanguineus* plantlets with microrhizomes obtained *in vitro* required more time for acclimatization than those obtained directly from the *in vitro* microtubers. Thus, the plantlets that sprouted from the tubers did not present this problem of water loss during acclimatization because their development was entirely *ex vitro*.

**Anatomical analysis.** The microtubers of *C. atrosanguineus* obtained *in vitro* were anatomically similar to the *C. pringlei* tuber. Even though they are two different species, the anatomy is practically the same, with slight variations

due to the environments under which the tubers developed. The *in vitro* microtubers were subjected to continuous high relative humidity which promotes larger cells while in contrast the wild tubers were stressed by such intermittent environmental factors as seasonality and periodic drought.

Secondary growth in the storage organs that come from a stem is carried out with the formation of rays from increased mitotic activity of the interfascicular cells of the vascular tissue. The development of these storage organs is typically characterized by the division of the cells of the vascular cambium, as well as proliferation and massive filling with reserve carbohydrates, such as starch, in *Solanum tuberosum*, *Eleocharis dulcis* (Burm. f.) Trin. ex Hensch., *Dioscorea* sp., *Amorphophallus paeoniifolius* (Dennst.) Nicolson, and *Colocasia esculenta* (L.) Schott (Sharma & Kaushal 2016). Although *Cosmos* does not contain starch as the main reserve carbohydrate (data not shown), the rhizome, a modified stem, shows similarities in the thickening process of these tuberous structures. According to Angyalossy *et al.* (2016), during tuber development, a thickening intended to increase circumference occurs in the secondary phloem, the axial parenchyma, the radial, or both, and the epidermis.

In beets, for example, it has been observed that the most accelerated growth occurs in the first eight weeks; mitotic activity decreases once the maturation process begins (Bellin *et al.* 2007). The maturation of tuberous tissue could explain the observed changes in the organization of vascular tissue and the reduction of the axial parenchyma in the outermost areas of the wild tuber *C. pringlei*. In other storage organs, it has been reported that progressive lignification of vascular regions can limit radial growth by restricting cell expansion (Rukundo *et al.* 2013, Villordon *et al.* 2014, Lobato *et al.* 2018). However, in the species analyzed in this study, lignification was not detected in the pith, and lignified elements were mainly restricted to tracheal elements, suggesting that the regulation of tuberous growth does not depend exclusively on lignification. In this context, the formation of the tuber in *C. atosanguineus* seems to be associated with the presence of the cambium activity and a high parenchyma expansion capacity, rather than with the direct absence of lignification, indicating that multiple anatomical and physiological factors contribute to the development of the storage organ.

This study establishes an efficient protocol for the propagation and conservation of *Cosmos atosanguineus*, an endemic Mexican species whose sterility limits its natural reproduction. It was determined that supplementing the culture medium with 1 mg/L NAA is the optimal condition for the mass induction of microrhizomes, achieving the highest formation rate of these structures per explant.

Furthermore, it was demonstrated that increasing the sucrose concentration to 12 % acts as a fundamental metabolic signal that triggers the tuberization of microrhizomes. This thickening process is a direct response to carbohydrate incorporation into storage tissues rather than a merely osmotic effect, as confirmed through comparison with mannitol treatments. Anatomical characterization revealed that the cellular organization and secondary growth patterns of the microtubers obtained *in vitro* are remarkably similar to those of wild *C. pringlei* tubers, validating the biological identity of these structures.

Finally, the use of tuberized microrhizomes represents a strategic advantage for *ex vitro* acclimatization. Unlike conventional plantlets, tuber-derived propagules exhibit functional and reduced transpiration rates from the onset of sprouting, allowing the omission of gradual humidity control processes and significantly reducing the risk of mortality due to water stress during transplanting. Taken together, these results provide a robust biotechnological tool for the commercial multiplication and germplasm preservation of this unique species.

## Acknowledgements

This paper contributes to the first author's Ph.D. requirements at the Posgrado en Ciencias Biológicas, UNAM; we thank the program for its support. We also thank the editors for their kind suggestions for improving the manuscript.

## Literature cited

- Angyalossy V, Evert R, Marcati C, Oskolski A, Terrazas T, Kotina E, Lens F, Mazzoni-Viveiros S C, Angeles G, Machado S R, Crivellaro A, Rao K S, Junikka L, Nikolaeva N, Baas P. 2016. IAWA List of Microscopic Bark Features. *International Association of Wood Anatomists Journal* **37**: 517-615. DOI: <https://doi.org/10.1163/22941932-20160151>
- Bellin D, Schulz B, Soerensen TR, Salamini F, Schneider K. 2007. Transcript profiles at different growth stages and tap-root zones identify correlated developmental and metabolic pathways of sugar beet. *Journal of Experimental Botany* **58**: 699-715. DOI: <https://doi.org/10.1093/jxb/erl245>
- Brijesh H, Ajjappala B. 2023. Micropropagation strategies in medicinally important turmeric (*Curcuma* sp.): Current research and future challenges. *Journal of Applied Biology & Biotechnology* **11**: 1-8. DOI: <https://doi.org/10.7324/JABB.2023.65814>
- Castro-Castro A, Vargas-Amado G, Harker M, Rodríguez A. 2014. Macromorphological and cytogenetic analysis of the genus *Cosmos* (Asteraceae, Coreopsidae), with a key to its identification. *Botanical Sciences* **92**: 363-388. DOI: <https://doi.org/10.17129/botsci.111>
- Chappell AL, Koym JW, Scheuring DC, Miller JC. 2020. Incorporation of Mannitol in Tissue Culture Media for Long-Term Storage of Potatoes at Moderately Low Temperature and Effect on Subsequent Micropropagation. *American Journal of Potato Research*. **97**: 439–446. DOI: <https://doi.org/10.1007/s12230-020-09780-6>
- Chirangini P, Sharma GJ. 2005. In vitro propagation and microrhizome induction in *Zingiber cassumunar* (Roxb.)-an antioxidant-rich medicinal plant. *Journal of Food, Agriculture & Environment* **3**: 139-142.
- Chittaragi DC, Menon JEV, Anoop EV. 2022. Histochemical analysis and storage behaviour of Ginger (*Zingiber officinale* Roscoe) under Zero-Energy Cool Chamber (ZECC). *Plos One* **17**: e0265320. DOI: <https://doi.org/10.1371/journal.pone.0265320>
- de Paiva Neto VB, Otoni WC. 2003. Carbon sources and their osmotic potential in plant tissue culture: does it matter? *Scientia Horticulturae* **97**: 193-202. DOI: [https://doi.org/10.1016/S0304-4238\(02\)00231-5](https://doi.org/10.1016/S0304-4238(02)00231-5)
- Debnath SC. 2005. Strawberry sepal: Another explant for thidiazuron-induced adventitious shoot regeneration. *In Vitro Cellular & Developmental Biology-Plant*. **41**: 671–676. DOI: <https://doi.org/10.1079/IVP2005688>
- Du J, Groover A. 2010. Transcriptional Regulation of Secondary Growth and Wood Formation. *Journal of integrative Plant Biology* **52**: 17-27. DOI: <https://doi.org/10.1111/j.1744-7909.2010.00901.x>
- Duan Y, Xue T, Li J, Teng J, Zhang A, Sheng W, Zhu Y, Chang L, Xue J. 2015. In vitro induction of yacon tuberous root and identification of genes associated with tuberous root expansion. *Journal of Animal and Plant Sciences* **25**: 1753-1763.
- Dubois M, Gilles K, Hamilton JK, Rebers PA, Smith F. 1951. A colorimetric method for the determination of sugars. *Nature* **168**: 167. DOI: <https://doi.org/10.1038/168167a0>
- Ferreira PJJ, Luiz OF, Zucoloto M, das Graças TA, do Carmo PM, Antonio TM. 2020a. Yacon (*Smallanthus sonchifolius*), propagation from rhizophores with different numbers of buds. *Revista de la Facultad de Ciencias Agrarias* **52**: 52-63.
- Ferreira P, Luiz F, Zucoloto M, Oliceira M, Amaro R, de Oliveira A. 2020b. Yacon potato propagation from herbaceous cuttings with different numbers of buds. *International journal of agriculture and natural resources* **47**: 46-57. DOI: <http://dx.doi.org/10.7764/ijanr.v47i1.2134>
- Ghaheri M, Kahrizi D, Bahrami G. 2017. Effect of mannitol on some morphological characteristics of in vitro *Stevia rebaudiana* Bertoni. *Biharean Biologist* **11**: 94-97.
- Haupt AW. 1930. A gelatin fixative for paraffin sections. *Stain Technology* **5**: 97-98. DOI: <https://doi.org/10.3109/10520293009115555>
- Hennion N, Durand M, Vriet C, Doidy J, Maurousset L, Lemoine R, Pourtau N. 2019. Sugars en route to the roots. Transport, metabolism and storage within plant roots and towards microorganisms of the rhizosphere. *Physiologia Plantarum* **165**: 44-57. DOI: <https://doi.org/10.1111/ppl.12751>

- Hind N, Fay MF. 2003. Plate 461. *Cosmos atrosanguineus* Compositae. *Curtis's Botanical Magazine* **20**: 40-48. DOI: <https://doi.org/10.1111/1467-8748.00369>
- Hong-jiu L, Cai-ping H, Pei-jiang T, Xue Y, Ming-ming C, Zhi-hui C. 2020. Response of axillary bud development in garlic (*Allium sativum* L.) to seed cloves soaked in gibberellic acid (GA3) solution. *Journal of Integrative Agriculture* **19**: 1044-1054. DOI: [https://doi.org/10.1016/S2095-3119\(20\)63156-2](https://doi.org/10.1016/S2095-3119(20)63156-2)
- Hooker WJ. 1861. *Cosmos diversifolius* var. *atro-sanguineus*. *Curtis's Botanical Magazine* **87**: 5227.
- Hosoki T, Kobayakawa H, Ohta K. 2003. Micropropagation of chocolate cosmos (*Cosmos atrosanguineus*) by repeated division of nodes/axillary shoots and adventitious shoots from microshoots. *Acta Horticulturae* **625**: 261-264. DOI: <https://doi.org/10.17660/ActaHortic.2003.625.30>
- Jiménez ML. 2015. The cultivation of the dahlia. *Tropical crops* **36**: 107-115.
- Jing S, Jiang P, Sun X, Yu L, Wang E, Qin J, Zhang F, Prat S, Song B. 2023. Long-distance control of potato storage organ formation by SELFPRUNING3D and FLOWERING LOCUS T-like 1. *Plant Communications* **4**: 100547. DOI: <https://doi.org/10.1016/j.xplc.2023.100547>
- Johansen DA. 1941. Plant Microtechnique. *Nature* **147**: 222. DOI: <https://doi.org/10.1038/147222b0>
- Kapoor P, Rao IU. 2006. In vitro rhizome induction and plantlet formation from multiple shoots in *Bambusa bambos* var. *gigantea* Bennet and Gaur by using growth regulators and sucrose. *Plant Cell, Tissue and Organ Culture* **85**: 211-217. DOI: <https://doi.org/10.1007/s11240-005-9074-y>
- Kerk NM, Sussex IM. 2012. Roots and Root Systems. In: *Encyclopedia of Life Sciences*. Ed. Chichester: John Wiley & Sons. 1-10. DOI: <https://doi.org/10.1002/9780470015902.a0002058.pub2>
- Khaleghi A, Khalighi A, Sahraroo A, Karimi M, Rasoulnia A, Ghafoori IN, Ataei R. 2008. In vitro Propagation of *Alstroemeria* cv. 'Fuego'. *American-Eurasian Journal of Agricultural & Environmental Sciences* **3**: 492-497.
- Kolachevskaya OO, Sergeeva LI, Flokova K, Getman IA, Lomin SN, Alekseeva VV, Rukavtsova EB, Buryanov YI, Romanov GA. 2017. Auxin synthesis gene *tms1* driven by tuber-specific promoter alters hormonal status of transgenic potato plants and their responses to exogenous phytohormones. *Plant Cell Reports* **36**: 419-435. DOI: <https://doi.org/10.1007/s00299-016-2091-y>
- Kondhare KR, Patil AB, Giri AP. 2021. Auxin: An emerging regulator of tuber and storage root development. *Plant Science* **306**: 110854. DOI: <https://doi.org/10.1016/j.plantsci.2021.110854>
- Kozak D. 1991. Shoot regeneration from various parts of *Narcissus* cv. Carlton through tissue culture. *Prace Instytutu Sadownictwa i Kwiaciarnictwa w Skierniewicach. Seria B, Rośliny Ozdobne*, **16**: 41-48.
- Kozak D, Pogroszewska E, Szmagara M. 2013. The influence of type and orientation of explants on in vitro growth and development of *Cosmos atrosanguineus* (Hook.) Voss. *Acta Scientiarum Polonorum Hortorum Cultus* **12**: 41-53.
- Labrooy C, Abdullah TL, Stanslas J. 2020. Influence of N6-benzyladenine and sucrose on in vitro direct regeneration and microrhizome induction of *Kaempferia parviflora* Wall. Ex Baker, an important ethnomedicinal herb of Asia. *Tropical Life Sciences Research* **31**: 123-139. DOI: <https://doi.org/10.21315/tlsr2020.31.1.8>
- Lipavská H, Vreugdenhil D. 1996. Uptake of mannitol from the media by in vitro grown plants. *Plant Cell, Tissue and Organ Culture* **45**: 103-107. DOI: <https://doi.org/10.1007/BF00048751>
- Ljung K, Nemhauser JL, Perata P. 2015. New mechanistic links between sugar and hormone signalling networks. *Current Opinion in Plant Biology* **25**: 130-137. DOI: <https://doi.org/10.1016/j.pbi.2015.05.022>
- Lobato MC, Daleo GR, Andreu AB, Olivieri FP. 2018. Cell Wall Reinforcement in the Potato Tuber Periderm After Crop Treatment with Potassium Phosphite. *Potato Research* **61**: 19-29. DOI: <https://doi.org/10.1007/s11540-017-9349-9>
- Medina RD, Faloci MM, González AM, Mroginski LA. 2007. In vitro cultured primary roots derived from stem segments of cassava (*Manihot esculenta*) can behave like storage organs. *Annals of Botany* **99**: 409-423. DOI: <https://doi.org/10.1093/aob/mcl272>
- Misalová A, Durkovic J, Priwitzer T, Lengyelová A, Hladká D, Lux A. 2009. Changes in leaf organisation, Photosynthetic performance and wood formation during ex vitro acclimatisation of black mulberry (*Morus nigra* L.). *Plant biology* **11**: 686-93. DOI: <https://doi.org/10.1111/j.1438-8677.2008.00166.x>



- Mohammed M, Munir M, Ghazzawy HS. 2023. Design and evaluation of a smart ex vitro acclimatization system for tissue culture plantlets. *Agronomy* **13**: 78. DOI: <https://doi.org/10.3390/agronomy13010078>
- Murashige T, Skoog F. 1962. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum* **15**: 473-497. DOI: <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
- Natarajan B, Kondhare KR, Hannapel DJ, Banerjee AK. 2019. Mobile RNAs and proteins: Prospects in storage organ development of tuber and root crops. *Plant Science* **284**: 73-81. DOI: <https://doi.org/10.1016/j.plantsci.2019.03.019>
- Nayak S. 2000. In vitro multiplication and microrhizome induction in *Curcuma aromatica* Salisb. *Journal of Plant Growth Regulation* **32**: 41-47. DOI: <https://doi.org/10.1023/A:1006307316393>
- Oku T, Ti H, Yagi F, Nakamura I, Mii M. 2008. Hybridisation between chocolate cosmos and yellow cosmos confirmed by phylogenetic analysis using plastid subtype identity (PSID) sequences. *Journal of Horticultural Science & Biotechnology*. **83**: 323-327. DOI: <https://doi.org/10.1080/14620316.2008.11512386>
- Orlikowska T, Sabata I, Kucharska D. 2000. The effect of leaf and shoot tip removal and explant orientation on axillary shoot proliferation of *Codiaeum variegatum* Blume var. *pictum* Muell.-Arg. cv. *excellent*. *Scientia Horticulturae*. **85**: 103-111.
- Ortega-Larrocea P, Chávez MV, Bye R. 1997. Micropropagation and ex vitro establishment of *Cosmos atrosanguineus* (Hook.) A. Voss. in the Botanical Garden of the Institute of Biology of the UNAM. *Amaranth* **10**: 1-9.
- Ortgies KE. 1861. *Bidens atrosanguinea* Ortgies. *Gartenflora* **10**: 406-409, t. 347.
- Pelacho AM, Mingo-Castel AM. 1991. Effects of photoperiod on kinetin-induced tuberization of isolated potato stolons cultured in vitro. *American Potato Journal* **68**: 533-541. DOI: <https://doi.org/10.1007/BF02853770>
- Pumisutapon P, Visser RGF, de Klerk GJ. 2012. Moderate abiotic stresses increase rhizome growth and outgrowth of axillary buds in *Alstroemeria* cultured in vitro. *Plant Cell, Tissue and Organ Culture* **110**: 395-400. DOI: <https://doi.org/10.1007/s11240-012-0160-7>
- Quaresma LMA, Oliveira LF, Parajara CM, Silva NDM, Dalvi PL, Teixeira GA. 2020. Refrigerated storage period of rhizophores for yacon propagation. *Bioscience Journal* **36**: 301-306. DOI: <https://doi.org/10.14393/BJ-v36n0a2020-47817>
- Rajeswari V, Paliwal K. 2008. In Vitro Adventitious Shoot Organogenesis and Plant Regeneration from Seedling Explants of *Albizia odoratissima* L.f. (Benth.). *In Vitro Cellular and Developmental Biology-Plant*. **44**: 78-83. DOI: <https://doi.org/10.1007/s11627-008-9120-7>
- Rice G. 2017. The story of *Cosmos atrosanguineus*. *The Plantsman* **16**: 112-119.
- Rout GR, Palai SK, Samantaray S, Das P. 2001. Effect of growth regulator and culture conditions on shoot multiplication and rhizome formation in ginger (*Zingiber officinale* Rosc.) in vitro. *In Vitro Cellular & Developmental Biology - Plant* **37**: 814-819. DOI: <https://doi.org/10.1007/s11627-001-0135-6>
- Rukundo P, Shimelis H, Laing M, Gahakwa D. 2013. Storage root formation, dry matter synthesis, accumulation and genetics in sweetpotato. *Australian Journal of Crop Science* **7**: 2054-2061.
- Sairanen I, Novák O, Pencik A, Ikeda Y, Jones B, Sandberg G, Ljung K. 2012. Soluble carbohydrates regulate auxin biosynthesis via PIF proteins in *Arabidopsis*. *Plant Cell* **24**: 4907-4916. DOI: <https://doi.org/10.1105/tpc.112.104794>
- Sandoval ZE, Rojas LA, Guzmán RC, Carmona JL, Ponce SRM, León GC, Loyola BC, Vallejo ZMA, Medina AA. 2005. *Técnicas aplicadas al estudio de la anatomía vegetal*: cuaderno 38. DF, México: Instituto de Biología, Universidad Nacional Autónoma de México. ISBN: 9789703231317
- Seabrook JEA, Cumming BG, Dionne LA. 1976. The in vitro induction of adventitious shoot and root apices on *Narcissus* (daffodil and narcissus) cultivar tissues. *Canadian Journal of Botany*. **54**: 814-819.
- Seminario JM, Valderrama EI, Manrique I. 2003. El yacón: fundamentos para el aprovechamiento de un recurso promisorio. Lima, Perú. Centro Internacional de la papa (CIP), Universidad Nacional de Cajamarca, Agencia Suiza para el Desarrollo y la Cooperación (COSUDE). [https://cipotato.org/wp-content/uploads/2014/07/Yacon\\_Fundamentos\\_password.pdf](https://cipotato.org/wp-content/uploads/2014/07/Yacon_Fundamentos_password.pdf) (Accessed July 15, 2025)
- Sharma TR, Singh B. 1995. In vitro microrhizome production in *Zingiber officinale* Rosc. *Plant Cell Reports* **15**: 274-277. DOI: <https://doi.org/10.1007/BF00193735>

- Sharma HK, Kaushal P. 2016. Introduction to Tropical Roots and Tubers. In: Sharma HK, Njintang NY, Singhal RS, Kaushal P. eds. *Tropical Roots and Tubers: Production, Processing and Technology*. John Wiley & Sons, pp. 1-33. DOI: <https://doi.org/10.1002/9781118992739.ch1>
- Shirgurkar MV, John CK, Nadgauda RS. 2001. Factors affecting in vitro microrhizome production in turmeric. *Plant Cell Tissue Organ Culture* **64**: 5-11. DOI: <https://doi.org/10.1023/A:1010645624618>
- Shuangying Y, Liu H, Yiqing L, Xiaodong C. 2024. In Vitro Microrhizome Production, Genetic Homogeneity Assessment, and Field Performance Evaluation in Ginger. *Agronomy* **14**: 747-765. DOI: <https://doi.org/10.3390/agronomy14040747>
- Sunitibala H, Damayanti M, Sharma GJ. 2001. In vitro propagation and rhizome formation in *Curcuma longa* Linn. *Cytobios* **105**: 71-82.
- Suárez GL, Mederos VVR. 2011. Apuntes sobre el cultivo de la yuca (*Manihot Esculenta* Crantz). Tendencias actuales. *Cultivos tropicales* **32**: 27-35.
- Thangavel K, Ravichandran P, Ebbie M, Manimekalai V. 2014. In vitro microrhizome production in *Decalepis hamiltonii*. *African Journal of Biotechnology* **13**: 1308-1313. DOI: <https://doi.org/10.5897/AJB2013.12969>
- Vargas-Amado G, Castro-Castro A, Harker M, Villaseñor JL, Ortiz E, Rodríguez A. 2013. Geographic distribution and richness of the genus *Cosmos* (Asteraceae: Coreopsidae). *Revista Mexicana de Biodiversidad* **84**: 536-555. DOI: <https://doi.org/10.7550/rmb.31481>
- Villordon AQ, Ginzberg I, Firon N. 2014. Root architecture and root and tuber crop productivity. *Trends in Plant Science* **19**: 419-425. DOI: <https://doi.org/10.1016/j.tplants.2014.02.002>
- Wilkinson T, Wetten A, Prychid C, Fay MF. 2003. Suitability of cryopreservation for the long- term storage of rare and endangered plant species: a case history for *Cosmos atrosanguineus*. *Annals of Botany* **91**: 65-74. DOI: <https://doi.org/10.1093/aob/mcg009>
- Yang Y, Zhu J, Sun L, Kong Y, Chen J, Zhu M, Xu T, Li Z, Dong T. 2023. Progress on physiological and molecular mechanisms of storage root formation and development in sweetpotato. *Scientia Horticulturae* **308**: 11158. DOI: <https://doi.org/10.1016/j.scienta.2022.111588>
- Zahid NA, Jaafar HZE, Hakiman M. 2021. Alterations in Microrhizome Induction, Shoot Multiplication and Rooting of Ginger (*Zingiber officinale* Roscoe) var. Bentong with Regards to Sucrose and Plant Growth Regulators Application. *Agronomy* **11**: 320. DOI: <https://doi.org/10.3390/agronomy11020320>
- Ziv M, Halevy AH, Shillo R. 1970. Organs and plantlets regeneration of *Gladiolus* through tissue culture. *Annals of botany* **34**: 671-676. <https://doi.org/10.1093/oxfordjournals.aob.a084400>

**Associate editor:** Martín Mata Rosas

**Author contributions:** MAVV, conceptualization, study design, methodology, and data evaluation, writing; RBB, conceptualization, data evaluation, writing, and reviewing; CMFO, data evaluation, writing, and reviewing; YPR, data evaluation, writing, methodology and reviewing; MTOM, writing, reviewing, and editing; JGOM, conceptualization, supervision, investigation, and editing; VMCA, conceptualization, investigation, funding acquisition and editing. All authors wrote, read, and approved of the final manuscript.

**Supporting Agencies:** The authors thank the Consejo Nacional de Ciencia y Tecnología (CONACyT), nowadays, the Secretaría de Ciencia, Humanidades, Tecnología e Innovación (SECIHTI), for the Ph.D. scholarship No. 480708 awarded to the first author. Laboratory expenses were supported by Universidad Nacional Autónoma de México (Jardín Botánico del Instituto de Biología and Facultad de Estudios Superiores Iztacala). R.B. gratefully acknowledges the critical support for the initial stage of the conservation project of *C. atrosanguineus* provided by Graham Pattison (NCCCP) as well as Michael Fay and Timothy Wilkinson (Royal Botanic Gardens Kew).

**Conflict of interest:** The authors declare that there is no conflict of interest, financial or personal, in the information, presentation of data, and results of this article.