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TÍTULO: *In vitro* sugarcane (*Saccharum spp.)* Micropropagation

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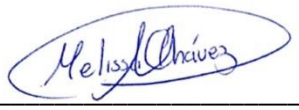

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Dedicatoria

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RESUMEN

La caña de azúcar (*Saccharum spp.*) es el insumo principal del "Ingenio Azucarero del Norte", empresa productora de azúcar en Ecuador. Con el objetivo de resolver los inconvenientes de calidad, patógenos y decadencia genética de la variedad CCC 01-1940, debido al cultivo convencional, se buscó desarrollar e implementar un protocolo para su micropropagación, en el Ingenio Azucarero del Norte. La desinfección evaluó 6 tratamientos con concentraciones de hipoclorito de sodio (0.5%, 0.75% y 1%) y tiempos de inmersión (5 y 10 minutos). En 3 semanas, se registró el porcentaje de supervivencia, contaminación, oxidación y viabilidad. El mejor tratamiento se usó en la Brotación, que evaluó 6 tratamientos con diferentes concentraciones de 6-Benzyladenina (6-BAP) y Kinetina, (0.5mg/L y 1mg/L). En un mes se registraron los mismos parámetros de la desinfección y el número de brotes. El Enraizamiento evaluó 16 tratamientos con diferentes concentraciones de Medio Murashige and Skoog (media y completo), 6-BAP (0.5mg/L) y ácido naftalenacético (0, 1, 2 y 3 mg/L). En 3 semanas se registró el porcentaje de supervivencia, contaminación, oxidación, apariencia, altura aérea, número y longitud de raíces. Finalmente, en Aclimatación se analizaron 3 tratamientos: T_{vp}(turba de vermiculita y perlita (1:1), T_{CP}(turba de fibra de coco y perlita (1:1)) y T_{PM} (Peat moss, turba de vermiculita y vermiculita (2:1:1)). En 35 días se registró la supervivencia, contaminación, longitud aérea y raíz, área foliar, número y distancia entre nudos, masa fresca y masa seca de la planta, hojas, tallo y raíz. Los resultados indicaron que los tratamientos adecuados para desinfección usan 0.5% o 0.75% NaClO por 10 min; tratamientos con 0.5mg/L 6-BAP + 1 mg/L KIN favorece la brotación; tratamientos con ½ MS + 1mg/L NAA o 3mg/L NAA favorecen el enraizamiento y las plantas mejores aclimatadas estuvieron en un sustrato de turba de vermiculita y perlita (1:1, v/v%).

Palabras clave: Caña de azúcar, cultivo *in vitro*, micropropagación, sustrato, Modelo Logístico.

ABSTRACT

Sugarcane (*Saccharum spp.*) is fundamental to the "Ingenio Azucarero del Norte," a sugar production company in Ecuador. To solve drawbacks in quality, susceptibility to pathogens, and genetics due to overuse of variety CC 01-1940, this study worked to develop and implement a protocol for micropropagation at the Ingenio Azucarero del Norte. The Disinfection phase evaluated 6 treatments with different sodium hypochlorite concentrations (0.5%, 0.75% and 1%) and immersion times (5 and 10 minutes). After 3 weeks, survival, contamination, oxidation, and viability percentages were recorded. The plants with best results were used to test the sprouting stage, where 6 treatments were evaluated, with concentrations of 6-Benzyladenine (6-BAP) and Kinetin, between 0.5mg/L and 1mg/L. In 1 month, the same parameters than of disinfection phase were recorder, and the number of sprouts. The rooting phase analyzed 16 treatments, with two concentrations of Murashige and Skoog medium (half strength and complete), 6-BAP 0.5mg/L, and α-naphthaleneacetic acid (0, 1, 2, and 3 mg/L) varied. In 3 weeks, we recorded survival, contamination, and oxidation percentages, likewise, appearance, aerial height, number, and roots length. Finally, the acclimatization stage examined 3 treatments: T_{vp}(vermiculite peat and perlite (1:1), T_{CP}(coconut fiber peat and perlite (1:1)) and T_{PM}(Peat moss, vermiculite peat and vermiculite (2:1:1:1)). Survival and contamination were recorded until 35 days. In addition, aerial, root length, leaf area, number and distance between nodes, fresh and dry mass of the plant, leaves, stem, and root were measured at the final. The results indicated that suitable treatments for disinfection used 0.5%NaClO or 0.75% for 10 min; treatment with 0.5mg/L 6-BAP + 1 mg/L KIN favored sprouting; treatments with ½ MS + 1mg/L NAA or 3mg/L NAA favored rooting and the best-acclimatized plants were in a peat substrate of vermiculite and perlite (1:1, v/v%).

Key words: sugarcane, *in vitro* culture, micropropagation, substrate, Logistic model.

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1. INTRODUCTION

The constant growth of human population obliges for the search and invention of more efficient methods to expand crop production, and their applications towards the increase of yields and reduction of manufacture times. *In vitro* plants fertilization could help in solving this issue.

Sugarcane (*Saccharum spp*.) is an important crop in tropical regions because of its multiple derivatives, such as sugar. Many companies in the sugar industry provide of these products to families in a global scale. More specifically, in the case of Ecuador, Ingenio Azucarero del Norte Compañía Economía Mixta (IANCEM) is one of the most relevant national companies producing mainly sugar, in addition to molasses and cane residues, in the Province of Imbabura, located in the northern region of the country (1). IANCEM has a capacity of process about 360 000 tons of sugarcane per year, resulting in the production of approximately 720 000 50kg-bags of white sugar and 100 000 2kg-bags of brown sugar. Variety CC01-1940 of sugarcane, registered in Centro de Investigación de la Caña de Azúcar del Ecuador (CINCAE), is key for the productivity and quality of IANCEM products. This variety is recognized for its high sucrose concentration levels and the resistance quality to common pathogens of sugarcane (2). However, the quality of its seed has presented deficiencies lately, and moreover, an increase in its susceptibility to pathogens has been reported too.

In vitro micropropagation is a biotechnological technique that offers the revitalization of plants, enhancing vigorousness, and increasing the number of identical plants free of diseases and pathogens. Sugarcane crops obtained from micropropagation have some advantages, including the rejuvenation and rapid multiplication of a larger number of plants, free from pathogens. Plants obtained by this mean present excellent genetic quality and phytosanitary form (3). Thus, the aim of this work was to obtain an *in vitro* micropropagation protocol for the sugarcane variety CC01-

1940 and establish the basis for the development of plant culture studies in the IANCEM research laboratory.

1.1. PROBLEM STATEMENT

The manufacturing of sugarcane-derived products is one of the most prominent industries in the province of Imbabura, located in the north of Ecuador. Given that the performance of the cultivar varieties is critical for the efficiency of the production, some difficulties involving genetic decadence and proneness to disease in current crops represent complications for the local sugar industry.

Conventional cultivation in this industry presents some difficulties such as the poor quality of seed material, multiple problems with pests and diseases, which are results of the variety aging of the variety. In the case of IANCEM, the current seed quality could be a cause for certain difficulties, such as the susceptibility to pathogens and low productive potential of crops. To get a solution, tissue culture techniques might be applied for regeneration and revitalization of the variety with the additional benefits that it offers. Thus, to obtain a variety with better performance in sugar production it is important to implement technologies that make the production faster and efficient.

In this sense, IANCEM seeks to have its own laboratory of plant breeding. Even tough, there are some efforts towards implementing tissue culture in sugarcane, it is necessary the adaptation of a protocol for the specific climate conditions of the laboratory and plant varieties. In this way, to increase sugarcane production, IANCEM requires the implementation of vegetal culture laboratory, starting by the micropropagation of sugarcane *in vitro*. Additionally, this *in vitro* technique would provide the basis for the use of vegetal biotechnology on a large scale of one of the main sources of income such as sugarcane.

In this study, the sugarcane variety called CC 01-1940 was introduced to *in vitro* conditions. This variety was selected due to its resistance to pests and its adaptation to variable climate conditions.

1.2. OBJECTIVES

1.2.1. General Objective

- To develop and implement a basic protocol for the *in vitro* micropropagation of the sugarcane variety CC 01-1940 at the Ingenio Azucarero del Norte laboratory.

1.2.2. Specific Objectives

- To introduce *in vitro* conditions in explants from sugarcane variety CC 01-1940.
- To determine the correct concentration of phytohormones and other growth parameters required for suitable outcomes regarding shoot induction, multiplication and rooting of *in vitro* micropropagation of sugarcane CC 01-1940.
- To define adequate acclimatization conditions for *in vitro* plants for better survival under *ex-vitro* condition.

2. LITERATURE REVIEW

2.1. Sugarcane biology

Sugarcane was carried from Europe to Latin America in 1498, but the large-scale production and commercialization of sugarcane started in the XX century due to the new technologies and tools developed to extract sugar (4). This plant grows in tropical and subtropical regions of the world and its crops cover 26 million hectares worldwide (5). According to botanical taxonomy, sugarcane belongs to the grass, family Gramineae (currently denominated as Poaceae), subfamily Panicoideae, supertribe Andropogoneae, subtribe Saccharineae, and genus *Saccharum*. The

current varieties are hybrids of different *Saccharum* genus species; for that, sugarcane crops recently received the name of *Saccharum spp. hybrid*.

This monocotyledonous plant grows in tropical and subtropical regions of the world and it can achieve 5m of height and 6cm of the stem thickness. The 1ideal soil pH range is between 5.5 to 7.8, and suitable temperatures for cultivation are around 16 to 30°C (6).

Sugarcane is recognized for its ability to store high concentrations of sucrose, or sugar, which is the main product accumulated in the internodes of the stalk. Some factories use this sucrose as raw material for human food, ethanol production by fermentation, low polluting fuel, as in the Brazilian sugar industry, and for paper production (7). The phenological stages of sugarcane are: germination, tillering and establishment (until 3 months), intense growth and stalk elongation (until 7 months) and ripening (11 months) (5). In sugarcane development, the vigorous plant will have a rapid and sustained initial growth. It will produce new shoots, and the leaf canopy will expand to reach the light. Young shoots will do more work in terms of photosynthesis. Growth at this point is slow until they develop their leaves and can photosynthesize on their own. Young stems will be lost when the leaf cover does not allow light to pass through, but this seemingly large number represents less than 5% of the entire crop production.

At this point, temperature and humidity play a crucial role. Farmers must take care of the amounts of water provided to the crop because it can reduce the growth rate and even stop it. The stem is the essential organ, as this is where sugars are stored. The stem contains several nodes separated by internodes. The node is the rigid, fibrous portion of the stem; it is formed by the growth ring, the root band, the leaf scar, the bud, and the waxy ring. Sugarcane leaves originate at the nodes but are distributed in alternate positions along the stem as it grows. The root band gives rise to the primordial roots. The shoot gives rise to the new stems, then it is a critical stage.

In the apical part of the stem, the internodes are shorter compared to the basal part. Characteristics such as diameter, color, shape, length, and growth habit may depend on the variety and the agroecological conditions of the production area and crop management (8).

The tillering stage is characterized by an increase in the number of shoots or stems after germination or cutting. During the first five months, growth is fast and produces up to 30 stems/m, but its elongation is minimal. The tillering decreases due to competition for light and nutrients; resulting in the death of many stems. After the sixth month, the population stabilizes (8).

Although sugarcane is considered a perennial crop, its growth continues for only some crop cycles. The time to harvest depends on each variety but is generally around 12 to 14 months (8). Regrowth can occur after the stalk is cut, as the buds germinate depending on humidity and temperature conditions.

2.2. Optimal growth conditions

The first stage of cultivation requires the control of several external factors, such as the adequate water source, as well as good aeration for adequate respiration (9). The development is susceptible to any water deficit; then by the 4th and 5th month, the nodes formation, and the stem elongation is favored by temperature, high humidity, and high solar radiation, as the plant achieves a maturation period. When the sugarcane begins to ripen 2 or 3 months before harvest, irrigation stops, plant growth is interrupted, and sucrose begins to accumulate (9). The ripening process takes place from the stalk base to the apex, so the basal part has a higher sugar content than the upper part (10).

The correct development of sugarcane must consider some factors, such as:

- Humidity ranges from 83 to 71% throughout the plants' lives and helps the sucrose content grow above 45% of the dry matter's total weight. The latter is influenced by internal humidity (9).
- Luminosity is the main factor guiding photosynthesis in this C4 type plant; if it is reduced, sugar storage decreases, and a decrease in leaf starch is observed. If the light power decreases, the size of primary stems increases, and the secondary stems growth slow down, root growth stops, and the appearance of leaves changes.
- Temperature plays a significant role in the maturation of sugarcane, influencing the decrease in growth and sucrose accumulation. At this stage, it is recommended that the temperature is maintained between 12 to 14°C, as higher temperatures will result in the sucrose degradation to glucose and fructose (9).
- Nutrients have a significant influence on the development and maturation of the plant. For example, nitrogen increases sugarcane production, but it harms the juices in excess (9).

2.3. Sugarcane importance in the Industry of Ecuador

Sugarcane is a plant for cultivation in tropical and subtropical zones, preferably hot and humid environments. The cultivation of its varieties is confined to 30° north and south latitude. In this context, environmental Ecuadorian conditions allow the reframed expansion of this crop.

Sugarcane is an important for energy production and food (11). Due to its high capacity to accumulate sucrose in high levels in stems, its high yield makes it one of the highest cultivated crops. In 2011, Brazil harvested about 625 million tons of sugarcane in just eight million hectares (11). It makes Brazil the maximum producer, compared with reports stating around 1,558 million tons in the whole world produced in 2012 (6).

Given that sugar and other sugarcane-derived products have a high demand as a staple food in our society, the sugarcane crops are important for Ecuador from economic point of view. The no availability of sugarcane free elite stock because of multiple diseases, pests, changes in adaptive temperatures, and other conditions results in significant economic loss for our local sugar industries. Therewith, the increases in the use of sugar results in a challenging situation for small producers, larger industries, and researchers. Besides, the implementation of new breeding methods for rapid genetic improvement of these species is required, as it would take up to 15 years to complete a selection cycle (12).

In Ecuador, the sugarcane crop overcame 6'620,206 ton, in 2019, reaching the 90,211 ha of this crop surface with a yield of 74.30 t/ha (13). In the same year, there were at least 11,786 people working for the sugar cane growth and production. Likewise, Ecuador exported raw sugar in high quantities, reaching 30'000,000 ton in 2019 (13).

2.4. Micropropagation

Micropropagation is the production of full, viable plants from vegetative tissues or parts of plants, through aseptic and controlled environmental and nutritional conditions. This methodology is used to maximize the production and obtain vigorous, selected and pathogen free plants (14). Over the last thirty years, some *in vitro* techniques such as micropropagation have been widely used in horticulture and agriculture to propagate plants with commercial and economic interest (15). One of the first studies in the cell and tissue culture of sugarcane was implemented in 1969 in Hawaii, where techniques to induce callus formation and differentiation were developed. After that, hormones use solved the rooting and shooting formation problems with different hormonal requirements (16).

Micropropagation, also called *in vitro* cloning, comes from vegetal tissue culture, one of the vegetal biotechnology bases. It consists of the plant asexual propagation using vegetal tissue culture techniques. Some advantages include no diseases in vegetal material, genetic homogeneity known also as clonal lines, and reinvigoration (or *in vitro* rejuvenation) (6). Micropropagation using the apical meristem is a popular method of clonal propagation, which is successful and viable for producing identical plants free of pathogens and with a much faster multiplication rate. *In vitro* culture is also helpful for germplasm storage. As a consequence, some sugarcane industries have opted to use this technique primarily to facilitate safe and rapid movement through quarantine restrictions (12).

Sugarcane is a genetically complex crop, and the biotechnology techniques give an advantage to its propagation (3). There are heterogeneous sugarcane varieties that are commonly multiplied by cuttings. But at present, micropropagation is the only proven means of rapid, quality large-scale production, disease-free plants. *In vitro* multiplication can be completed through callus culture, axillary bud, and shoot tip culture (15).

2.5. Stages of micropropagation

2.5.1. Selection of mother plants

The selection of adequate mother plants avoids diseases and pathogens, which guarantees a high genetic quality for the next plants. The explants were collected from field-grown plant. Six months old plants were selected and obtained the cauline apex of 60cm. This part is then used in the establishment *in vitro* phase (6). For that, the mother plants selected should look vigorous and healthy. This phase aims to improve the implantation efficiency and the posterior development of *in vitro* cultures. The initial plant material is known as explant, and its aseptic condition could reduce exogen contamination risks. The physiological status of the selected plant has a significant

influence. The nutritional and hormonal requirements depend on the different physiological ages of the mother plant.

2.5.2. The establishment of explants

In the process of disinfection, certain exogen pathogens are eliminated such as the bacteria that causes ratoon stunting disease (RSD) *(Leifsonia xyli subsp. xyli*) and the sugarcane leaf scald (caused by *Xanthomonas albilineans* (Ashby) Dowson) are destroyed (3). With the selection of the most vigorous explants, together with a proper sterilization protocol, it is possible to start with the axenic culture that are viable physiologically for the next step, the multiplication.

In general, it is better if the explants are taken from young plants and their active development zones because less differentiated tissues allow easy regeneration *in vitro.* A short explant has low contamination risks, but the regeneration is complex. In contrast, in large explants the risks increase but the regeneration and growth are faster. Microorganisms live on the surface of plant tissues and could be held in stomata, lenticels, trichomes, and others, where their elimination is difficult. Contaminants can cause significant losses in posterior phases. All contaminants near apical meristem should be eliminated (6).

2.5.3. Shoot multiplication

The multiplication stage should guarantee the shoots propagation and genetic stabilization. This phase attempts to produce a great number of projections from established propagules. The propagules, now new shoots, should be separated to grow in different fresh mediums under sterile conditions. The maximum genetic material establishment allows the multiplication of more subcultures and keeps an adequate propagation coefficient in time (6).

2.5.4. Elongation and rooting

The shoots develop a radial system and acquire an optimal height. Phytohormones such as auxins like Naphthaleneacetic acid (NAA) promote rooting, while gibberellins (GAs) play a role in elongation. The plants will pass to the next phase once they achieve an adequate height and have a profuse radial system (6).

2.5.5. Acclimatization

Acclimatation is the final stage in micropropagation, where the steps above are validated for their efficiency and the quality of the resultant plants are measured (6). Its objective is the survival of plants after transplanting and the *ex-vitro* initiation growth, probably in a field or plant nursery. After rooting, explants are very sensitive to environmental changes, and it is necessary the gradual light increase and relative humidity decrease to manage the environmental conditions where sugarcane grows.

In addition, the prunings in the first stage are essential for aeration and inhibiting the proliferation of pathogens (17). Factors such as the growth and plant development are indicators of good quality under respective conditions. On the other hand, the substrate selection for the acclimatization stage is essential for the growth of transplanted plants. The fair mixture contains an organic part that provides plants with macro and micronutrient complexes necessary for survival; and the inorganic part that allows for drainage.

2.5.6. Phytohormones

Phytohormones are organic compounds commonly synthesized by higher plants that regulates the physiological plant processes. In general, phytohormones support in the promotion of multiplication, rooting and elongation. Commonly phytohormones used are:

- Auxins are essential in plant activities such as stem growth, root formation, inhibition of lateral buds, abscission of leaves and fruits, and embryogenesis. (18). In this work, α-naphthaleneacetic acid (NAA) was used.
- Cytokinins are produced in root meristems and through the xylem to the shoot. They stimulate cell division in plants, allowing growth and delaying tissue aging. Two of them, kinetine (KIN) and 6-Benzyladenine (BAP), were used in this work.

2.5.7. Culture media

Toshio Murashige and Folke Skoog, in an article published in Physiologia Plantarum, describe a culture medium capable of providing essential nutrients for tobacco tissue growth *in vitro*. After more than half a century, the Murashige and Skoog (MS) medium is still widely used and has been the current basis for propagation systems (19). The growth and development of *in vitro* plants are determined by genetic constitution, supplements in the media, and physical factors. The adequate culture media to allow plant growth should contain the following:

- Water: constitutes more than 90% of the culture medium, and it must be double-distilled to work with meristems.
- Carbon source: such as sucrose, because all cultures are heterotrophic, they need a carbon source since the explants will not carry out their photosynthesis.
- Mineral nutrients: essential group after sugars. Concentrated stock solutions are generally used. Nitrogen and potassium stand out among the macro and micronutrients, and iron should be incorporated as a chelating agent.
- Vitamins such as thiamine are essential for good crop growth.
- Phytohormones: organic compounds synthesized by higher plants act differently depending on where they are produced. Based on studies by White, Skoog, and co-workers

realized the relationship and importance of auxin and cytokinin hormones during *in vitro* stimulation of roots and shoots from tobacco callus culture.

Likewise, the physical factors control is also crucial to the plant development, such as light, temperature, pH, O2 and CO2 concentrations, and other organic substances (18).

2.6. Sugarcane variety CC 01-1940

Sugarcane variety CC 01-1940 results from the polycrossing of Colombian varieties such as CCSP 89-1997 with the types CC 91-1867 and CC 91-1583 (20) .The variety is capable of adaptation to hard climatic conditions. RioPaila Agricola S.A. (2013) reports that this variety tolerates the humidity and its germination is excellent even in wet and dry clay soils. It possesses a long, erect stem, and the internode arrangement forms a smooth zig zag. The internode is cylindrical, with a length of 10 to 13 cm and a diameter of up to 43mm (9). It is resistance property for diseases such as Brown rust (caused by *Puccinia melanocephala*), Orange rust (caused by *P.kuehnii*), mosaic rust and less in yellow leaf virus (ScYLV) (20). Other studies suggest that this variety is reasonably resistant to *Diatraea spp*. and susceptible to *Aenolamia varia* (9).

It has vigorous growth, and its tillering is between 9-13 stems per vine. Per linear meter, the plant has an average of 12 stems. (21). This parameter should consider the location of varieties that each variety has its potential for expression depending on the site where it is planted and the agroecological zone that governs it. In Ecuador, a significant number of the sugarcane varieties are registered in Centro de Investigación de la Caña de Azúcar del Ecuador (CINCAE), which also reported that this variety is resistant to brown rust (22). This variety registered a high productivity potential under wetlands due to the high sucrose content, and it has demonstrated great height and development, influencing excellently to present a higher cane tonnage per hectare (2).

3. METHODOLOGY

3.1. Study area

The research took place in the fields and Research laboratory of the Ingenio Azucarero del Norte (IANCEM) from June 2021 until September 2022. The geographic localization of this factory is at 0°28′ 52.93” N and 78° 05′ 52.15” W, in Imbabura province, Ecuador. The mother plants were obtained from Hacienda Tababuela field, which geographic localization is 0°23′ 53” N and 78°07′ 53” N, in Imbabura province, Ecuador. The CC01-1940 variety is the variety selected for the development of a micropropagation protocol, which genetic material were provided by IANCEM. This variety was brought from the sugar cane research center of Colombia (CENICAÑA).

3.1.1. Laboratory

The experiments were carried out in the research laboratory of the Ingenio Azucarero del Norte, which is divided in three areas: biotechnology, vegetal culture, and germination area (Figure 1). In the biotechnology area (Figure 1D), mother plant samples were received and washed with detergent, alcohol, and water; this space was also used for the culture media establishment, explants washing, solutions preparation, and autoclaving. The vegetal culture area (Figure 1C) was used to wash explants with sodium hypochlorite and water, and then established to culture media. Additionally, this aseptic area was employed for multiplication, rooting, and washing at acclimatization stage. Finally, the explants and plants were kept in the germination area (Figure 1B), which has the adequate light conditions with lamps of 2200kA, a photoperiod of 16 hours, and a regulated temperature of 22 ± 2 °C. In acclimatization, the plants went the last area for two weeks before moving to the greenhouse (Figure 1E).

Figure 1. Distribution of Research Laboratory of IANCEM. A) Entrance of principal and Research Laboratory, **B)** Germination area, **C)** Vegetal culture area, **D)** Biotechnology area, and **E)** Greenhouse. (Blue area represents the Research Laboratory distribution)

3.2. Selection of mother plants

The project started in July 2021, when the mother plants were four months old. The apical sugarcane shoots of the CC01-1940 variety were used for the *in vitro* micropropagation. It comprises the selection and growth of the selected plant under optimal conditions. Mother plants were chosen randomly, but taking in consideration robust individual with good appearance. Sugarcane apical shoots were used as explants for the *in vitro* regeneration. Sixty centimeters of the plant head were cut in the field and then transferred to the laboratory with an average time of 1 hour.

Field samples were de-leafed in the sample receiving area of the laboratory using gloves, a scalpel, and 70% alcohol (Figure 1A). The cuts were 7 to 8 centimeters, with a considerable number of leaves and a diameter of approximately 1,5cm, next they were placed in distilled water and disinfected.

3.2.1. Micropropagation process

The study developed and implemented the micropropagation technique in the sugarcane variety CC01-1940. Figure 2 is a scheme of the steps followed in the micropropagation phases of sugarcane. The first step was the selection of mother plants, from which apical meristems were collected. The explant samples were disinfected in laboratory under aseptic conditions. After obtaining new shoot tips the multiplication was completed until an optimal number of plants and move to the rooting stage. Finally, rooted plants passed to the *ex-vitro* adaptation stage, where the plants went through acclimatization in the greenhouse.

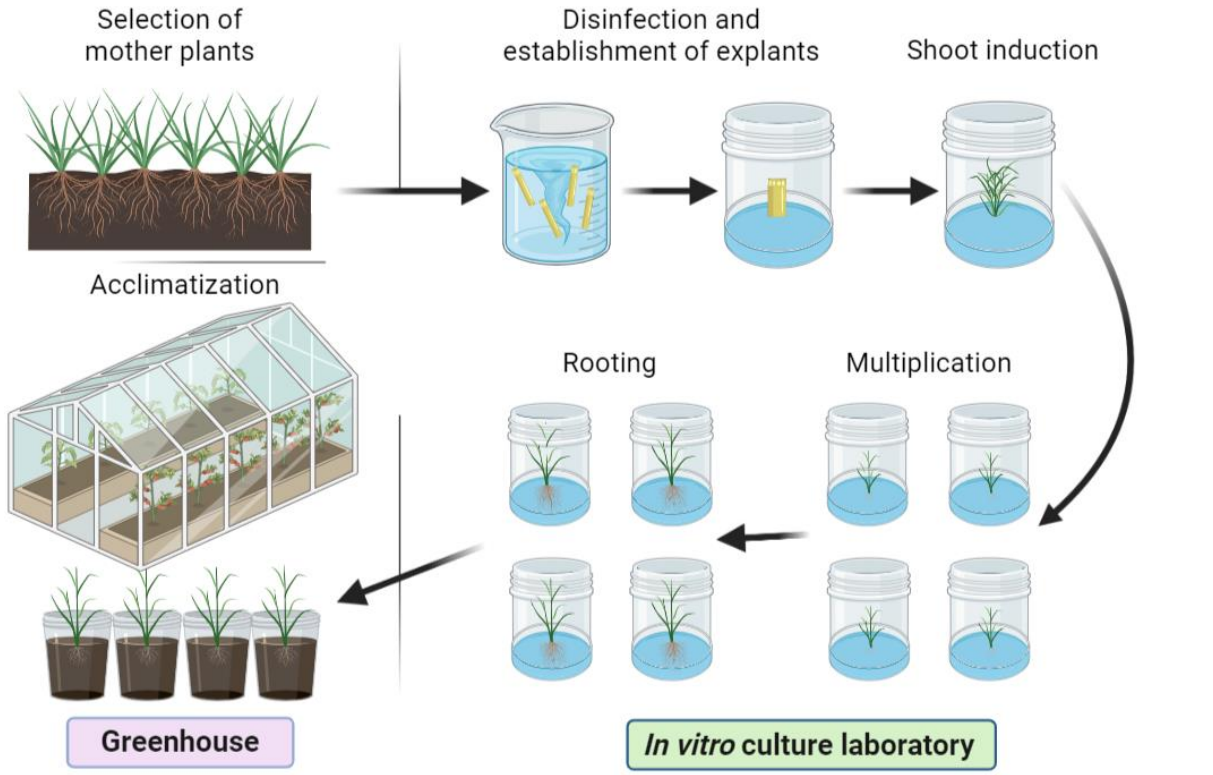

Figure 2. Phases of sugarcane micropropagation protocol. (Created with BioRender.com)

Regardless, all the stages are detailed in the following.

3.3. Disinfection

The first step was carried out in the Biotechnology Lab, where the explants were immersed in an antioxidant solution (citric acid + ascorbic acid, 2g/L) for 60 min; meanwhile, solutions of 0.5%, 0.75%, and 1% NaClO were prepared. Next, the explants were washed three times with distilled and tap water (alternating). Then, they were washed with a 30% (V/V) mild commercial detergent solution with Tween 80 2 drops/100mL of solution for 5 min with gentle agitation and rinsed three times with distilled water again (Figure 4B). Tween 80 or Polysorbate 80 is a non-ionic surfactant that reduces the surface tension of the disinfectant solution and allows better contact with the plant material; likewise, it is used widely to disperse and emulsify substances in food industry (23).

Subsequently, the explants were immersed in alcohol 70% (V/V) for 30 sec with gentle agitation and rinsed three times with sterile water removing residual alcohol from the explants surface. Finally, the shoots were transferred to the laminar flow cabinet at the vegetal culture Laboratory, and immersed in aqueous solutions of different sodium hypochlorite concentrations, described in Table 1, with Tween 80 2 drops/100mL with gentle agitation. Finally, the disinfected shoots were washed three times with autoclaved distilled water to remove all the sterilant traces and placed in a sterile 0.2 g/L citric acid solution for five min to inhibit premature oxidation.

To rid of any exogenous pathogen, the disinfection of sugarcane explants was carried out varying aqueous solution concentrations of sodium hypochlorite and immersion times. The treatments used of explants disinfection are detailed in Table 1:

Table 1. Treatments used for sugarcane disinfection, with NaClO and time variation.

Treatment	NaClO concentration (%)	Immersion time (min)
T1	0.5	10
T2	0.5	15
T3	0.75	10
T4	0.75	15
T5	1	10
T6	1	15

The nutritional requirements may differ according to the plant and its parts. However, plant tissue culture media should include the following components: micronutrients, macronutrients, source of carbon, vitamins, salts, amino acids or nitrogen supplements, solidifying agents and growth regulators to satisfy growth and morphogenesis of the plant (24). For the disinfection stage, the

culture media contained: Murashige and Skoog media with salts and vitamins (519), sucrose (30 g/L), Agar-agar (6 g/L) as a gelling agent, and cysteine (50 mg/L) to handle oxidation. The pH was fitted to 5.7 and autoclaved at 121 °C and 1.5 kg/cm^{2}pressure for 15 min. After three weeks of the establishment, percentages for survival, contamination, oxidation and viability were evaluated. The formula for each percentage indicator is showed in the Table 2.

Table 2. Parameters to evaluate the disinfection effectivity

Percentage of survival	**Percentage of contamination**
$\%S = \frac{\#\text{Survival explants}}{\#\text{Total explants of the treatment}} * 100$	$\%C = \frac{\#\text{Contaminated explants}}{\#\text{Total explants of the treatment}} * 100$
Percentage of oxidation	**Percentage of viability**
$\%O = \frac{\#\text{Oxidated explants}}{\#\text{Total explants of the treatment}} * 100$	$\%V = \frac{\#\text{Viable explants}}{\#\text{Total explants of the treatment}} * 100$

The parameters evaluated during the disinfection stage are presented in Figure 3. The first parameter considered all alive explants; that is, all alive explants would be considered as survivors even when experiencing contamination (Figure 3A). The overall contamination percentage evaluated the presence of any fungi, bacteria, or any other pathogen in the medium and explant (Figure 3B). The percentage of oxidation measured the existence of brown and black coloration at the base and aerial part of the explants (Figure 3C). Thus, the viability percentage only considered live explants with low oxidation and no contamination (Figure 3D).

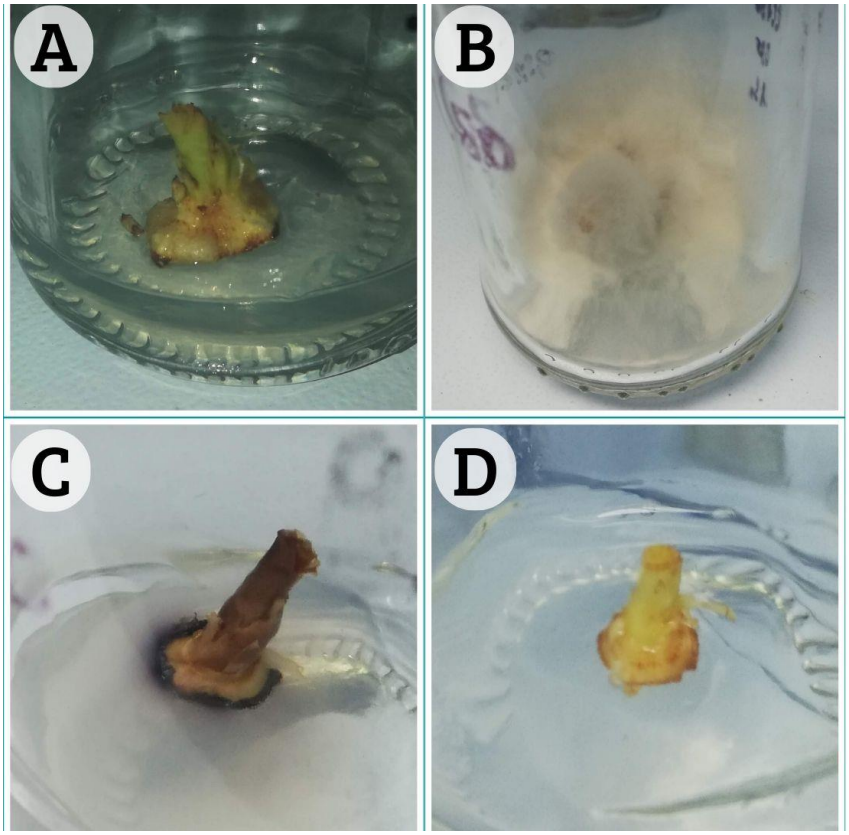

Figure 3. Parameters evaluated in the disinfection process. A) Survival explants after 1 week of the establishment. **B)** Contamination of culture media and explant after 1 week. **C)** Oxidation of explants, and **D)** Viable explant for the shoot induction.

3.3.1. Establishment of explants

All the processes in the laminar flow chamber were performed under strict aseptic conditions. The explants were cut with sterile forceps until 2cm of height with few leaves and around 1cm diameter in a base (Figure 4D). Additionally, the base were immersed in 1% of cysteine solution 50mg/L, removing the excess of liquid with paper tissue. Finally, the explants were implanted in the culture medium, contained in glass containers of 100ml capacity with 15ml of medium (Figure 4E).

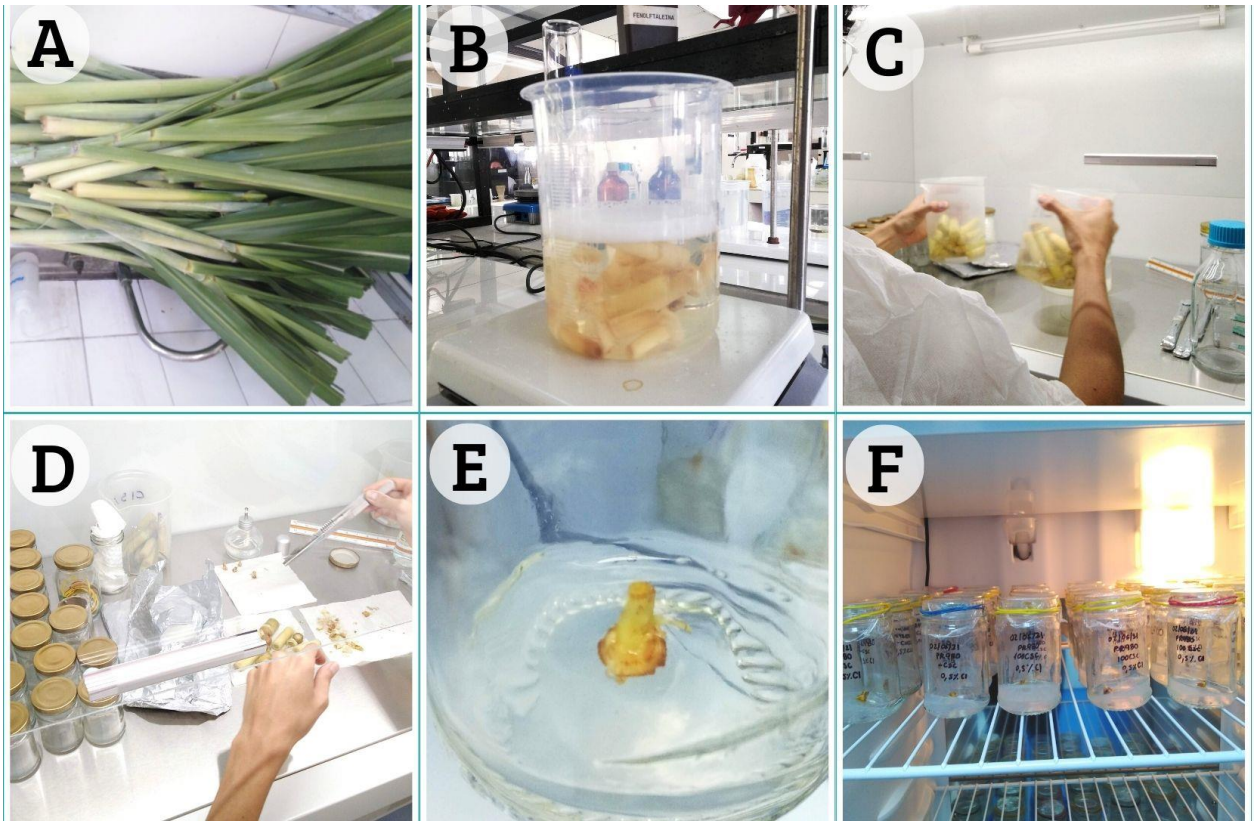

Figure 4. Disinfection process and establishment of sugarcane shoots to *in vitro* conditions. **A)** Selection of mother plants. **B)** Washing of explants with detergent and tween 80**. C)** Washing of explants with NaClO solutions under laminar flow cabinet. **D)** Establishment under germination chamber. **E)** Explants in culture media. **F)** Established shoots in the germination chamber.

The cultures were growth at 25 ± 2 °C, with a light intensity of 1000-3000 lux of white light lamps during 16 h, in the germination chamber TECNAL TE (4020Le-D) (Figure 4F). The establishment procedure was used in the shoot induction, multiplication and rooting stages.

3.4. Shoot induction and Multiplication

After determining the best treatment for disinfection, the establishment stage was accomplished. In this phase, the variation in the concentrations phytohormones, such as 6-Benzyladenine (6-BAP) and Kinetine (KIN), was carried out. Six treatments were implemented, along with the control treatment. The treatments for shoot induction are detailed in Table 3.

Table 3. Treatments for shoot induction of sugarcane *in vitro*

Treatment	Description
T1	0.5mg/L 6-BAP
T2	0.5mg/L 6-BAP; 0.5mg/L KIN
T3	0.5mg/L 6-BAP; 1 mg/L KIN
T4	1 mg/L 6-BAP
T5	1 mg/L 6-BAP; 0.5mg/L KIN
T6	1 mg/L 6-BAP; 1 mg/L KIN
T0	No phytohormones

After 42 days of establishment, the viable shoots with their corresponding sprouts, were passed to the multiplication phase. During all this stage, the explants with oxidized medium or a deeply black appearance (Figure 5A) were changed to fresh medium. In average, the medium was changed every 20 days (Figure 5B), which composition was the same as the introduction medium, with the difference in the concentration of phytohormones 6-Benzyladenine (6-BAP) and Kinetine (KIN). Likewise, temperature and photoperiod conditions were maintained as same as establishment.

In the shoot induction and multiplication stage, survival, contamination, oxidation, and viability percentages, and the number of new shoots were evaluated after 30 days (Figure 5C). The survival shoots continued in the multiplication and change at least 6 subcultures (Figure 5D).

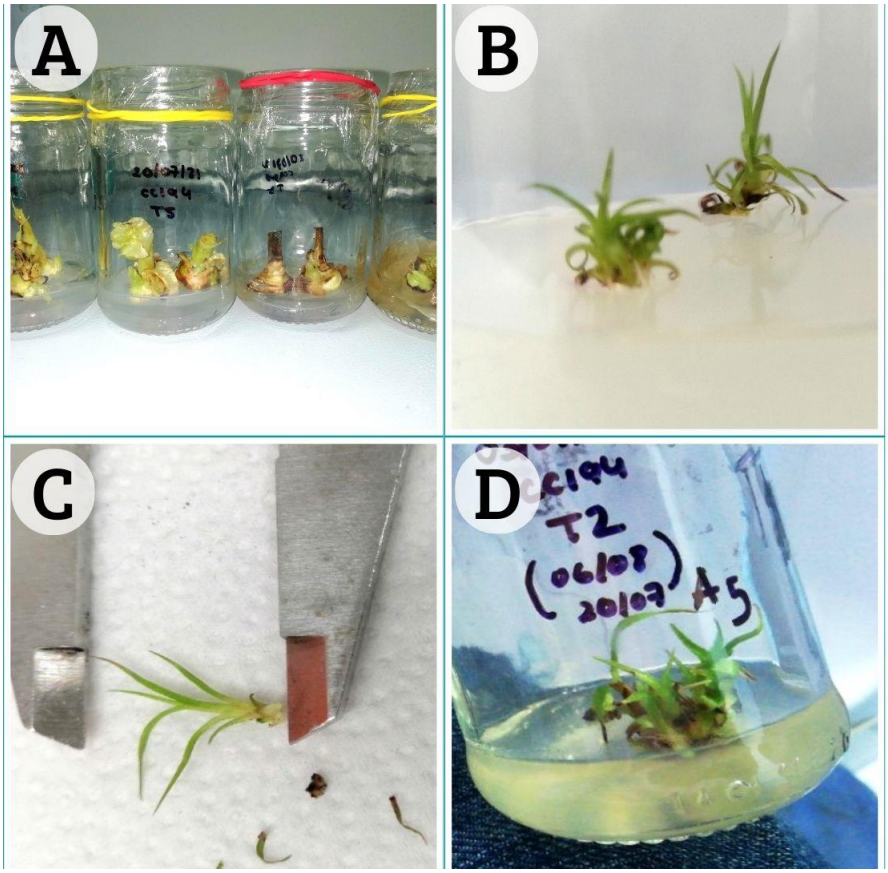

Figure 5. Shoot induction and multiplication *in vitro* of sugarcane. A) New shoots in explants. B) Sub-culturing of new shoot tips. C) Measuring the length of plants. D) Multiplication of new shoots.

The Figure 6 presents some pictures representing the parameters evaluated in the disinfection stage. The first parameter considered all alive explants; for example, an explant that was contaminated but survived could be counted among the percentage of survivors (Figure 5A). The contamination percentage evaluated the presence of fungi, bacteria, or any other pathogen in the

medium and explant (Figure 6B). In the Figure 6A, it can be seen the oxidation of explants and contamination presence (Figure 6B) after 15 days. The presence of brown and black coloration at the base and aerial part of the explants is recognized as oxidation. Thus, the viability percentage only considered live explants with low oxidation and no contamination (Figure 6B).

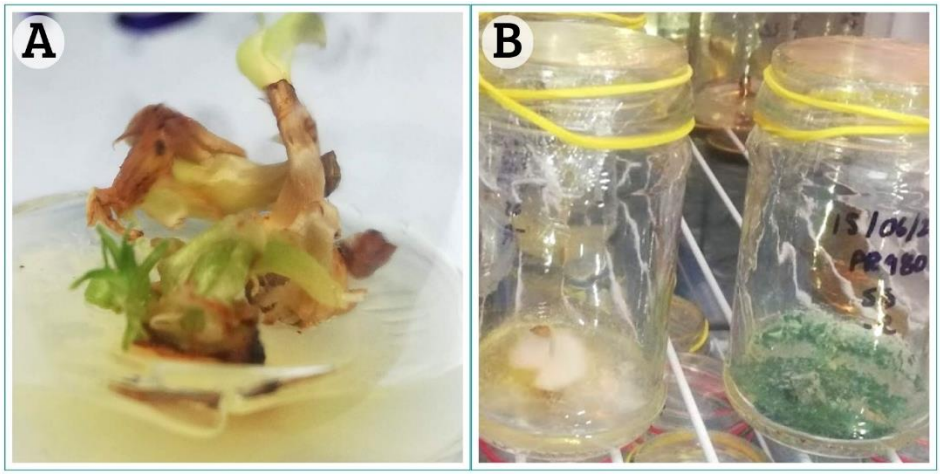

Figure 6. Parameters evaluated in shoot induction and multiplication stage.

3.5. Elongation and rooting

After reaching a good number of plants in multiplication, the best plants passed to the elongation and rooting stage (Figure 7A). The medium compounds for this stage vary in concentrations of phytohormones, such as 6-BAP and Naphthaleneacetic acid (NAA) and the basal medium (Murashige-Skoog salts with vitamins (519). However, all the mediums contained sucrose (30 g/L), Agar-agar (8 g/L) and 50mg/L cysteine. The treatments used are detailed in Table 4.

Table 4. Treatments for rooting and elongation of *in vitro* sugarcane Micropropagation

Treatments	Murashige-Skoog concentration Completed=1; Half =1/2	6-BAP mg/L	NAA mg/L
T1	1	0	0
T2	1	0	1
T3	1	0	2
T4	1	0	3
T5	1	0.5	0
T6	1	0.5	1
T7	1	0.5	2
T8	1	0.5	3
T9	½	0	0
T10	½	0	1
T11	½	0	2
T12	½	0	3
T13	½	0.5	0
T14	½	0.5	1
T15	½	0.5	2
T16	½	0.5	3

After two weeks, the oxidation percentages, contamination, and viability percentages were evaluated. Likewise, root length, number of roots and plant length were evaluated (Figure 7, C and D). After rooting, all the survival and plants with good appearance were placed in a medium without phytohormones until the next stage.

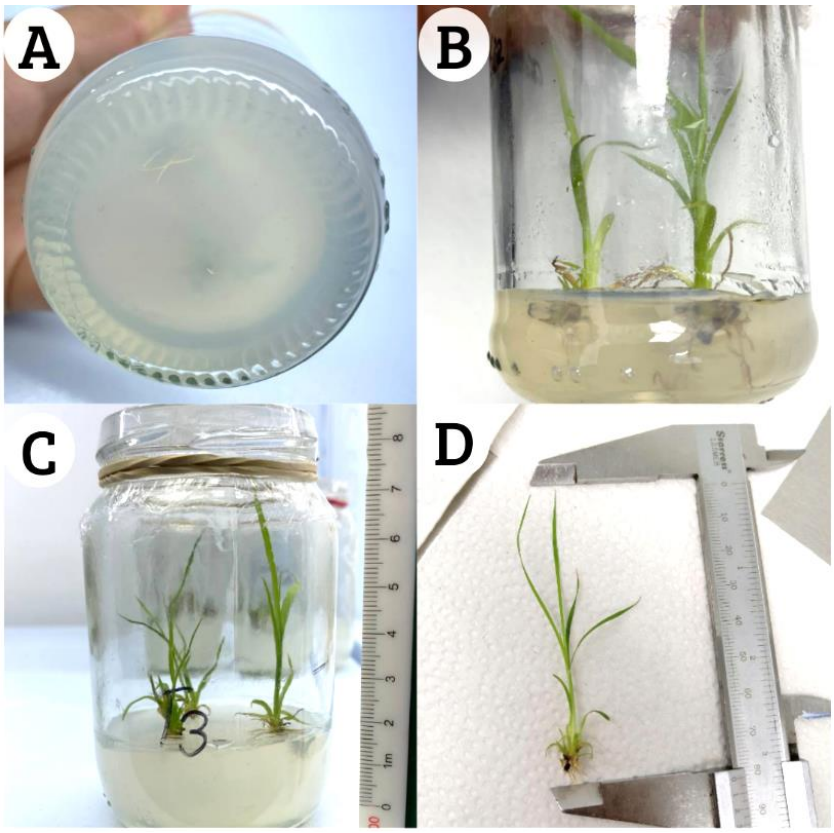

Figure 7. ***In vitro*** **sugarcane rooting and elongation process. A)** New roots in shoots. **B)** and **C)** Elongation and rooting of sugarcane**. D)** Measuring the plant length and root length.

Starting from this point, the multiplication stage could be conducted for as long as possible. Thus, the medium used for the following plants was MS basal medium, 30g/L sucrose, 6g/L Agar-agar, 0.15g/L cysteine, 0.15g/L citric acid, 0.5mg/L NAA, 0.1mg/L GA3, and 500ul PPM. In the multiplication of new shoots, a solution of 1 g/L of cysteine, 1g/L of citric acid, and 0.5 ml/L of PPM were used in the change of medium.

3.6. Acclimatation

The next step for the *in vitro* plants is the gradual adaptation to *ex vitro* conditions, starting from greenhouse to open-field conditions for autotrophic life. The acclimatization stage began in July 2022, where different effects of substrates on plant survival and development were tested. The project was based on measuring some anatomical and physiological indicators of the plant to recognize the most efficient treatment. This study considered the use of three treatments: Peat of vermiculite and perlite (1:1, v/v%); Coconut coir and perlite (1:1, v/v%); and Peat moss, peat of vermiculite and vermiculite (2:1:1, v/v%), summarized in Table 5 below.

Table 5. Substrates evaluated for sugarcane acclimatization

Treatment	Coding	Composition
T1	T_{vp}	Peat of vermiculite and perlite (1:1, v/v%)
T2	T_{CP}	Coconut coir and perlite (1:1, v/v%)
T3	T_{PM}	Peat moss, peat of vermiculite and vermiculite (2:1:1, v/v%)

The acclimatization process is shown in Figure 8. The *in vitro* plants were transplanted to *ex-vitro* conditions in a laminar flow chamber to plastic cups (previously decontaminated) with 25 g of the substrate (Figure 8A). Plants with 5 to 10 centimeters of the foliar area were considered for transplanting. Roots were washed with warm autoclaved distilled water (around 60 °C or less) (Figure 8A). The senescent leaves were removed, and the remaining dry tips were pruned. Next, the plants were weighed and measured from the neck to the longest leaf and root using millimetric paper.

The autoclaved substrates were distributed in plastic cups, and properly irrigated with autoclaved distilled water, before and after the transplant. The plants were taken to the incubation area during the first days, under a photoperiod of 16 hours of light and 8 hours of darkness, under an artificial light intensity of 1000-3000 lux. The plants were covered with a plastic dome to maintain a high relative humidity percentage (RH%) of 74-90% and a temperature of 22°C for the first week (Figure 8B). Next, the plants were passed gradually to the greenhouse conditions (Figure 8B). In this way, the light intensity and the temperature increased while the RH% decreased. During this time, the plants received proper irrigation of distilled water until soil saturation (around 80%).

Pruning at the time of transplanting and every two weeks was also considered to eliminate dead leaves and dry tips and inhibit contamination. Fine-tipped scissors, sprayed with 70% alcohol were used to remove the dry tips or leaves with signs of pathogens, such as stains. In the transplanting days were applied Tachigaren® fungicide 1g/L, rooting agent (Raizante) 1mg/L, and anti-stress (AGRIMELAZA) 10ml/L (Figure 8A). The plants were properly irrigated with anti-stress 5 and 15 days after transplanting, for a total of three times. Likewise, the fungicide was applied eight days after transplanting, and inorganic fertilizer (Rancho Alegre) 0.2g per plant, was applied by soil one time seven days after transplanting with water irrigation.

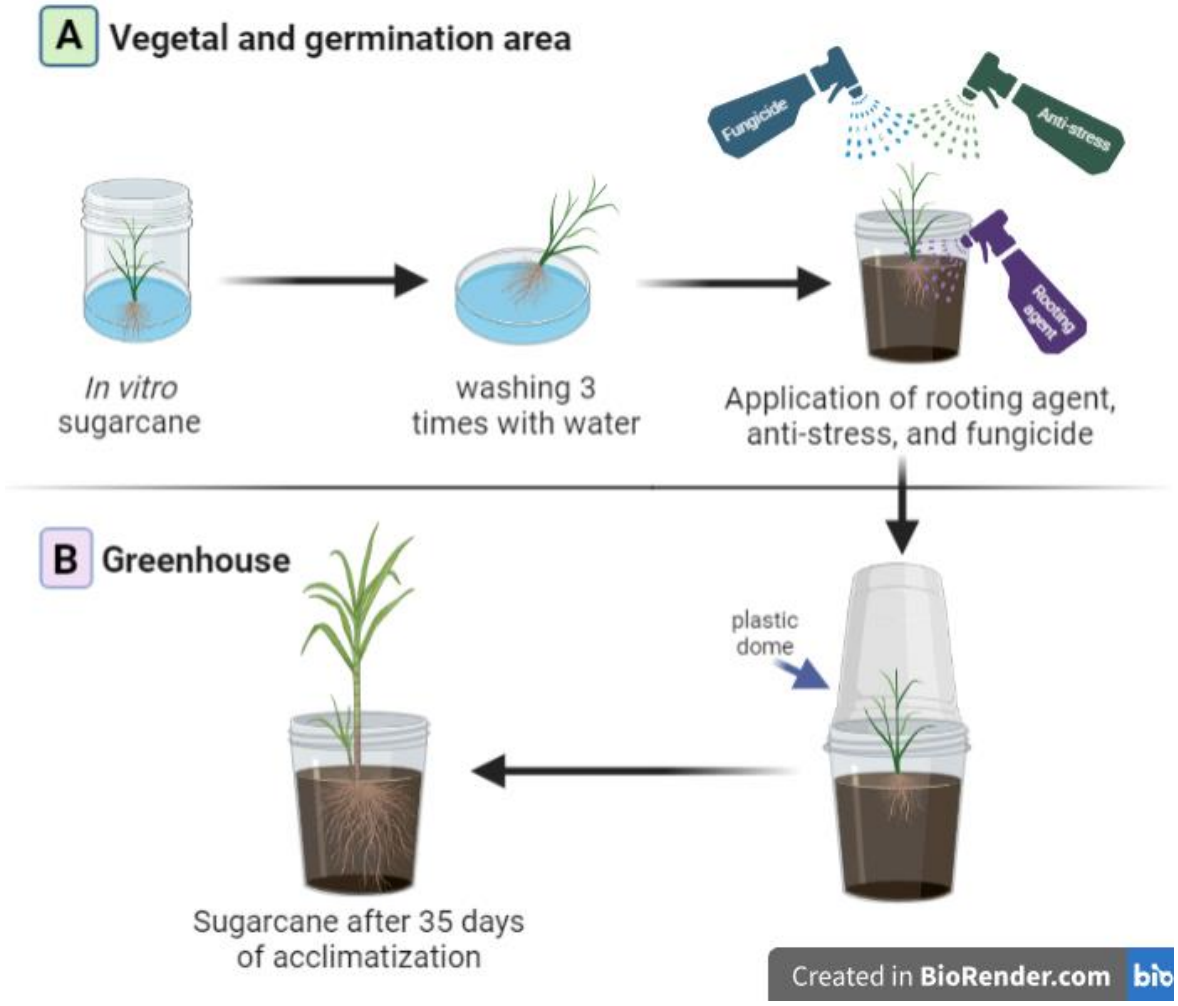

Figure 8. Acclimatization process of sugarcane *in vitro* protocol. (Created with BioRender.com)

To identify the adequate substrate treatment, every 7 days were recorded data of survival percentage, contamination percentage and appearance until day 35. For example, Figure 9 shows the contamination of substrates and plant in acclimatation; while Figure 10 presents the rank of appearance of sugarcane after 35 days. In the day of transplanting, we registered the weight of the whole plant, the length of the foliar area, and the length of the root. After 35 days, all the parameters mentioned before were measured, including the number of nodes and the length, foliar area, weight of leaves, stem and roots, and the fresh and dry mass.

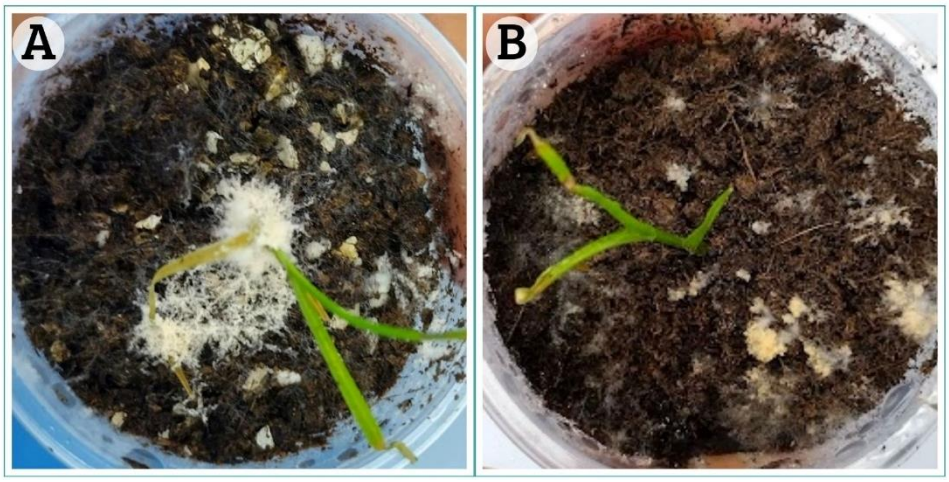

Figure 9. Contamination is acclimatization of sugarcane. **A)** Contamination in T_{PM} : Peat moss, peat of vermiculite and vermiculite (2:1:1, v/v%). **B)** Contamination in T_{vp}: Peat of vermiculite and perlite (1:1, v/v%)

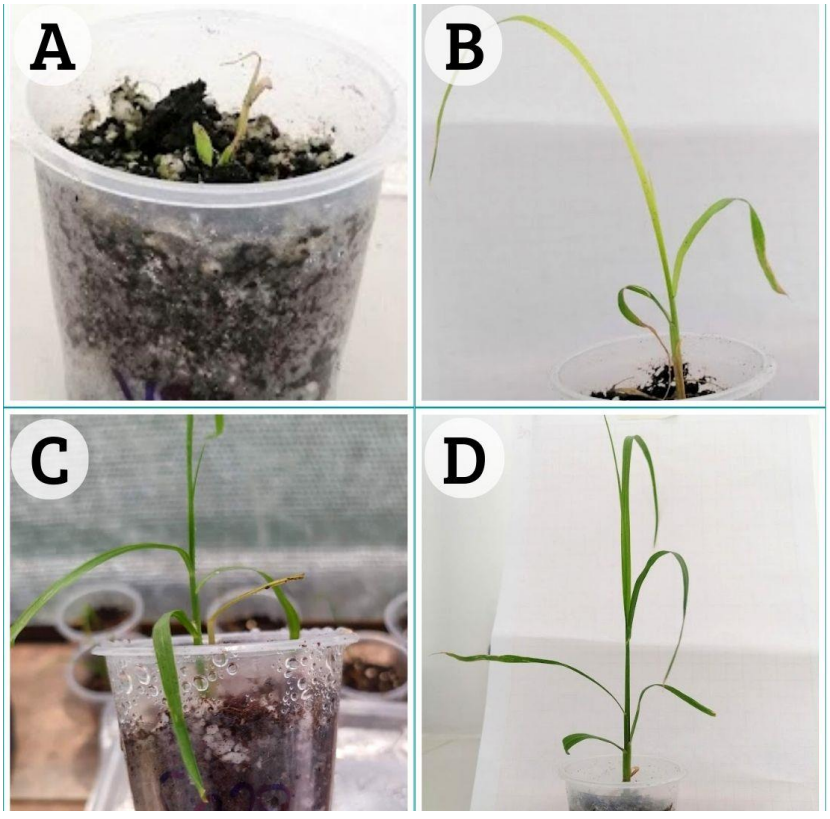

Figure 10. Appearance parameter evaluation of sugarcane after 35 of acclimatization. Appearance 0, 1, 2 and 3 (A, B, C, and D, respectively).

3.6.1. Sterilization of materials and equipment

To avoid contamination, tools and equipment were cleaned before usage. Likewise, all sources of microbiological or chemical contamination by water and laboratory areas were monitored. The transplanting chamber was cleaned with 70% alcohol and placed under ultraviolet light for 20 minutes, to irradiate the substrates, plastic cups (with holes in the base), petri dishes, napkins, tweezers, and autoclaved water for washing. The substrates were autoclaved at 120 ºC at 1.0-atmosphere pressure for 20 minutes. Before placement, all domes were cleaned with detergent, alcohol 70% and sprayed with Tachigaren® fungicide 1 g/L.

3.6.2. Greenhouse conditions

The greenhouse in which the acclimatized plants were rested is 2.02 meters long, 1.85 meters wide, and 2.5 meters high. This space was covered by polyethylene plastic and a 65% Shade Cloth Net. To maintain the temperature (T) and the relative humidity percentage (RH%), the greenhouse had a semi-automated system, with 20°C to 35°C, and controlled ventilation during the day, while a heater was implemented at nights. Humidity in air and substrate was maintained with the greenhouse watering system, working 2 minutes every 30 min, from 7 am to 7 pm. These parameters were recorded by a wireless system composed of a smart hygrometer and controlled by the Life Smart App (Version 4.4.2) by Volcano Technology Limited.

3.7. Statistical analysis

A Complete Randomized Experimental Design (CRD) was performed in the disinfection, shoot induction, rooting, and acclimatization stages. Depending on the phase, one explant, shoot, or plant was one experimental unit (EU). Therefore, the disinfection and shoot experiment had six treatments (EU=8) with three replicates. Rooting managed sixteen treatments (EU=8) with three

replicates each, where Treatment 9 was taken as the control. Then, acclimatization established three treatments (EU=20) with three replicates.

The results were examined by a simple classification system, a Logistic model (Logit) ($p \leq 0.05$), and the analysis of the odds ratio performed in RStudio (version 2022.07.0).

This study used a logistic regression model to predict the odds and probabilities of the response and looked at the effect of individual parameters on the answer (25). Thus, this model allows the analysis of more than two proportions of data sets whose responses are binary. Likewise, significant coefficient values are associated with p-value < 0.05. Therefore, treatments with important values affect the evaluated parameters. For instance, a positive number means that a specific event or parameter is increasing or happening. Conversely, a negative coefficient is associated with a reduction in the relative risk of that event.

On the other hand, the proportion is the number of successes in a trial with n events. Thus, the proportion of each treatment for the specific parameter is explained with the odds ratio. However, the odds ratio is related to relative risk when the parameter is rare, and this value is multiplied by the probability of that event occurring.

4. RESULTS

4.1. Disinfection

Disinfection was carried out to eliminate the highest percentage of pathogens and, simultaneously, be careful with the explants, trying not to cause high oxidation, consequently favoring survival and viability. After three weeks of establishment, some indicators of the effectiveness were evaluated, following the percentage of survival (%S), contamination (%C), oxidation (%O) and viability (%V). Results indicate that treatments T5 and T6 had the highest survival percentage

(100%) (Table 6), corresponding to the immersion of explants in 1% NaClO for 10 and 15 minutes, respectively. Likewise, the treatments with the lowest contamination percentage were T1 (0.5% NaClO; 10 minutes) and T3 (0.75% NaClO; 10 minutes), with 4.17% and 12.50% of contamination, respectively (Table 6). Thus, the lowest oxidation level of 0% and the highest viability percentage of 95.83% was in T3.

Table 6. Results of disinfection treatments, varying concentrations of NaClO and immersion times.

Treatment	% Survival	% Contamination	%Oxidation	%Viability
T1	95.83	4.17	12.50	54.17
T2	87.50	29.17	12.50	58.33
T3	87.50	16.67	0.00	95.83
T4	87.50	12.50	29.17	75.00
T5	100.00	25.00	20.83	66.67
T6	100.00	25.00	12.50	4.17

Note: Treatments vary in sodium hypochlorite (NaClO) concentration and immersion times. (T1: 0.5% NaClO; 10min, T2: 0.5% NaClO; 15min, T3: 0.75% NaClO; 10min, T4: 0.75% NaClO; 15min, T5: 1% NaClO; 10min and T6: 1% NaClO; 15min).

Until this point, only T3 had the best results oxidation and viability parameter. In addition, T3 survival percentage was 87% and presented contamination of 16.67%, which was suitable in contrast to the other treatments in this stage. Then, T1 had the lowest contamination percentage and a 95.8% of survival plants, but with this treatment viability went down to 54.17%. T5 and T6 had the best survival percentage, but the contamination reaches 25% for both, and the most deficient parameter was the viability percentage, with 66.67% and 4.17%, respectively. Some

observations showed an orange oxidation at the beginning in all explants, and then a brown to black oxidation appeared in plants of some treatments. However, the treatment with the highest oxidation of 29.17% was T4 (0.75% NaClO; 15min), which also had 12.5% of contamination and 75% of viability.

For the statistical analysis, logistic regression model was employed. Table 7 presents the coefficients with their standard errors (SE), where the intercept was significant for all the parameters. In other words, it is significant where the rest of the treatments are in the reference levels. Only the contamination variable has one statistically significant treatment; it was T2 by a positive influence in this variable.

Table 7 Disinfection of sugarcane with variation of NaClO concentration and time of immersion, after 3 weeks.

Treatment	Survival	Contamination	Oxidation	Viability
Intercept	3.14 ± 1.02 **	-3.14 ± 1.02 **	-1.95e+00 ± 6.17e-01 **	0.89 ± 0.45 *
T2	-1.19 ± 1.19	2.25 ± 1.12 *	6.11e-01 ± 7.96e-01	-0.72 ± 0.61
T3	-1.19 ± 1.19	0.74 ± 1.26	-1.66e+01 ± 1.33e+03	-0.55 ± 0.61
T4	-1.19 ± 1.19	0.74 ± 1.26	-4.52e-01 ± 9.63e-01	0.45 ± 0.67
T5	16.43 ±2195.15	2.04 ± 1.12	1.06e+00 ± 7.63e-01	-0.19 ± 0.62
T6	16.43 ± 2195.15	2.04 ± 1.12	-4.07e-15 ± 8.73e-01	-0.19 ± 0.62

Note: Values are coefficients ± SE. * = statistically different ($p<0.05$) (T1: 0.5% NaClO; 10min, T2: 0.5% NaClO; 15min, T3: 0.75% NaClO; 10min, T4: 0.75% NaClO; 15min, T5: 1% NaClO; 10min and T6: 1% NaClO; 15min).

The coefficients were used to calculate exponentiates and interpret them as odds ratios. Table 8 shows the odds ratios and confidence intervals, considering the survival and contamination. To understand the statistical explanation of the odds ratio, plant was considered as an experimental unit. Thus, for one unit increase in T5 and T6, the odds of survival increase by a factor of 1.36E+07:1 in each treatment. In contrast, by one unit increase in T2, T3, and T4, the odds ratio of death is 3.33:1. In contamination, the major odds ratio in survival per one unit of increase was in T2, by a factor of 9.47:1. On the other hand, the major odds of death by contamination were in the intercept, when the remains of the treatments were in the reference levels.

Table 8. Survival and contamination data of disinfection treatments of sugarcane, odds ratio.

Treatment	Survival			Contamination		
	OR	**CI 2.5%**	**CI 97.5%**	**OR**	**CI 2.5%**	**CI 97.5%**
Intercept	2.30	4.85	411.56	0.04	0.00	0.21
T2	0.30	1.44	2.58	9.47	1.49	185.89
T3	0.30	1.44	2.58	2.09	0.19	46.89
T4	0.30	1.44	2.58	2.09	10.19	46.89
T5	1.36e+07	5.69e-70	$e^{4406.73}$	7.67	1.17	151.78
T6	1.36e+07	5.69e-70	$e^{4406.73}$	7.67	1.17	151.78

Note: OR: Odds ratio, **CI:** Coefficient interval. (T1: 0.5% NaClO; 10min, T2: 0.5% NaClO; 15min, T3: 0.75% NaClO; 10min, T4: 0.75% NaClO; 15min, T5: 1% NaClO; 10min and T6: 1% NaClO; 15min).

In the Table 9, it can be seen that the major increase in the odds ratio per unit increase in oxidation parameter was in T5 by a factor of 2.88:1. In contrast, one unit increase in T3 means no oxidation by a factor of 16.52E+6:1, the major reported. In the viability, excluding the intercept, the major

odd ratio was in T4, where, by one unit of increase, the odds of viability were 1.56:1; in contrast, by one unit increase in T2, the odds of no viability were 2.04:1.

Table 9. Oxidation and viability data of disinfection treatments of sugarcane, odds ratio

Treatment	Oxidation			Viability		
	OR	**CI 2.5%**	**CI 97.5%**	**OR**	**CI 2.5%**	**CI 97.5%**
Intercept	0.142	0.03	0.41	2.43	1.05	6.28
T2	1.84	0.39	9.98	0.49	0.14	1.58
T3	6.05e-08	e^{-19418}	2.68e+27	0.58	0.17	1.89
T4	0.64	0.08	4.21	1.56	0.42	6.18
T5	2.88	0.69	10.49	0.82	0.24	2.81
T6	1	0.17	5.95	0.82	0.24	2.81

Note: OR: Odds ratio, **CI:** Coefficient interval. (T1: 0.5% NaClO; 10min, T2: 0.5% NaClO; 15min, T3: 0.75% NaClO; 10min, T4: 0.75% NaClO; 15min, T5: 1% NaClO; 10min and T6: 1% NaClO; 15min).

4.2. Shoot induction

The disinfection treatment used to move on to shooting stage was T3 (0.75% NaClO; 10min). This treatment was selected considering the high viability of explants with less contamination; also, it had an acceptable survival percentage after discarding the contaminated explants. In this context, viability represents the vigor of plant during time according to oxidation and contamination occurrence.

After one month, the same parameters of the disinfection phase (%S, %C, %O, and %V) were registered to evaluate the effectiveness of the selected disinfection treatment. This explants

establishment aimed to notice the influence of various phytohormones concentrations, such as 6-BAP and KIN. According to Table 10, T1(0.5mg/L 6-BAP) had the highest survival percentage of 96.67%, followed by 83.33% of T6 (1 mg/L 6-BAP; 1 mg/L KIN). Then, the 0% of contamination was registered in T2(0.5mg/L 6-BAP) and T5(1 mg/L 6-BAP; 0.5mg/L KIN). Additionally, T4 (1 mg/L 6-BAP) presented the lowest oxidation of 4.17%, followed by 10.83% of T1. Likewise, T3 (0.5mg/L 6-BAP) had the highest viability percentage of 35,83%, followed by T6 with 33.33% of survival.

Table 10. Results of established plants in treatments with different concentrations of 6-BAP and KIN.

Treatment	% Survival	% Contamination	%Oxidation	%Viability
T0	70.83	19.17	11.67	15
T1	96.67	16.67	10.83	32.5
T2	80	0	19.16	32.5
T3	54.83	12.5	22.5	35.83
T4	55.83	11.67	4.17	20.83
T5	70.83	0	16.67	29.17
T6	83.33	29.16	6.67	33.33

Note: Treatments vary in phytohormones concentration. (T0: no phytohormones, T1: 0.5mg/L 6-BAP, T2: 0.5mg/L 6-BAP; 0.5mg/L KIN, T3: 0.5mg/L 6-BAP; 1 mg/L KIN, T4: 1 mg/L 6-BAP, T5: 1 mg/L 6-BAP; 0.5mg/L KIN and T6: 1 mg/L 6-BAP; 1 mg/L KIN)

Furthermore, the number of shoots after one month were recorded, which did not exceed 0.66 shoots in T3 and 0.58 in T2. In general, the oxidation level and contamination in this stage were

low (Table 10), as well as the viability percentage, which did not pass 35% in any treatment. In general, the contamination percentage does not pass 29.16%.

Table 11 presents the coefficients, standard errors and the evaluation of each variable for 6 treatments. All the dependent variables had significant differences in the intercept, where all the treatments were in their reference levels. T1 had a significant coefficient in Survival, while T1, T2 and T3 had significant differences in the number of shoots variable.

Table 11. Establishment of sugarcane with variation of 6-BAP and KIN, after 1 month.

Treatment	Survival	Contamination	Oxidation	Viability	Nr. Shoots
Intercept	9.98 ± 0.44*	-1.43± 0.49 **	-0.20 ± 0.61 ***	-1.70 ± 0.54 **	-2.03±0.61***
T1	2.22 ± 1.11*	0.27 ± 0.74	5.87e-15 ± 0.86	1.07 ± 0.68	2.19 ± 0.73**
T2	0.71 ± 0.07	-18.13 ±2109.03	0.60 ± 0.79	0.50 ± 0.71	1.72 ± 0.73*
T3	-0.69 ± 0.59	-0.60 ± 0.79	0.83 ± 0.77	1.23 ± 0.68	2.35 ± 0.73**
T4	-0.68 ±0.59	-0.60 ± 0.79	-1.18 ± 1.19	0.50 ± 0.71	1.23 ± 0.75
T5	-9.94e-16±0.63	18.13 ±2109.03	0.33 ± 0.82	0.89 ± 0.69	1.40 ± 0.74
T6	0.71 ± 0.7	0.43 ± 0.67	-0.45 ± 0.96	1.07 ± 0.68	0.60 ± 0.79

Note: Values are coefficients ± SE * = statistically different ($p<0.05$) (T0: no phytohormones, T1: 0.5mg/L 6-BAP, T2: 0.5mg/L 6-BAP; 0.5mg/L KIN, T3: 0.5mg/L 6-BAP; 1 mg/L KIN, T4: 1 mg/L 6-BAP, T5: 1 mg/L 6-BAP; 0.5mg/L KIN and T6: 1 mg/L 6-BAP; 1 mg/L KIN)

Survival, contamination, and oxidation variables differed in their odds ratio in Table 12. In survival, one unit increase in T1 meant a 9.21:1 factor of increase, in contrast with T3 and T4, where one unit increase meant a 2:1 factor of no survival. In contamination, one unit increase in T6 referred to an increase factor of 1.54:1; in contrast to T2 or T5, one unit increase meant 75.18E+06:1 of no contamination in each treatment. In Oxidation, the major odd ratio was in T3 with a factor of a 2.30:1 per unit increase, while (excepting the intercept) T3 had a factor of 3.22:1 of no oxidation per unit increase.

Table 12. Survival, contamination and oxidation of sugarcane with variation of 6-BAP and KIN, odds ratio.

Treatment	Survival			Contamination			Oxidation		
	OR	**CI 2.5%**	**CI 97.5%**	**OR**	**CI 2.5%**	**CI 97.5%**	**OR**	**CI 2.5%**	**CI 97.5%**
Intercept	2.71	1.19	6.95	0.23	0.08	0.58	0.13	0.03	0.37
T1	9.21	1.46	179.95	0.76	0.17	3.27	1.00	0.17	5.90
T2	2.06	0.53	8.75	1.33e-08	$e^{-42454.77}$	1.01e+45	1.83	0.39	9.79
T3	0.50	0.15	8.75	0.54	0.10	2.51	2.30	0.53	12.03
T4	0.50	0.15	1.59	0.54	0.10	2.51	0.31	0.01	2.58
T5	1.00	0.29	3.46	1.33e-08	$e^{34018.65}$	1.01e+45	1.39	0.28	7.75
T6	2.03	0.53	8.75	1.54	0.42	6.02	0.64	0.08	4.19

Note: OR: Odds ratio, **CI:** Coefficient interval. (T0: no phytohormones, T1: 0.5mg/L 6-BAP, T2: 0.5mg/L 6-BAP; 0.5mg/L KIN, T3: 0.5mg/L 6-BAP; 1 mg/L KIN, T4: 1 mg/L 6-BAP, T5: 1 mg/L 6-BAP; 0.5mg/L KIN and T6: 1 mg/L 6-BAP; 1 mg/L KIN)

In Table 13, all treatments had a positive increase, but the major was in T3, with an increase of 3.43:1 per unit. Thus, the number of shoots had positive factors in all treatments, but the major was in T3 with 10.45:1.

Table 13. Viability and number of shoots of sugarcane with variation of 6-BAP and KIN, odds ratio.

Treatment	Viability			Nr Shoots		
	OR	**CI 2.5%**	**CI 97.5%**	**OR**	**CI 2.5%**	**CI 97.5%**
Intercept	0.18	0.05	0.47	0.13	0.03	0.37
T1	2.91	0.80	12.27	8.94	2.38	44.63
T2	1.65	0.41	7.26	5.62	1.47	28.03
T3	3.43	0.96	14.39	10.45	2.77	52.41
T4	1.65	0.41	7.27	3.41	0.85	17.30
T5	2.44	0.65	10.41	4.06	1.03	20.42
T6	2.91	0.80	12.28	1.83	0.39	9.79

Note: OR: Odds ratio, **CI:** Coefficient interval (T0: no phytohormones, T1: 0.5mg/L 6-BAP, T2: 0.5mg/L 6-BAP; 0.5mg/L KIN, T3: 0.5mg/L 6-BAP; 1 mg/L KIN, T4: 1 mg/L 6-BAP, T5: 1 mg/L 6-BAP; 0.5mg/L KIN and T6: 1 mg/L 6-BAP; 1 mg/L KIN)

From this phase, the new viable sprouts passed to being new shoots for the multiplication phase until to obtain a maximum of six subcultures of the more shoots (Figure 11, A and B).

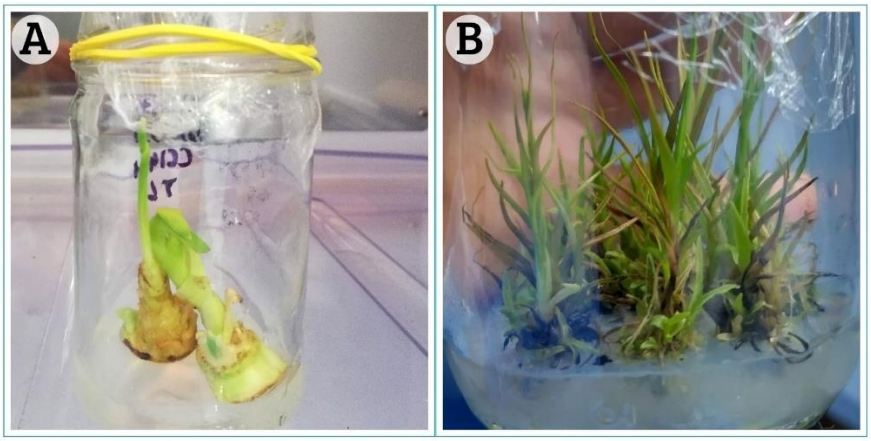

Figure 11. New shoot tips and multiplication of sugarcane *in vitro*. A) New shoots tips after two weeks of establishment. B) Multiplication of sugarcane.

4.3. Rooting

All shoots from the multiplication phase were used in this stage as if they were all homogeneous. Previously, all the plants were establishment in a basal medium without phytohormones. The rooting phase aimed to appear new roots, recording their length and number. Likewise, some parameters of previous phases (%S, %C and %O) were analyzed to enhance the plant management protocol through micropropagation. Thus, we searched alternatives for antioxidant application and plant management to obtain a great number of vigorous plants for acclimatization.

According to the Table 14, after two weeks, the treatments with 100% survival were T2 (MS + NAA 1.0mg/L), T7 (MS + BAP 0.5mg/L + NAA 2.0mg/L) and T14 (½MS + BAP 0.5mg/L + NAA 1.0mg/L). Then, T1 (MS), T3 (MS+ NAA 2.0mg/L), T4 (MS + KIN 3.0mg/L), T10 (½MS + KIN 1.0mg/L) until T14 had 0% contamination. Thus, the highest oxidation was 97.22%, corresponding to T9 (½ MS). The survival and contamination percentage at this phase were generally satisfactory; the minimum was 88.54% and the maximum 25%, respectively. However, all the treatments presented more than 75% of oxidation in their explants. Even though there was

no direct relation between phytohormones variation and these parameters, it was necessary to search for a new way to reduce the high oxidation by Phenolization.

Table 14. Rooting of sugarcane *in vitro* micropropagation after two weeks.

Treatment	% Survival	% Contamination	%Oxidation
T1	96.87	0	85.76
T2	100	9.37	75
T3	95	0	82.5
T4	100	0	93.75
T5	95.43	6.25	77.28
T6	96.88	12.5	87.5
T7	100	8.33	88.54
T8	96.88	8.33	76.98
T9	88.7	3.84	97.22
T10	96.87	0	89.58
T11	90.63	3.13	92.71
T12	88.54	0	81.59
T13	96.88	0	79.91
T14	100	0	81.77
T15	91.67	25	91.67
T16	92.5	8.33	88.54

Note: Survival, contamination and oxidation percentage of sugarcane in rooting phase after 2 weeks. (All the treatments are detailed in Table 4).

The logistic regression (Logit) of sugarcane survival, oxidation, contamination, and appearance in rooting of sugarcane *in vitro* is represented in Table 15. There were no significant values at survival; the intercept was the only significant in contamination and appearance variables. In the oxidation variable, T2, T5, T8, T12, and T13 had significant values, but they all influenced oxidation negatively, which means they influenced no oxidation.

Table 15. Rooting of sugarcane *in vitro* with variation of 5-BAP, NAA and MS, 2 weeks

Treatment	Survival	Oxidation	Contamination	Appearance
Intercept(T9)	2.14 ± 0.53	3.61 ± 1.01***	-2.89 ± 0.72***	-2.89 ± 0.73***
T1	1.33 ± 1.15	-1.88 ± 1.12	-17.68 ± 3086.4613	-17.68 ± 3086.46
T2	17.43 ± 1722.00	-2.67 ± 1.07*	0.41 ± 0.94	0.41 ± 0.94
T3	0.57 ± 0.90	-1.92 ± 1.12	-17.68 ± 3134.32	-17.68 ± 3134.32
T4	17.43 ± 1722.00	-1.12 ± 1.18	-17.68 ± 2839.13	-17.68 ± 2839.13
T5	0.88 ± 0.90	-2.41 ± 1.08*	0.3 ± 0.94	0.3 ± 0.94
T6	1.29 ± 1.15	-1.34 ± 1.18	0.62 ± 0.947	0.62 ± 0.95
T7	0.17 ± 1931.00	-1.70 ± 1.15	0.66 ± 0.95	0.66 ± 0.95
T8	1.29 ± 1.14	-2.51 ± 1.09*	0.62 ± 0.95	0.62 ± 0.95
T10	1.52 ± 1.14	-1.67 ± 1.12	-17.68 ± 2803.4177	-17.66 ± 2803.42
T11	0.06 ± 0.81	-0.97 ± 1.25	-0.48 ± 1.25	-0.48 ± 1.25
T12	0.09 ± 0.81	-2.18 ± 1.11*	-17.68 ± 3184.47	-17.68 ± 3184.47
T13	1.23 ± 1.15	-2.22 ± 1.11*	-17.68 ± 3237.11	-17.68 ± 3237.11
T14	17.43 ± 1679.00	-2.03 ± 1.09	-17.68 ± 2769.02	-17.68 ± 2769.02
T15	0.90 ± 1.15	-0.56 ± 1.44	1.04 ± 0.96	1.04 ± 0.96

T16	0.23 ± 0.80	-1.56 ± 1.14	0.52 ± 0.94	0.52 ± 0.94

Note: Values are coefficients ± SE * = statistically different ($p<0.05$) (All the treatments are detailed in Table 4).

In Table 16, the Length plant variable did not have significant values in the coefficients of treatments. However, the Number of roots had significant values in T1 and T8, the first with a positive influence and the second with a negative influence. The Length of roots had significant values at T10 and T12, with significant values, while T16 had significant values but with a negative influence.

Table 16. Rooting and plant growing of sugarcane *in vitro* with variation of 5-BAP, NAA and MS, 2 weeks

Treatment	Length plant	# of Roots	Length roots
Intercept (T9)	22.57 ± 7.82E+03	-0.54 ± 0.34	-1.89 ± 0.48***
T1	1.21E-08 ± 1.15E+04	0.11 ± 0.49*	0.38 ± 0.66
T2	-4.99E-08 ± 1.10E+04	1.01 ± 0.47	1.52 ± 0.58
T3	-6.14E-08 ± 1.16E+04	0.29 ± 0.49	0.95 ± 0.62
T4	-2.49E-10 ± 1.10E+04	0.59 ± 0.46	0.82 ± 0.6
T5	-19.55 ± 7.82E+03	-0.41 ± 0.48	-17.68 ± 1639.97
T6	-1.68E-10 ± 1.16E+04	-0.93 ± 0.56	-17.68 ± 1901.06
T7	-4.89E-08 ± 1.17E+04	-0.35 ± 0.52	-17.68 ± 1931.48
T8	-20.62 ± 7.82E+03	-0.73 ± 0.54*	-17.68 ± 1901.06
T10	-19.62 ± 7.82E+03	1.16 ± 0.47	1.27 ± 0.58*
T11	1.10E-08 ± 1.18E+04	0.27 ± 0.49	1.19 ± 0.62

T12	1.14E-08 ± 1.17E+04	0.86 ± 0.49	1.56 ± 0.6**
T13	-4.90E-08 ± 1.18E+04	-0.65 ± 0.55	-17.68 ± 1963.41
T14	-1.26E-10 ± 1.09E+04	-0.73 ± 0.51	-17.68 ± 1679.49
T15	-4.97E-08 ±1.29E+04	-0.96 ± 0.65	-1.16 ± 1.13
T16	-5.01E-08 ± 1.13E+04	-0.52 ± 0.51	-17.68 ± 1817.76**

Note: Values are coefficients ± SE * = statistically different ($p<0.05$) (All the treatments are detailed in Table 4).

In Table 17, the survival variable had high odds ratio in T2, T4, T7, and T14 had a factor of increase of 3.70E+07:1. In oxidation, all the odds ratios were > 0 (zero), and T8 had a factor of 12.5:1, which meant an influence of no oxidation per unit increase in this treatment. The major odd ratio in contamination was in T15, with a factor of 2.84:1 per unit increase. In contrast, T1, T3, T4, T10, T12, T13, and T14 had a factor of 47.39E+06:1 per unit increase but related to no contamination. In Appearance, T15 had a factor of 2.84:1 per unit increase in this treatment; in contrast, T1, T3, T4, T10, T12, T13, and T14 had a factor of 47.39E+06:1 per unit increase, but referring to no appearance.

Table 17. Odds ratio of plant and root growing of sugarcane *in vitro*, varying 6-BAP, NAA and MS, 2 weeks

Treatment	Survival			Oxidation			Contamination			Appearance		
	OR	CI 2.5%	CI 97.5%	OR	CI 2.5%	CI 97.5%	OR	CI 2.5%	CI 97.5%	OR	CI 2.5%	CI 97.5%
Intercept	8.50	3.39	28.48	37.00	8.03	657.08	0.06	0.01	0.18	0.06	0.01	0.18
T1	3.76	0.52	75.76	0.15	0.01	1.43	2.11E-08	$e^{-6190.6}$	7.07E+67	2.11E-08	$e^{-6190.6}$	7.07E+67
T2	3.70E+07	3.95E-36	$e^{3461.43}$	0.07	0.00	0.38	1.50	0.24	11.90	1.50	0.24	11.90
T3	1.76	0.32	13.38	0.15	0.01	0.97	2.11E-08	$e^{-6190.6}$	1.05E+69	2.11E-08	$e^{-6190.6}$	1.05E+69
T4	3.70E+07	3.95E-36	$e^{3461.43}$	0.32	0.01	2.67	2.11E-08	$e^{-6190.6}$	6.27E+61	2.11E-08	$e^{-6190.6}$	6.27E+61
T5	2.41	0.44	18.16	0.09	0.00	0.50	1.35	0.21	10.69	1.35	0.21	10.69
T6	3.65	0.51	73.43	0.26	0.01	2.16	1.86	0.29	14.86	1.86	0.29	14.86

T7	3.70E+07	2.34E-41	$e^{3461.43}$	0.18	0.01	1.32	1.93	0.30	15.40	1.93	0.30	15.40
T8	3.65	0.51	73.43	0.08	0.00	0.48	1.86	0.29	14.86	1.86	0.29	14.86
T10	4.59	0.64	92.10	0.18	0.01	1.25	2.11E-08	$e^{-6190.6}$	8.38E+60	2.11E-08	$e^{-6190.6}$	8.38E+60
T11	1.06	0.22	5.76	0.37	0.02	4.14	0.62	0.03	6.79	0.62	0.03	6.79
T12	1.10	0.22	5.96	0.11	0.01	0.71	2.11E-08	$e^{-6190.6}$	1.77E+70	2.11E-08	$e^{-6190.6}$	1.77E+70
T13	3.41	0.47	68.76	0.11	0.01	0.68	2.11E-08	$e^{-6190.6}$	3.43E+71	2.11E-08	$e^{-6190.6}$	3.43E+71
T14	3.70E+07	4.55E-35	$e^{3461.43}$	0.13	0.01	0.79	2.11E-08	$e^{-6190.6}$	8.23E+42	2.11E-08	$e^{-6190.6}$	8.23E+42
T15	2.47	0.34	50.09	0.57	0.02	14.85	2.84	0.44	23.02	2.84	0.44	23.02
T16	1.25	0.26	6.78	0.21	0.01	1.51	1.69	0.26	13.43	1.69	0.26	13.43

Note: OR: Odds ratio, **CI:** Coefficient interval. (All the treatments are detailed in Table 4).

In table 18, it can be seen following dependent variables: the Length of the plant, the number, and the Length of the roots. However, any treatment had a positive odd ratio in the Length plant variable. This way, T5, T8, and T10 had a factor of 307.69E+06:1 per unit increase, but representing the no occurrence of the variable Length plant. The number of roots variable had a high odd ratio in T10, with a positive factor of increase of 3.18:1, per unit increase in this treatment. In contrast, T15 had a factor of 2.63:1 per unit increase but influenced the no occurrence of the number of roots variable. Then, T12 had a factor of increase of 4.77:1 per unit increase in the length roots variable, in contrast to treatments such as T5, T6, T7, T8, T13, T14, and T16 with a factor of 47.61E+06:1, per unit increase, but referring to the no occurrence of length roots.

Table 18. Odds ratio of sugarcane rooting growing *in vitro*, varying 6-BAP, NAA and MS, 2 weeks

Treatment	Length plant			Number of Roots			Length Roots		
	OR	**CI 2.5%**	**CI 97.5%**	**OR**	**CI 2.5%**	**CI 97.5 %**	**OR**	**CI 2.5%**	**CI 97.5%**
Intercept(T9)	6.31E+09	e^{-15662}	e^{15662}	0.58	0.29	1.11	0.15	0.05	0.35
T1	1.00E+00	0.00	5.43E+213	1.11	0.42	2.93	1.47	0.40	5.59
T2	1.00E+00	0.00	5.57E+204	2.74	1.11	7.05	4.59	1.56	15.68
T3	1.00E+00	0.00	3.27E+215	1.33	0.51	3.51	2.58	0.79	9.36

T4	1.00E+00	0.00	5.57E+204	1.80	0.73	4.55	2.28	0.72	8.03
T5	3.25E-09	$e^{-168521}$	1.47E+292	0.66	0.26	1.69	2.10E-08	7.20E-280	1.10E+16
T6	1.00E+00	0.00	3.27E+215	0.40	0.12	1.16	2.10E-08	9.88e-324	5.00E+19
T7	1.00E+00	0.00	2.47E+217	0.70	0.25	1.92	2.10E-08	0.00	4.53E+19
T8	1.11E-09	0.00	5.03E+291	0.48	0.16	1.36	2.10E-08	0.00	6.88E+18
T10	3.01E-09	0.00	1.36E+292	3.18	1.28	8.23	3.55	1.19	12.18
T11	1.00E+00	0.00	2.40E+219	1.31	0.49	3.51	3.30	1.02	11.92
T12	1.00E+00	0.00	2.47E+217	2.37	0.91	6.40	4.77	1.54	16.89
T13	1.00E+00	0.00	2.40E+219	0.52	0.17	1.49	2.10E-08	0.00	3.45E+20
T14	1.00E+00	0.00	1.85E+202	0.48	0.17	1.28	2.10E-08	3.67E-284	1.06E+17
T15	1.00E+00	0.00	4.36E+240	0.38	0.10	1.27	0.31	0.02	2.13

T16	1.00E+00	0.00	2.76E+210	0.59	0.21	1.60	2.10E-08	3.2164 14e-310	2.79E+18

Note: OR: Odds ratio, **CI:** Coefficient interval. (All the treatments are detailed in Table 4).

The rooting induction and elongation of sugarcane were performed before the acclimatization (Figure 12, A and B) for all the plants.

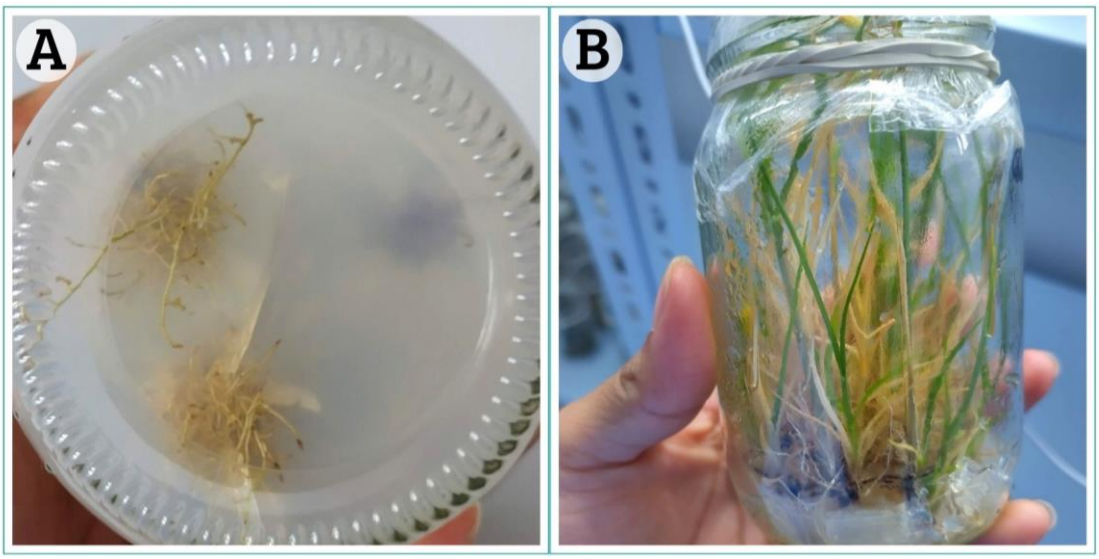

Figure 12. Results of rooting and elongation of sugarcane *in vitro*. A) Roots of sugarcane**. B)** Elongation of sugarcane.

4.4. Acclimatization

To estimate the effects of environmental conditions, change on plants from *in vitro* to *ex-vitro*, the survival and contamination percentage were ranked after five weeks. Table 19 shows that 64.12% survival using T_{vp} (Peat of vermiculite and perlite (1:1, v/v%)) as the highest in contrast to other treatments. Furthermore, the less contaminated plants correspond to 0% from T_{CP}(Coconut coir and perlite (1:1, v/v%)). The survival percentage of the other treatments was 36.67% for T_{PM} (Peat

moss, peat of vermiculite (2:1:1, v/v%)), and 33.33% for T_{CP}. On the other hand, 16.67% of contamination was obtained in T_{PM}, while T_{vp} had 3.5% contamination in the acclimatization experiment.

Table 19. Acclimatization of sugarcane after 5 weeks

Treatments	%Survival	%Contamination
$\boldsymbol{T_{vp}}$	64.12	3.5
$\boldsymbol{T_{PM}}$	36.67	16.67
$\boldsymbol{T_{CP}}$	33.33	0

Note:T_{vp}: Peat of vermiculite and perlite (1:1), T_{PM}: Peat moss, peat of vermiculite and vermiculite (2:1:1) and T_{CP}: Coconut coir and perlite (1:1), the proportions were v/v%.

Figure 13 shows the survival percentage recorded every seven days during 35 days. All the plants started from 100% on day 0, and until day seven, there were no significant differences among treatments. After 14 days, the survival started to differ between the three treatments; T_{PM} declined under 80%, T_{CP} had just more than 80%, and T_{vp} did not present a big difference. In the third week, T_{vp} had declined more, and the other treatments kept a similar percentage. In the fourth week, the plants of all the treatments had declined; T_{CP} and T_{PM} had just a little more than 60% of plants, while T_{vp} achieved 80% survival. Finally, after 35 days, T_{CP} and T_{PM} achieved the percentages described in Table 9 (33.33% and 36.67%, respectively), and T_{vp} achieved 64.12%.

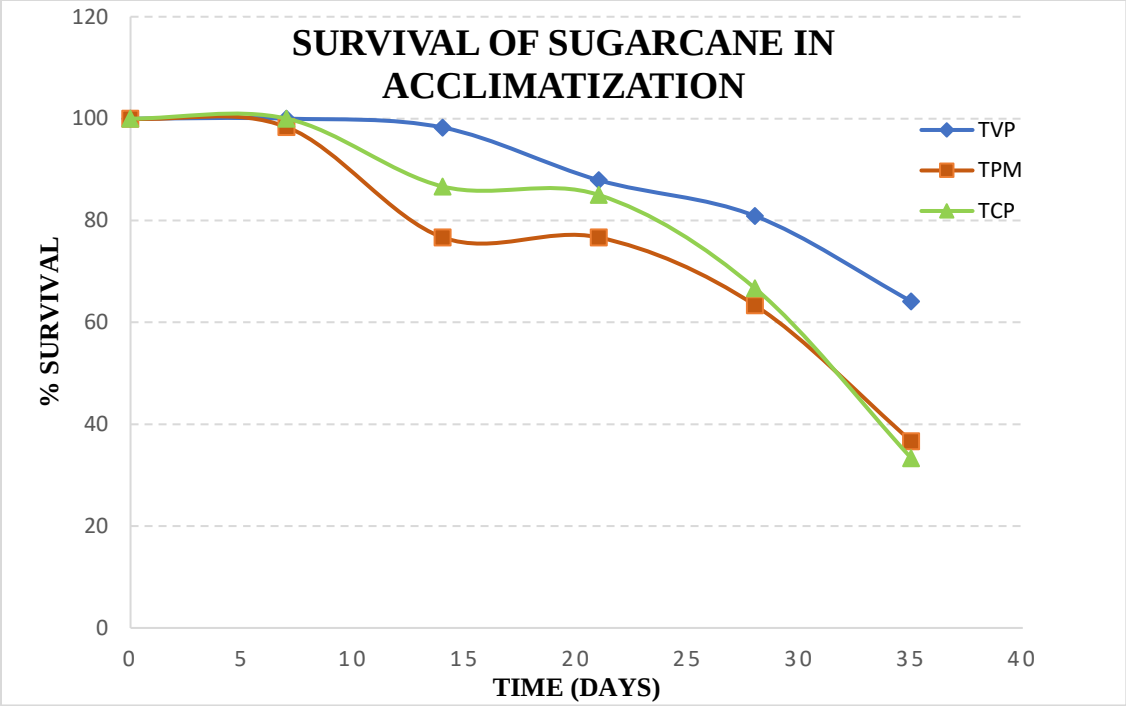

Figure 13. Survival percentage of sugarcane in acclimatization during 35 days. (T_{vp}: Peat of vermiculite and perlite (1:1), T_{PM}: Peat moss, peat of vermiculite and vermiculite (2:1:1) and T_{CP}: Coconut coir and perlite (1:1), the proportions were v/v%))

According to Figure 14, the contamination percentage recorded during 35 days of Acclimatization of sugarcane shows that after seven days of the transplanting, the treatment with the highest contamination was T_{PM} (40%), while the other treatments did not pass 5%. Seven days later, the contamination percentage was down for T_{PM}, which achieved 26.66%, T_{vp} kept the same, and T_{CP} changed to 0%. T_{PM} reduced to 16.66% of contamination from the third to the fifth week, while the other treatments remained almost constant.

The overcome contamination percentage was found in T_{PM} in the first week, reducing its percentage gradually after the second application of the fungicide Tachigaren. In the same way, the T_{PM} and T_{vp} remained almost constant during the 35 days. Discussing the Figure 14, T_{vp}

obtained a significant number of plants after 35 days with less contamination, in contrast with T_{PM}, which had a survival of 36.67% and the same treatment obtained the worst contamination during Acclimatization. T_{CP}, for its side, only obtained less contamination of 0% in general, and its plants obtained 33.33% survival.

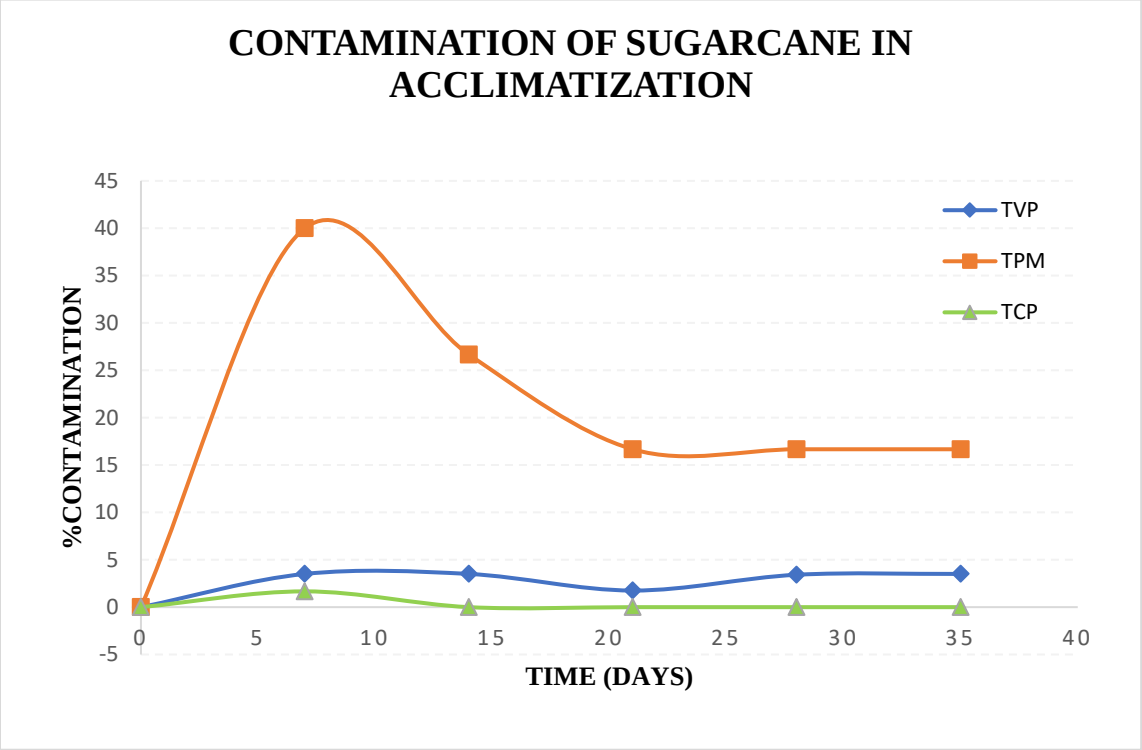

Figure 14. Contamination of sugarcane in acclimatization during 35 days. T_{vp}: Peat of vermiculite and perlite (1:1), T_{PM}: Peat moss, peat of vermiculite and vermiculite (2:1:1) and T_{CP}: Coconut coir and perlite (1:1), the proportions were v/v%.

T_{PM} was taken as the control to calculate a logistic model (Logit). Table 20 presented data on survival, contamination, and appearance of Acclimatization of sugarcane after 35 days, where it can be seen significant differences with T_{vp} in survival, with a positive value. Thus, the T_{vp} also showed a significant difference in the contamination variable but with a negative value. Finally, T_{vp} was also significant in the appearance variable, with a positive influence.

Table 20. Survival, contamination and appearance of sugarcane in Acclimatization, 35 days

Treatment	Survival	Contamination	Appearance
Intercept	-0.55 ± 0.26*	-1.61 ± 0.35 ***	-0.55 ± 0.27 *
$\boldsymbol{T_{vp}}$	1.17 ± 0.38 **	-1.76 ± 0.79 *	1.17 ± 0.38 **
$\boldsymbol{T_{CP}}$	-0.15 ± 0.38	-17.96 ± 1388.33	-0.30 ± 0.39

Note: Values are coefficients ± SE * = statistically different (p<0.05) (T_{vp}: Peat of vermiculite and perlite (1:1) and T_{CP}: Coconut coir and perlite (1:1), the proportions were v/v%))

According to Table 21, the high odd ratio in the survival variable was in T_{vp}, which implied an increase factor of 3.21:1 per unit increase. In contamination, all odds ratios were under 0; for instance, T_{CP} had odds of no contamination of 62.89E+06:1 per unit increase at that treatment. Finally, by one unit increase in T_{vp}, the odds of appearance variable increase to 3.21:1; in contrast, the odds of 1.35:1 of T_{CP} to no appearance.

Table 21. Odds ratio of survival, contamination and appearance of sugarcane acclimatization, 35 days.

Treatment	Survival			Contamination			Appearance		
	OR	**CI 2.5%**	**CI 97.5%**	**OR**	**CI 2.5%**	**CI 97.5%**	**OR**	**CI 2.5%**	**CI 97.5%**
Intercept	0.58	0.34	0.97	0.2	0.10	0.38	0.58	0.34	0.97
T_{vp}	3.21	1.54	6.87	0.17	0.03	0.69	3.21	1.54	6.87
T_{CP}	0.86	0.41	1.83	1.59e-08	0.00	7.47e+36	0.74	0.34	1.58

Note: OR: Odds ratio, **CI:** Coefficient. (T_{vp}: Peat of vermiculite and perlite (1:1) and perlite (1:1), the proportions were V/V%))

After 35 days of acclimatization, biomass distribution in the sugarcane plants of three treatments was evaluated. Table 22 summarizes the fresh, and dry mass mean of the plants classified by the used treatments. The major fresh and dry weight plants corresponded to T_{vp}, with 0.57g and 0.15, respectively. The major fresh weight mass is in the roots, comparing to the distribution of mass in other parts of the plant. However, the dry weight of leaves, roots, and stems was similar in all the treatments.

Table 22. Comparison of the fresh and dry mass of sugarcane after acclimatization with varying substrates, 35 days.

Treatment	Fresh weight of plant (g)	Dry weight of plant (g)	Fresh leaves weight (g)	Dry leaves weight (g)	Fresh stem weight (g)	Dry stem weight (g)	Fresh roots weight (g)	Dry roots weight (g)
T_{vp}	0.57 ± 0.09	0.15 ± 0.11	0.15 ± 0.02	0.02 ± 0.01	0.14 ± 0.02	0.02 ± 0.01	0.21 ± 0.03	0.02 ± 0.01
T_{CP}	0.49 ± 0.11	0.04 ± 0.02	0.16 ± 0.06	0.02 ± 0.01	0.12 ± 0.02	0.01 ± 0.01	0.18 ± 0.04	0.01 ± 0.01
T_{PM}	0.53 ± 0.13	0.11 ± 9.08	0.16 ± 0.04	0.02 ±0.01	0.14 ± 0.03	0.02 ± 0.02	0.18 ± 0.04	0.02 ± 0.01

Note: Values are means ± SE (T_{vp}: Peat of vermiculite and perlite (1:1), T_{PM}: Peat moss, peat of vermiculite and vermiculite (2:1:1) and T_{CP}: Coconut coir and perlite (1:1), the proportions were V/V%))

In Table 23, it is registered the mass increment and growth of sugarcane. Taking into account the data of plants on the day of transplanting and the last day of acclimatization, it can be seen the following results. There was no big difference in the fresh plant weight among the treatments; however, their highest value was 0.51g of increment in T_{PM}. The last treatment also had a major increment in plant length, with 15.48cm, followed by 15.39cm of T_{CP}. The major increment of the root length was registered in T_{CP}, with 5.97g.

Table 23. Effect of different substrates on fresh mass increment and growth of sugarcane, 35 days.

Treatment	Plant fresh weight (g) Δ	Plant length (cm) Δ	Root length (cm) Δ
T_{vp}	0.48 ± 0.08	14.78 ± 1.32	5.78 ± 0.51
T_{CP}	0.48 ± 0.12	15.39 ± 2.18	5.97 ± 0.77
T_{PM}	0.51 ± 0.15	15.48 ± 2.47	4.97 ± 0.65

Note: Values are means ± SE (T_{vp}: Peat of vermiculite and perlite (1:1), T_{PM}: Peat moss, peat of vermiculite and vermiculite (2:1:1) and T_{CP}: Coconut coir and perlite (1:1), the proportions were V/V%))

Table 24 shows other dependent variables related to the growth of sugarcane in acclimatization after 35 days (Figure 15). T_{vp} and T_{PM} registered the major foliar area of 3.20 cm^2. In the other variables, T_{vp} presents considerable values such as 6.86cm in root length, 4.16 in the number of leaves, 2.81 in the number of nodes, and 3.50 in the length of nodes.

Table 24. Effect of different substrates on leaves, roots and nodes of sugarcane in acclimatization, 35 days.

Treatment	Foliar area (cm^2)	Root length (cm)	Number of leaves	Number of nodes	Length of nodes (cm)
T_{vp}	3.20 ± 0.39	6.86 ± 0.54	4.16 ± 0.20	2.81 ± 0.16	3.50 ± 0.21
T_{CP}	2.70 ± 0.51	5.73 ± 0.81	3.72 ± 0.35	2.5 ± 0.28	3.44 ± 0.35
T_{PM}	3.20 ± 0.40	5.83 ± 0.64	3.95 ± 0.30	2.71 ± 0.26	3.46 ± 0.30

Note: Values are means ± SE (T_{vp}: Peat of vermiculite and perlite (1:1), T_{PM}: Peat moss, peat of vermiculite and vermiculite (2:1:1) and T_{CP}: Coconut coir and perlite (1:1), the proportions were V/V%))

Figures 15 and 16 show the acclimatization process and results of 3 substrates evaluated after 35 days. First, the viable plants moved to acclimatization were washed with water and irrigated with a rooting agent, fungicide, anti-stress, and water. Next, the adaptation, rooting formation, and elongation of explants (Figures 15, C, D, and E, respectively).

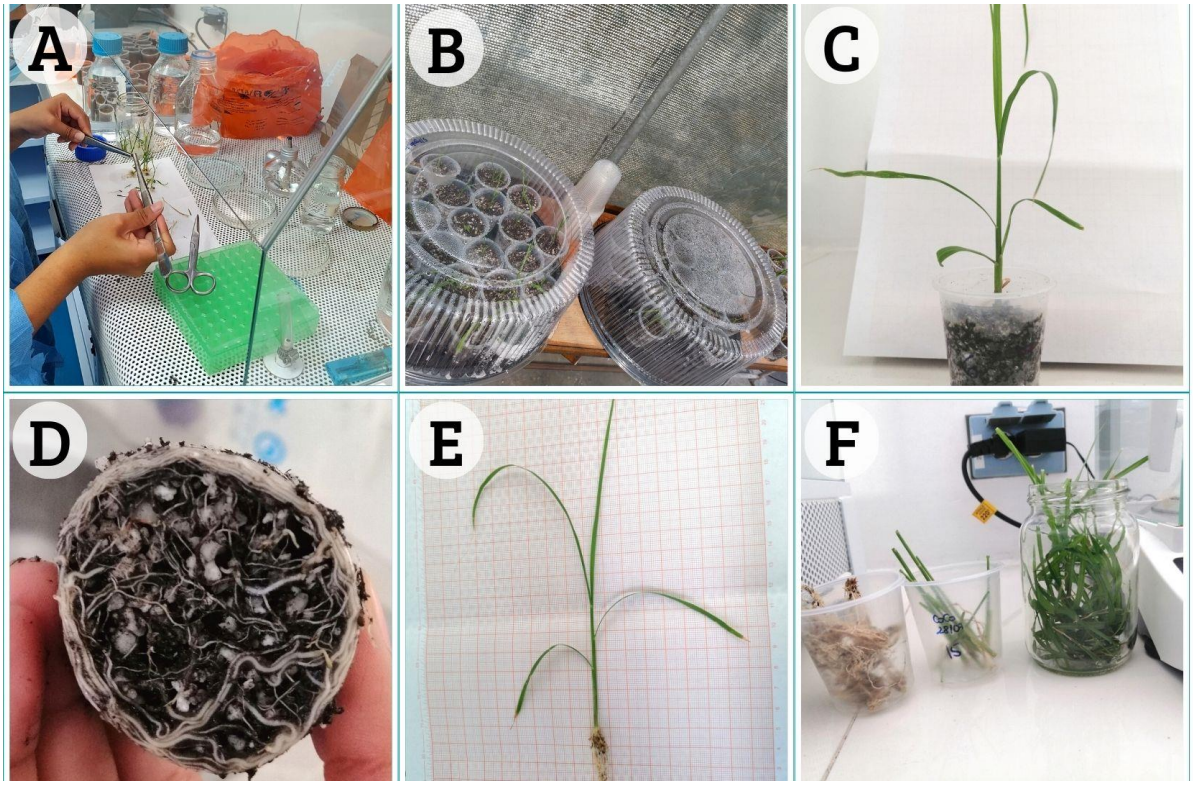

Figure 15. Acclimatization process of sugarcane. A) *In vitro* sugarcane transplanted to *ex vitro* conditions. **B)** Sugarcane covered with plastic to maintain the relative humidity. **C)** Sugarcane plants after 35 days. **D)** New roots formation in *ex vitro* conditions. **E)** and **F)** Measuring some parameters of sugarcane after acclimatization.

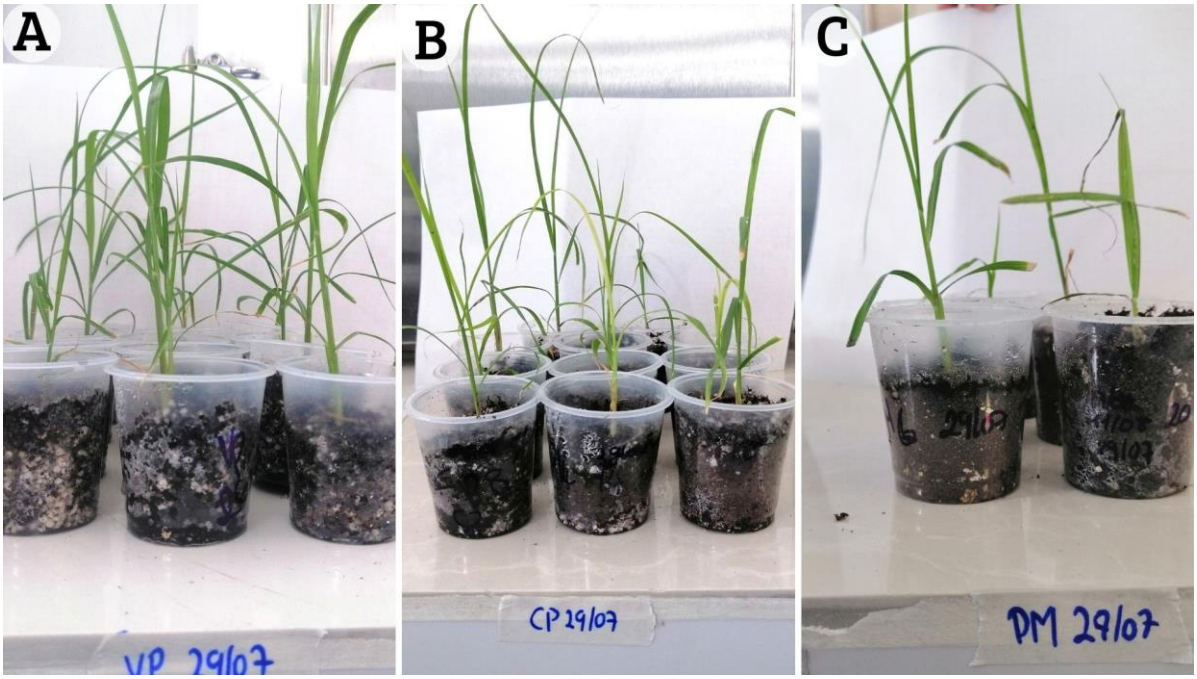

Figure 16. Acclimatization of sugarcane after 35 days. A) T_{vp}: Peat of vermiculite and perlite (1:1). **B)** T_{PM}: Peat moss, peat of vermiculite and vermiculite (2:1:1) and **C)** T_{CP}: Coconut coir and perlite (1:1)

5. DISCUSSION

5.1.Disinfection of sugarcane

The exogen and endogen pathogens affect the plant tissues of certain sugarcane varieties, causing contamination and damage. Multiple studies report sugarcane disinfection methods, but they can only be applied to some varieties. Exudation is another type of contamination produced when the plant tissues are injured during the disinfection process; this defense mechanism can have a deleterious effect on growth that includes necrosis (26). Our study identified it as the oxidation of leaves and the base of the explants. Then, the contamination and the severe oxidation were considered as factors that impact the explant viability. In this way, the implementation of a disinfection protocol was completed for the sugarcane variety CC01-1940. One of the principals

aims in the testing of some disinfection treatments was to select the suitable concentration of NaClO and immersion time, which resulted in less contamination and oxidation, providing high survival and viability at the same time.

Sodium hypochlorite (NaClO) is a disinfectant substance widely used in plant culture establishment, in specific for surface disinfection because of its strong oxidizing properties against bacteria, viruses, and fungi (27). In this study, T1 (0.5%NaClO; 10min) registered the lowest contamination of 4.17%, an oxidation of 12.5%, a high survival rate of 95%, and viability of 54% (Table 6). Similarly, the exposure of shoot tips to T3 (0.75%;10min) resulted in 16.67% of contamination, 0% oxidation, a survival rate of 85.7%, and the highest viability of 95.17%. Analogous protocols on different species, such as a hybrid of peach x almond and ginger shoots, revealed similar results. For *Garfi x Nemared* (peach x almond) *in vitro*, the greatest establishment employed 0.75% NaClO for 12 min for the disinfection (28). In the same way, ginger shoots explants had the highest mean number of clean explants with 0.50% v/v Sodium hypochlorite at 20 minutes (29).

Largely, sugarcane disinfection protocols use NaClO in higher concentrations to manage the contamination and effective disinfection for the *in vitro* establishment. However, in this study, shoot tips immersed in 1% NaClO for 10min (T5) or 15min (T6) resulted in 100% of survival but also obtained the highest contamination and the lowest viability with one of the prominent oxidations (Table 6). Indeed, Rangel et al. (2016) showed that in varieties ITV 92-1424, Laica 82-2220, and Q28-2, the greatest disinfection was obtained with 2% NaClO for 20min, resulting in the best outcomes in survival with less oxidation and quality contamination. Likewise, the disinfection of the variety Co 0118 used 6% NaClO to establish the meristem shoot tip for 10min, resulting in high survival and lower contamination (30).

At this point, sodium hypochlorite could act as an aseptic over tissue culture. However, 1% of NaClO exposition could damage meristematic tissues in this variety, inhibiting the viability of explants and not working well for avoiding contamination. That could be because NaClO also reacts with water, resulting in HClO, a lethal compound for DNA (30). Even though in other sugarcane varieties, higher concentrations of NaClO led better results, in the variety CC01-1940, it worked as previously described.

Nevertheless, the problem drawbacks with T1 are due to its low viability, probably due to oxidation; in comparison, T3 had higher contamination. It is common that during the plant culture initiation, there were high oxidation levels of phenolic substances released by the young tissues into the medium. The resulting dark oxidation generally causes abnormal growth during cell development or even the mortality of the shoot tips. This mechanism is associated with stresses by environmental changes, wounds, and growth regulators (31). Thus, the frequent change of medium and the use of antioxidants such as cysteine, citric acid, and abscisic acid in the establishment is frequently practiced to control the adverse effects of phenolic substances (26).

On the other hand, contamination can be controlled with the application of antibiotics in the culture media. Using cefotaxime at a concentration of 500mg/L can aid in controlling bacterial contamination without causing adverse effects on the sugarcane grown in the multiplication stage and even improve somatic embryogenesis and regeneration (32,33). Additionally, other antibiotics used in the sugarcane culture are streptomycin, tetracycline, and aureofungin (31).

The growth and development of *in vitro* plants are determined by the genetic constitution of the plant, nutrients (water, macronutrients, micronutrients, and sugars), as well as physical factors such as light, temperature, pH, O2 and CO2 concentrations, and other organic substances (18). Besides, phenolization and contamination are related to plant age and the conditions in which the

tissues were collected from the mother plants. In this study, the plants were not taken from a plant bank under controlled conditions but from outdoor cultivation.This also could influence in the successful disinfection for establishment *in vitro*. Nevertheless, another alternative could be other disinfectant products used in sugarcane disinfection recommended by other authors, such as Calcium hypochlorite and magnesium chloride (34).

5.2.Viability of shoot induction

In micropropagation, the shoot induction and the posterior multiplication are essential for achieving the large-scale yield of commercial cane (35). The success of shooting depends on the suitable media composition and supplementation of specific phytohormones concentration to allow the shoot induction in a determined time. Thus, some parameters of the disinfection phase were evaluated to follow up on the quality of the disinfection treatment chosen.

Data of shoot induction presented in Table 13 showed that T3 (0.5mg/L 6-BAP + 1 mg/L KIN) had the major odds ratio of increasing the number of shoots per plant increase in the treatment by a factor of 10.45:1, followed by T1 (0.5mg/L 6-BAP) with an odds ratio of 8.94:1. In the literature there are studies that relate more to T1 than to T3. Thus, similar results to T1 have been described by Geetha (2000), who found that 0.5mg/L 6-BAP was effective in producing shoots in the sugarcane variety Co Si 95071. Later, Gill et al. (2004) also reported comparable good results in shoot regeneration in varieties Co.J. 83 and Co.J. 86 using 0.5mg/L 6-BAP.

According to other studies, lower levels of 6-BAP also work for great shoot induction. Some examples are the shooting induction of the variety CC-06791 in a medium with 0.4mg/L 6-BAP (14), variety Q28-2 using 2.5uM of 6-BAP (34), and variety C91-301 under phytohormones concentration of 1.3uM 6-BAP in the media (36). In general, the use of a single phytohormone

would be enough for shooting induction. Even though the use of more than one cytokinin is considered for the induction of new shoots in a few varieties, such as Co 449 and Co 678, which had the major shoot tip induction using 8.8uM BAP + 1.1uM Kinetine (37). Contrastingly, in varieties, ITV 92-1424 and Q28-2, the length of the new shoot tips was even higher without phytohormones supplementation (34).

Cytokinins are growth regulators that promote cell proliferation; thus, there are widely used in multiplication mediums (38). In this way, the cytokinins 6-benzylamineopurine (6-BAP) and kinetin (KIN) are generally used for rapid shoot multiplication (39). Both phytohormones promote protein synthesis and play an important role in cell cycle control and division; they overcome apical dominance and stimulate lateral shoot multiplication (40). Our study demonstrated the influence of the use of cytokinins in shoot induction. It can be also observed that the new shoot tips development depends on the morphogenic capacity of the CC01-1940. Besides, the use of 0.5mg/L of BAP is the common factor in the treatments T1 and T3, but in the last, it can be seen that the combination with another cytokinins (for example kinetin) can result in a small better quantity of new shoots.

Additionally, T1 presented a significant difference in survival, as presented in Table 10; even though this is not related to the use of phytohormones, it says a lot about the effectiveness or weakness of the disinfection process. Despite the low effectiveness of disinfection methods, this is compensated when reaching the multiplication stage through sub-culturing.

5.3. Rooting

Once a considerable number of shoots have been produced, the rooting induction can be carried out. In general, this phase is treated under *in vitro* conditions, while the elongation also is

promoted. This step is regulated essentially by the sucrose and growth regulators in special auxins (for example NAA) (41). Thus, the aim of the rooting induction phase was to determine the suitable culture media that promoted the greatest root formation and elongation of the plants. The principal dependent variables taken were the length of the plant number and the length of roots; additionally, parameters related to viability and appearance were measured.

According to Tables 16 and 18, the treatments that positively influenced the rooting were T10 and T12, which had ½ MS media supplemented with 1mg/L and 3mg/L of NAA, respectively, and carried the best increase odds ratio of 3.18:1 and 4.77:1, respectively, for the rooting formation. Both treatments had resulted in great root formation in other studies. In this way, the variety of sugarcane N14 and Pr103 also had similar results to T10 (in this study), with the highest number of roots per shoot and average root length in 1/2 MS supplemented with 1mg/L NAA (42,43). Furthermore, the rooting induction of sugarcane in varieties CP77400 showed frequencies of root formation up to 90% with ½ MS+1mg/L NAA (Ali et al., 2008). In addition, similar studies to T12 were described by Tesfa (2016), who used the genotypes sugarcane N52 and N53 and showed a good performance in half-strength liquid MS supplemented with 3mg/L NAA. Thus, the rooting of sugarcane L. cv-Nayana also had a profuse rooting under half-strength MS supplemented with 3mg/L NAA (15).

Other studies also demonstrated that a high concentration of NAA can induce root formation in a great way. In the sugarcane genotype Isd 31, the maximum average root length was obtained with 5mg/L NAA in ½ MS medium (44). Furthermore, for the sugarcane genotype N52, the better conditions for rooting were 5mg/L NAA + 50g/L sucrose in half-liquid MS media (45).

The common factor in these studies is the use of 1/2 MS basal medium and the use of growth regulator NAA. Even though varieties ITV 92-1424, Laica 82-2220, and Q28-2 did not present

elongation and rooting induction effect by 1/2 MS medium (34), our study demonstrated that half-strength MS basal salts worked best for the rooting induction in sugarcane CC01-1940 compared to complete MS.

On the other hand, the physiological action is not detailed yet, but the auxin signaling works in the consequence signal transduction cascade responsible for root induction. Auxin activity induces the adventitious root formation through the increase of cell division and the root primordia initiation. Auxin acts in the hydrolysis of starch and the conduction of sugars and nutrients toward the cutting base (46). Furthermore, Auxins like NAA are widely used in the micropropagation of sugarcane because of their rhizogenic efficacy related to the high-stability plant culture (43). Even though some studies consider NAA as the best growth regulator for root formation, high concentrations of NAA, around 7mg/L, cause an inhibitory effect in rooting and plant elongation (43,45). That is because the NAA at higher concentrations is related to the inhibitory effect in rooting and elongation due to ethylene induction (47).

Furthermore, in our study, the treatments with 0.5mg/L of 6-BAP negatively influenced the rooting formation, and in general, there were no good results in the treatments supplemented with a combination of auxin and 6-BAP. That was because cytokinin influence positively in cell proliferation, but it also inhibits root formation; for that, some authors recommend an intermediate step with free phytohormones medium before (26,38).

5.4. Acclimatization conditions

After the entire *in vitro* process, the plants were transferred to the soil and they were subjected to a substantial modification in environmental conditions, which can be extremely stressful and critical due to the high rate of deaths if suitable precautions are not carried out (26). Selecting a

proper substrate for acclimatization is fundamental for the success of the whole micropropagation process. An adequate substrate supports the plant and maintains an adequate pH, with sufficient porous that allow aeration and liquids drainage (48).

Particularly, the success of acclimatization depends on some conditions that an adequate substrate can support, like humidity, water retention and nutrients; for that, development and adaptation of plants to the substrate were evaluated. In this study, only the plants in the substrate peat of vermiculite and perlite (1:1, V/V%) (T_{vp}) achieved more than 60% survival with considerable contamination of 3.5% after 35 days. The highest fresh and dry mass accumulations were 0.57g and 0.15g (respectively) in the treatment T_{vp}.

The mass distribution was major in fresh roots in all treatments, but there were no significant differences among treatments in roots, stems, and leaves, in dry mass. Likewise, as shown in Table 23, the aerial length was longer than the roots in all treatments, but the major increase in plant weight and length in fresh was in substrate with peat moss, peat of vermiculite and vermiculite (2:1:1) (T_{PM}), and the increase of root length was major in substrate coconut coir and perlite (1:1) (T_{cp}).

Survival and contamination

The acclimatization showed similar results in other reports using mixed substrates. Rangel (2016) registered good results in the survival of varieties ITV 92-1424, Laica 82-2220, and Q28-2 in mixed substrates of peat and perlite (1:1, v/v) after six weeks. Another variety of sugarcane with successful acclimatization in a similar substrate was C91-301 (36). In this study, substrate peat of vermiculite and perlite (1:1, V/V%) (T_{vp}) positively influenced the survival and appearance of the logistic model, but negatively influenced the contamination. Other researchers use this substrate

mix to acclimate different plant species. For example, the acclimatization of grapevine (*Vitis vinifera*) employed peat and perlite (1:1, v/v%), obtaining good results (49). On the contrary, Caamal and Bello (2014) obtained good results with the mixture of peat moss, peat of vermiculite, and vermiculite (2:1:1, V/V%) in the adaptability of varieties CP 94-1674; SP 83- 5073; SP 80-3280, and SP 80-1816; however, in our study the plants at the same mixture suffered a huge decline in their survival of around 60% (Figure 8) and resulted in high contamination of 16.66% after 35 days.

Mass accumulation

Largely, the parameters measured coincide with better results with substrate peat of vermiculite and perlite (1:1, V/V%) (T_{vp}). In this substrate, one key component is perlite, commonly used in compost because it maintains aeration and permeability. Perlite arrives from the inert volcanic lava at 800ºC and keeps sanity in the acclimatization phase (50). Since the quality of this substrate, it is capable of having a good relative humidity, aeration, and sanity that confers successful acclimatization to the plants. The results also were due to the high content of organic matter in this substrate that helps in humidity retention; then, the plants absorb the water and increase their Biomass (51).

Root length

The moisture retention capacity is similar in all substrates, so, the dry matter in roots was similar in leaves and stems. Thus, roots need at least 10% of aeration in the soil to absorb the major nutrients from the substrate (50). Indeed, root development needs a great porosity in the substrate like coconut coir and perlite (1:1, V/V%), which had the highest difference in the root length of 5.97cm.

Plant weight and length

Substrate with peat moss, peat of vermiculite and vermiculite (2:1:1) (T_{PM}) had better humidity retention, followed by T_{cp} and substrate peat of vermiculite and perlite (1:1, V/V%) (T_{vp}). For that, the last two treatments presented a high length in the growth of the roots, while T_{PM} presented a slightly higher plant length and plant weight, and their roots had less length after 35 days. On the contrary, substrate coconut coir and perlite (1:1) (T_{cp}) presented the highest increase in the length of roots.

The vascular connections have deficiencies from the root microshoots *in vitro*. This water uptake restriction makes it necessary to have high humidity and provide continuous irrigation during acclimatization (48). Water retention allows the development of the superficial part of the plant, and maintaining a good relative humidity in the environment is important for this phase. However, when this humidity is excessive, pathogens such as fungi thrive in the substrate, leading to getting diseased plants and eventually death, like in the case of plants under T_{PM}. To converse this contamination, fungicide Tachigaren 30SL was employed in all the treatments, which could help plant growth, increasing the root induction by adding an extra chemical rooting agent. Consequently, all plants absorb nutrients and physiological activity (52).

Use of biostimulators

In general, a substantial number of plants do not survive the transfer to open environmental conditions because in the greenhouse and field it is challenging to control the humidity, temperature, light, and septic environments (50). Some bio stimulants were employed in this study to support this transfer process to *ex vitro* conditions. They were: a rooting agent (Raizante) on the day of the transplant, an inorganic fertilizer (Rancho Alegre) after one week, and an anti-stress

(Agrimelaza) used during the first two weeks, all of this was performed to support the adaptation process of plants and to reduce severe environmental shock.

Owing to the sugarcane root plasticity, it can gradually adapt to different environmental stresses and form deep roots due to its long growth cycle (53). Also, the distribution and architecture of the roots depend on soil moisture. Under drought stress, roots seek moisture by proliferating to deeper levels to extract and hold more soil volume for water absorption (54). In this way, using a rooting agent allowed for accelerated root formation so that the plant could take nutrients from the substrate and support itself faster. Applying a rooting agent (Raizante) induces the bio stimulation of roots through amino acids, polysaccharides, and nutrients that provide the energy to plant development (55).

Even though the plants could absorb nutrients from the substrate, rooting agent, and anti-stress, in the first weeks, it was necessary to add an inorganic fertilizer to supply the fundamental substances for plants, such as phosphorus, nitrogen, and potassium, over time. Potassium, for example, is one of the regulators of osmotic pressure and maintains water inside the cells, coordinates the opening and closing of stomata (53). Additionally, the decrease in potassium affects nutrient uptake by the roots (53).

Particularly, the use of anti-stress may improve environmental stress tolerance and help the survival rate (56). Although there is no evidence that this product (Agrimelaza) has been used previously in the acclimatization of sugar cane, it may support the plant against stress. However, its high sugar content (57) could have been a source of contamination, especially in the T_{PM} environment, due to the high humidity of this substrate.

Luminosity

At this point, photosynthesis is paramount to survive when becoming autotrophic is forced. The luminosity intensity also plays an important role in survival; however, it should be less intense in the first days considering the low metabolic activity of the plant after transplanting (58). The stomata play an important role in this adaptation; in the *in vitro* conditions, they are countless and nonfunctional, but after the exposition to the gradual luminosity increase, they increment notably and make them functional (59).

Water

Sugarcane is a plant with C4 metabolism that requires abundant water for growth and development. Thus, adequate control of this factor is fundamental for acclimatization success. Thus, the ideal substrate should be sterile and retain high humidity with adequate drainage (26).

In this study, fresh root biomass was better in all the treatments than in leaves and stems due to a hydric deficit, which drives the energy toward roots. This water stress was more elevated in T_{vp}; consequently, leaf area, leaves number, nodes number and distance were lower than in the other treatments. It is important to clarify that these differences were minimal, then all treatments suffered similar water deficits. Regardless, because of a water deficit, the Leaf relative water content (RWC) could decrease, and the water uptake could not happen to maintain the turgor (53).

Cellular dehydration due to drought leads to the accumulation of osmolytes such as proline, sugars, alcohols, glycine betaine, and reactive oxygen species (ROS). Therefore, the cell structure and metabolism are affected, leading to the degradation of vital proteins and enzyme activities (53). Fortunately, the sugarcane variety CC01-1940 has tolerance to dry soils (21). Drought tolerance

involves some changes in plant metabolism and functioning. The plant closes its stomata and reduces CO2 concentration, then ROS stimulates the mechanisms that drive oxidative stress (53).

For further studies, these water deficit stress factors could be evaluated to measure the dimension of this situation and how to solve it.

6. CONCLUSIONS

- In our study, the development and implementation of *in vitro* micropropagation of sugarcane variety CC01-1940 was successfully achieved.
- The disinfection process considered two adequate treatments using sodium hypochlorite: T1 (0.5% NaClO; 10 min) and T3 (0.75% NaClO; 10 min), because T1 presented the lowest contamination of 4.1% and a great survival of 95%; and T3 presented no oxidation and the higher viability of 95%, after 3 weeks.
- The adequate concentration for the shoot induction was MS basal medium supplemented with 5mg/L 6-BAP + 1 mg/L KIN of T3. Our study also demonstrated that half strength MS basal salts worked best for the rooting induction of sugarcane in this study, compared to complete MS; this medium can be supplemented with 1mg/L NAA (T10) or 3mg/L NAA (T12), whose odds ratios of rooting increase by a factor of 3.18:1 and 4.77:1, respectively.
- The adequate substrate for the acclimatization of sugarcane variety CC01-1940 was T_{vp} (Peat of vermiculite and perlite (1:1, v/v%)). This substrate presented the necessary humidity, aeration and porosity to obtain the best results in the biomass, length and development of the sugarcane plants in this study. Even though it is necessary to manage in a better way the water irrigation.
- The micropropagation method using the described treatments in each phase had 2.16 times the odds of survival of other used treatments in this study.

7. RECOMMENDATIONS

- The acclimatization environment should be improved to consider the morphological, physiological, and structural changes in all the plants in the first weeks and after 35 days. Further studies could help to understand the nutritional requirements of these varieties according to the climate conditions in the IANCEM.
- Water irrigation needs to be managed better to avoid problems that drought carries on, like the limitation of the metabolic processing pathways of plants.

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