Chapter 6

In Vitro Propagation of Sugarcane for Certified Seed Production

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Additional information is available at the end of the chapter

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Abstract

Micropropagation of sugarcane is important to obtain pathogen-free plants, genetically homogeneous and invigorate. The micropropagation procedure is divided into stages for the sake of better understanding. Micropropagation for large-scale sugarcane production using a temporary immersion system (TIS) is described. In addition, the aim of this chapter is to report, from the laboratory to the field, the best way to establish and use basic seed (primary seed), semicommercial seed (foundation or secondary seed) and commercial seed production. In conclusion, commercial sugarcane micropropagation enables the massive multiplication of plants to obtain certified vitroplants and increase the sugarcane and sugar productivity per unit area.

Keywords: biotechnology, micropropagation, temporary immersion system, vitroplants, seed production

1. Introduction

Sugarcane cultivars (*Saccharum* spp. hybrids) are grown mainly for sugar, ethanol and subproducts. Its large-scale production makes it an important crop in the tropical and subtropical regions of many countries [1]. Despite the importance of sugarcane cultivation, its production is generally characterized by low yield in the field due to, among other causes, virtually zero renewal of plantations because of the lack of vegetative materials certified free of pests and diseases. According to Flores [2] and Lal et al. [3], sugarcane varieties age over the years, losing their productive power, which can deteriorate and eventually disappear from the commercial crop. In many countries, most vegetative seed is propagated by conventional methods

by sowing bud-containing cuttings. However, this technique does not ensure the sanitation and rejuvenation of the selected varieties in the field. An alternative to this problem is the use of Plant Tissue Culture (PTC) techniques [4]; this plant biotechnology tool allows the establishment, manipulation and development, under artificial and controlled conditions, of cells, tissues or organs and is very useful for the regeneration of rejuvenated, genetically homogeneous plants free of pests and diseases.

In sugarcane, *in vitro* propagation or cloning of plants uses PTC techniques to obtain a constant supply of plant material, unlike conventional vegetative propagation, which is seasonal in nature. Sugarcane micropropagation has allowed the rapid multiplication of new varieties, rejuvenation of old deteriorated varieties and sanitation of diseased varieties [3] and has also facilitated the exchange of in vitro plant material. Currently, semiautomation of micropropagation by temporary immersion systems (TISs) offers a practical strategy to reduce production costs [5]. TISs are semiautomated bioreactors designed for the mass propagation of cells, tissues, embryos or organs using liquid medium [6]. TISs have been shown to be a powerful tool for sugarcane propagation [7].

A micropropagation-based crop has prominently better quality than a conventionally raised one. According to Sawant et al. [1], sugarcane micropropagation increases productivity in the field by up to 25%, while Pérez et al. [8] mention that the combined effect of in vitro sanitation and rejuvenation is expected to increase sugar yields by between 10 and 15% per unit area. These advantages have allowed the commercial exploitation of micropropagation in the sugar industry worldwide. This technology is now used to supplement commercial sugarcane production in many countries including Brazil, India, the USA and Cuba [4].

Certified vitroplants obtained from in vitro propagation systems are used in the field for the production of quality seed. This technology helps farmers to enhance crop productivity. The aim of this chapter is to report, from the laboratory to the field, a seed production system comprising basic seed (primary seed), foundation (secondary seed) and commercial seed production to obtain a commercial crop.

2. *In vitro* propagation

Sugarcane micropropagation enables the identical production of the selected cultivars using PTC techniques. PTC refers to growing and differentiation of cells, tissues and organs isolated from the mother plant, on artificial semisolid or liquid media under aseptic and controlled conditions. The small organs or pieces of tissue used in PTC are called explants. PTC medium provides inorganic nutrients and usually a carbohydrate to replace the carbon which the plant normally fixes from the atmosphere by photosynthesis. When carbon is supplied with sucrose and kept in low light conditions, micropropagated plantlets are not fully dependent on their own photosynthesis.

To date, sugarcane micropropagation has shown great productive potential [3]; it is being used in commercial laboratories to obtain certified plant material.

The sugarcane micropropagation process is carried out in the following stages [9].

Stage 0: mother plant selection. Donor cultivars are selected and conditioned to be used to initiate in vitro cultures. It should be considered that the mother plant corresponds to the selected variety; in many cases, there is a varietal mixture in commercial cane plantations. The genetic purity of the variety should be certified by the breeder or research organization identified for the maintenance of the variety.

Stage I: in vitro establishing. The choice of the apical meristems (explants) and their disinfection is carried out to initiate an aseptic in vitro culture. Apical meristem culture produces virus-free sugarcane plants. The meristem remains in an active state during the vegetative growth phase, and the meristem cells are in a permanent totipotent state.

Stage II: multiplication. It is at this stage that mass propagation is performed, obtaining many new shoots from minimal amounts of tissue. Based on our experience, we recommend making no more than eight subcultures because above that level the length and number of shoots decrease. In addition, the likelihood of genetic variants occurring increases.

Stage III: elongation and rooting. The shoots must form their root system and at the same time increase their size to facilitate their manipulation and adaptation to the acclimatization conditions.

Stage IV: acclimatization. This process is carried out in a greenhouse. It consists of a slow reduction of the relative humidity and gradual increases in the luminous intensity for a better adaptation to the external environment. The greenhouse infrastructure must ensure control of both relative humidity and light entry.

It is important to mention that the elongation and rooting stage varies according to the method being utilized; it is not always necessary when semiautomation of micropropagation by TISs is used.

Conventional micropropagation of sugarcane in semisolid media has been reported [10, 11]. However, to reduce the labor required and increase efficiency, temporary immersion systems (TISs) have been successfully used to improve in vitro sugarcane multiplication [12–16]. The principle of these systems is the immersion of explants for a determined time and frequency.

We have implemented different TISs for commercial sugarcane micropropagation. Sugarcane meristems (cv. Mex 69–290) were collected from field-grown plants and cultured following the protocol of Jiménez et al. [17] The 3-cm-long sugarcane shoots after three subcultures (30 d each) were used as explant. Explants (two shoots each) were placed in the Temporary Immersion Bioreactor (TIB™, Cuba), the Recipient for Automated Temporary Immersion (RITA™, France), the Gravity Immersion Bioreactor (GIB, Mexico) and the SETIS™ Bioreactor (Belgium) containing MS [18] medium supplemented with 30 g/L sucrose, 1 mg/L Kinetin (Sigma Chemical Company, MO, USA), 0.6 mg/L 3-indoleacetic acid (IAA, Sigma Chemical Company, MO, USA) and 0.3 mg/L 6-benzylaminopurine (BAP, Sigma Chemical Company, MO, USA). The pH of the culture medium was adjusted to 5.8 with 0.1 N sodium hydroxide and then autoclaved at 1.2 kg/cm^2 for 15 min at 120°C. Three replicates were used in all experiments. TISs were incubated at 24 ± 2°C and were maintained under fluorescent light (40–50 μmol m^{-2} s^{-1}) and a photoperiod of 16 h. Immersion frequency was according to Lorenzo et al. [12] After 30 d of incubation, the number and length of shoots per explant were assessed.

A completely randomized experimental design was used for all experiments. Results were statistically analyzed by one-way analysis of variance (ANOVA) and Tukey's comparison of means test ($p \leq 0.05$) using SPSS statistical software (version 22 for Windows).

When evaluating the different TISs in sugarcane during in vitro propagation, significant statistical differences were observed for the number and length of shoots per explant. The bioreactors with the highest number of shoots per explant were TIB, GIB and SETIS, with 38, 40 and 41 shoots/explant, respectively, followed by RITA, with 32 shoots/explant. Regarding shoot length, the bioreactors with the longest length were TIB, GIB and SETIS with 8.6, 10.7 and 9.8 cm in length, followed by RITA with 6.0 cm in length (**Table 1**).

Semiautomation of sugarcane micropropagation using TISs is a strategy to reduce production costs. The TIB, GIB and SETIS bioreactors showed good performance in the formation of the length and number of shoots; probably their size, among other factors, favors the development of explants. On the other hand, RITA, due to its limited capacity, did not allow an increase in length and number of new shoots. Commercial sugarcane micropropagation by TISs is shown in **Figure 1**.

TIS	No. of shoots/explants	Shoot length (cm)
RITA	32.8 ± 0.58 b	6.0 ± 0.27 b
TIB	38.8 ± 0.67 a	8.6 ± 0.43 a
GIB	40.0 ± 0.55 a	10.7 ± 0.33 a
SETIS	41.0 ± 0.40 a	9.8 ± 0.47 a

Values represent mean ± SE (standard error). Means with different letters per column represent statistical difference (Tukey, $p \leq 0.05$).

Table 1. Sugarcane (*Saccharum* spp. hybrid cv. Mex 69-290 micropropagation by different temporary immersion systems (TISs)).

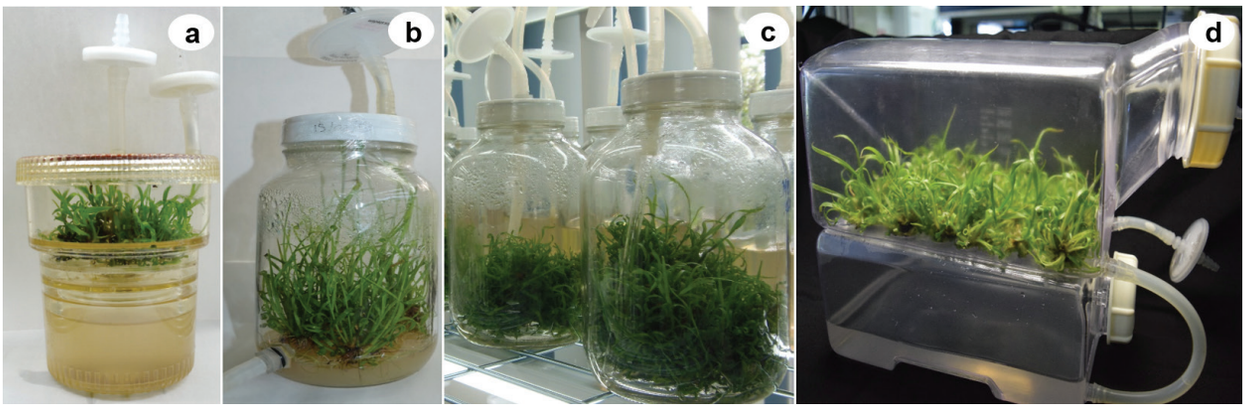

Figure 1. Sugarcane micropropagation by temporary immersion systems. (a) Recipient for automated temporary immersion (RITA™), (b) gravity immersion bioreactor (GIB), (c) Temporary Immersion Bioreactor (TIB) and (d) SETIS™ bioreactor, after 30 d of incubation.

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3. Genetic homogeneity

The genetic or epigenetic variation obtained by different in vitro propagation systems is called somaclonal variation [19]; it is a problem that affects commercial micropropagation, where it is necessary to maintain the maximum genetic homogeneity of the regenerated individuals with respect to the mother plant.

The causes of somaclonal variation are not well understood and have not been fully elucidated [20]. However, some factors that determine the frequency of somaclonal variation include the in vitro regeneration system, the type and concentration of growth regulators applied, and the number of subcultures [21]. Consequently, it is important to determine the optimal number of subcultures that can be made from an explant for each sugar cultivar to be micropropagated.

Martínez-Estrada et al. [22] determined by inter-simple sequence repeat (ISSR) markers that no more than eight subcultures should be done due to the existence of polymorphism between the subcultures produced by a Temporary Immersion Bioreactor (**Figure 2**), since above eight subcultures the length and number of shoots decrease.

Genetic homogeneity and plant health are two important quality aspects that must be addressed before the seedlings are distributed outside the laboratory. According to Lal et al. [3], contamination of cultures is a severe problem that not only reduces the frequency of shoot culture initiation from the source explants but also the total number of shoots produced at various cycles of cultures. Plant tissues could also be cultured in the presence of bacterial and/or fungal contaminants. Therefore, a phytosanitary diagnosis should be required.

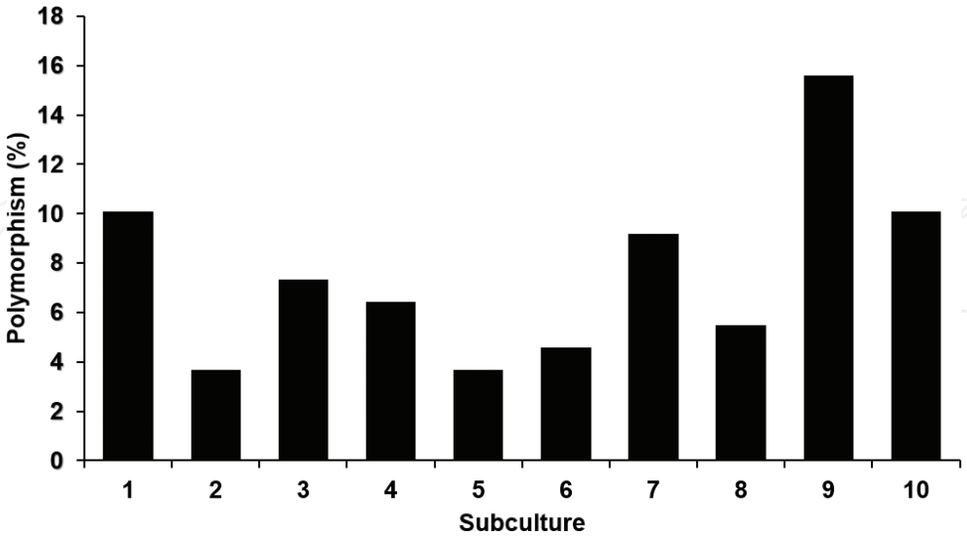

Figure 2. Effect of subculturing on polymorphism percentage of shoots of sugarcane (cv. Mex 69–290) using Temporary Immersion Bioreactors assessed by inter-simple sequence repeat (ISSR) markers. Each bar represents the polymorphic percentage of subcultures 1–10.

4. Phytosanitary diagnosis

Diseases represent one of the main factors that affect sugarcane production. Knowledge of phytosanitary status and the correct identification of phytopathogens are key to reducing losses due to diseases. In this regard, it is essential to carry out a phytosanitary diagnosis at an early stage to ensure the phytosanitary quality of the seedlings obtained in the laboratory.

In many countries where sugarcane is an important part of the economy, plant health departments (PHDs) are responsible for certifying the procedures for obtaining pest- and disease-free sugarcane vitroplants. The PHD determines the requirements necessary for accreditation of micropropagation laboratories engaged in in vitro culture of sugarcane, whose legal basis is determined based on Plant Health Laws. To obtain certification, a laboratory must meet a series of requirements that demonstrate technical competence, satisfactory infrastructure and sanitary capacity to produce plant material in vitro.

Accreditation of micropropagation laboratory: micropropagation laboratory should be accredited by an appropriate authority to ensure technical competence and satisfactory infrastructure.

Common name	Scientific name
Sugarcane scale	*Aulascaspis tegalensis*
Sugarcane leafhopper	*Perkinsiella saccharicida*
Sugarcane leafhopper	*Pyrilla perpusilla*
Spotted stalk borer	*Chilo partellus*
Sugarcane borer	*Eldana saccharina*
Purple stem borer moth	*Sesamia inferens*
Kenya mealybug	*Planococcus kenyae*
Giant moth borer	*Castnia licoides*
Sugarcane downy mildew	*Peronosclerospora sacchari*
Sugarcane gumming disease	*Xanthomonas campestris pv. vasculorum*
Ratoon stunting disease	*Leifsonia xyli* ssp. *xyli*
Bacterial canker	*Dickeya chrysanthemi*
Bacterial wilt	*Pantoea stewartii*
Leaf scald of sugarcane	*Xanthomonas albilineans*
Virosis	*Sugarcane Streak Virus*
Virosis	*Sugarcane Sereh virus*
Sugarcane smut	*Sphacelotheca erianthi*
Sugarcane smut	*Sphacelotheca macrospora*
Sugarcane smut	*Ustilago scitaminea*

Table 2. Pests and diseases which plant material produced *in vitro* must be free of.

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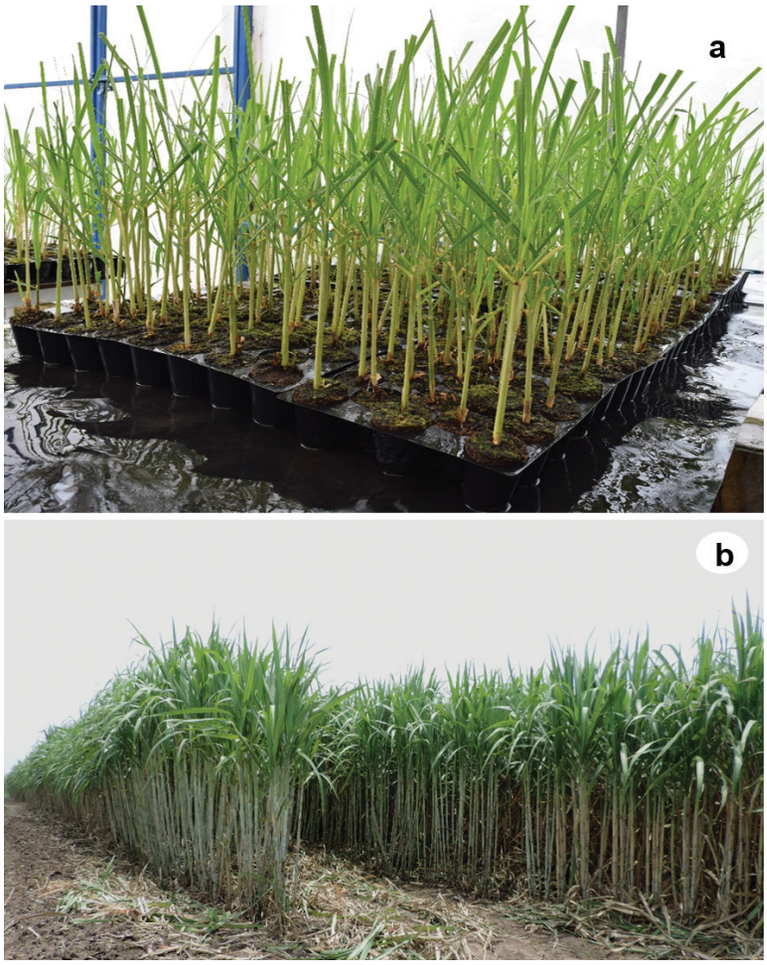

Figure 3. Certified vitroplants obtained from laboratory. (a) Vitroplants ready for field transfer and (b) field planting.

To determine plant health, samples of sugarcane shoots are taken *in vitro* and sent to the Phytosanitary Diagnostic Centers authorized to verify the plant health. *In vitro* propagated plants should be indexed for freedom from viruses and virus-like diseases through enzyme-linked immunosorbent assay (ELISA) and molecular methods [23].

According to the phytosanitary diagnosis report and based on the Plant Health Law, the diagnosis must be negative for the main sugarcane diseases (**Table 2**).

After obtaining healthy in vitro plants, a certification is issued so that vitroplants can be used for the establishment of certified basic seed nurseries (**Figure 3**).

5. Seed production

Seed production system comprises basic seed (primary seed), semicommercial seed (foundation or secondary seed) and commercial seed production.

The unit area of the seed nursery should be approximately one-tenth of the area that is planned to be renewed each year in the commercial plantations. This plantation system is used because

the multiplication rate is around 1:10 (10 internodes/stem). Sugarcane stem used for seed production is composed of a series of internodes; each internode forms a new plant. Harvesting of each seed nursery takes place after 7–10 months of development, when the plants have the required physiological conditions to obtain seed (internodes). Physiological maturity also depends on the type of variety used. After the harvest, heat treatment of internodes helps to get rid of several diseases and pests. According to Jalaja et al. [23], for seed heat treatment, thermohydrotherapy is recommended. Internodes are immersed in water maintained at 50°C for 2–2.5 h. Fungicides and bactericides are mixed in hot water to eliminate diseases. Proper thermohydrotherapy and pesticide application ensure the eradication of diseases and insect pests. Each seed nursery is described below.

The basic seed nursery is established with vitroplants. The health status of the seeds should be adjusted to those of each country. In seed nurseries, two phytosanitary assessments should be carried out to detect off-types (mutations or varietal mixture) and to remove plants infected with diseases or pests: the first, at 4 months of age, and the second, immediately before the seed cut, at which time stem samples are randomly taken. With approximately 10,000 vitroplants/ha, with an average distance between furrow and plant of 1 × 1 m, it is possible to establish basic seed nurseries. However, the sowing density depends on the type of variety and cropping system.

The *semicommercial seed* nursery is planted with material from the basic seed or with material from the ratoon of another semicommercial seed nursery that has been heat treated. The area of this field is, in general, 10 times greater than that of the basic seed nursery; as in this one, at 4 months of age, a phytosanitary evaluation must be done, and at the time of the cut, samples must be taken for phytosanitary diagnosis.

The *commercial seed* nursery is established with material from the template or the first ratoon of a semicommercial seed nursery. The area is at least 10 times greater than that of the semicommercial seed nursery. Although in this case it is not necessary to thermally treat the material, it is recommended and the same phytosanitary evaluations should be carried out. This seed nursery must have a sanitary state like that presented by the semicommercial seed nursery.

In the seed production system, it is important that the growing area has some type of irrigation so that the seedlings do not suffer from stress. Seed has to have a high water content and good nutritional status. In addition, plants must receive all the care and practices required for good development. In order to ensure the proper development of each seed nursery, a technological package is required that includes crop fertilization and adequate pest, disease and weed control.

In each seed nursery, the following criteria must be met:

- *Plant health*: plants free of pests and diseases.
- *Genetic purity of material*: high varietal purity, there being no more than one variety within the seed nursery (varietal mixture).
- *Genetic homogeneity*: clonal fidelity, identical plants without somaclonal variants (mutations).

Finally, the commercial plantation comes from the template or ratoon of a certified commercial seed nursery. This area is 10 times greater than that of the commercial seed. Commercial

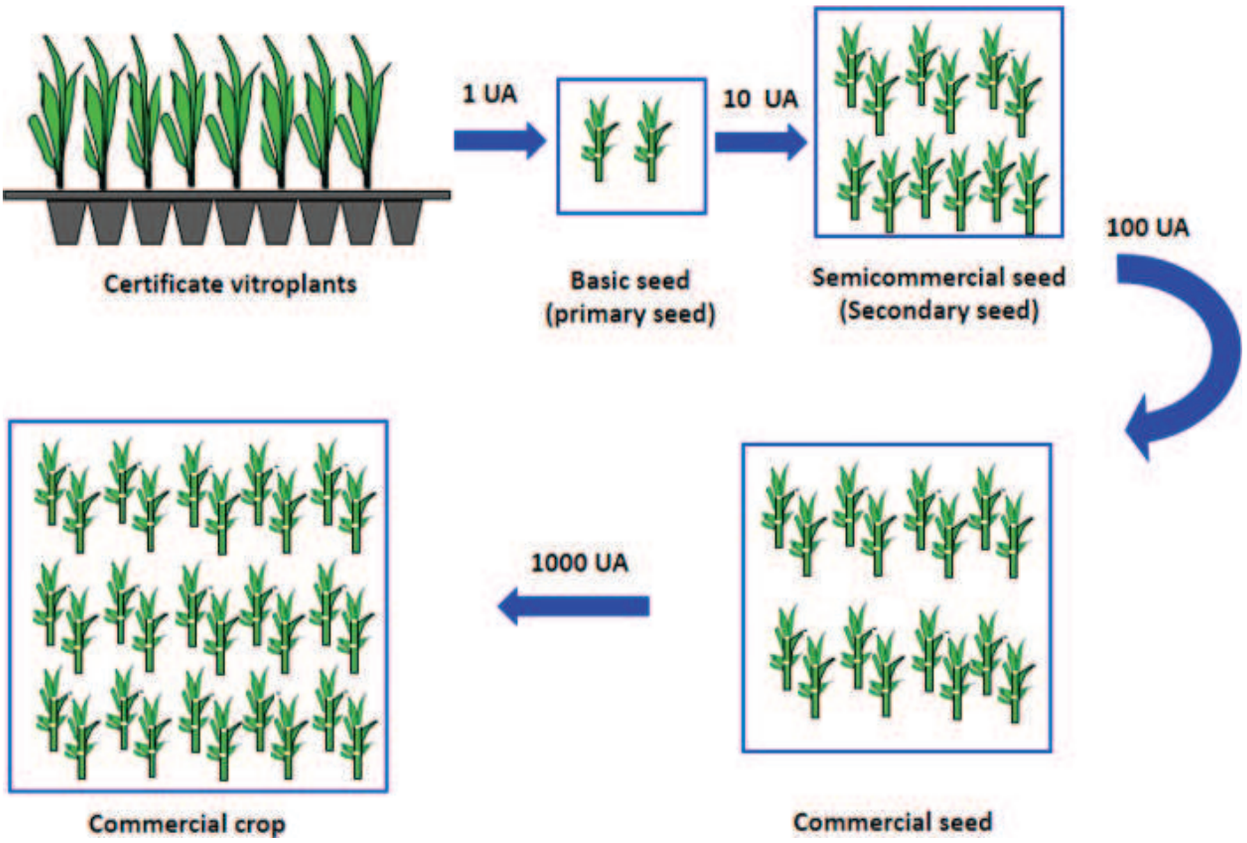

Figure 4. Seed production system comprising basic seed (primary seed), foundation (secondary seed) and commercial seed production to obtain a commercial crop per unit area (UA).

seed should preferably be located near the commercial crop to minimize transportation costs and damage during transport. The sowing time of the commercial crop determines the time for establishing the seed nursery. This is because there are early, intermediate and late crop-cycle cultivars; other factors include the availability of irrigation or whether it is subject to a seasonal sowing calendar. The fields for this purpose must be chosen from among the best according to the physical and chemical conditions of the soil and water availability. For the commercial crop, a change of seed is required once every 4 or 5 years. This change of seed favors the repopulation of the plantation, as well the rejuvenation and health of the crop, which recovers its productive potential. It is important to mention that canes from the ratoon crop, however, should not be used for seed production [23]. This is because ratoon cropping involves growing a fresh crop from the suckers of the plant crop without replanting (second crop). Therefore, a decline in cane yield in successive ratoon crops has been reported; the causes for this decline are poor ratoon management, inherited differences in potential productivity and an increasing incidence of diseases which results in a gappy stand [24]. **Figure 4** summarizes the procedure for the seed production system to obtain a commercial crop.

In conclusion, commercial sugarcane micropropagation enables the massive multiplication of plants to obtain certified vitroplants and increase the sugarcane and sugar production per unit area. This technology helps farmers to enhance their crop productivity. In addition, a sugarcane micropropagation laboratory can be used for in vitro conservation of germplasm,

application of biotechnology for genetic improvement programs and easy transportation during exchange of in vitro plant material between countries.

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