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# Production of healthy seed cane in Tucumán, Argentina

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**ABSTRACT**

Since 2001, the Estación Experimental Agroindustrial Obispo Colombres (EEAOC) has been working on the "Vitroplantas" Project. Ninety thousand sugarcane plantlets of commercial varieties are produced annually through *in vitro* meristem culture, in order to eliminate systemic diseases caused by bacteria and viruses. Sanitation of plant material is achieved through *in vitro* culture of apical meristems from donor plants, previously hot water treated and grown for three years under greenhouse conditions, with anti-aphid screens. Systemic diseases are evaluated in both meristem donor plants and micropropagated plantlets using different molecular diagnostic techniques. It is well known that *in vitro* plant tissue culture can produce somaclonal variations, which consist of genetic changes in cultured cells and tissues. In order to guarantee that seedlings propagated *in vitro* are identical to the parental variety, molecular markers that quantify and detect somaclonal variation are routinely applied. Thus, the aim of this project is to guarantee healthy and genetically pure plantlets for their plantation in the field. After *in vitro* micropropagation and testing, plantlets undergo an acclimatization process in an especially adapted greenhouse at the EEAOC. In order to avoid dehydration of plantlets, this process takes place in a greenhouse with very high relative humidity (RH=80%-100%) and low light intensity. After acclimatization, two other stages of conventional field propagation (Basic and Registered fields) are carried out before the seed cane is finally distributed among sugarcane growers. The implementation of the "Vitroplantas" Project has greatly improved the health and yields of sugarcane plantations in Tucumán, Argentina.

**Key words:** genetic purity, micropropagation, plant health, meristem culture.

**RESUMEN**
**Producción de caña semilla saneada en Tucumán, R. Argentina**

En el año 2001, la Estación Experimental Agroindustrial Obispo Colombres (EEAOC) puso en marcha el Proyecto Vitroplantas. Se producen anualmente 90.000 plantines saneados de variedades comerciales de caña de azúcar mediante cultivo *in vitro* de meristemas, con la finalidad de eliminar enfermedades sistémicas causadas por bacterias y virus. El saneamiento del material vegetal se consigue mediante el cultivo *in vitro* de meristemas apicales de plantas donadoras, previamente hidro-termotratadas y mantenidas durante tres años en invernadero en condiciones controladas. Las enfermedades sistémicas son evaluadas mediante diferentes técnicas de diagnóstico molecular, tanto en las plantas donadoras de meristemas como en los plantines micropropagados. Por otro lado, el cultivo *in vitro* de tejidos vegetales puede producir variaciones somaclonales, que consisten en cambios genéticos en las células y tejidos cultivados. Con el fin de garantizar que los plantines propagados *in vitro* sean idénticos a la variedad parental, se utiliza una metodología basada en marcadores moleculares para detectar y cuantificar la variación somaclonal. Es así que el objetivo de este proyecto es garantizar la sanidad y la pureza genética de los plantines micropropagados antes de su plantación a campo. Al finalizar el proceso de micropropagación *in vitro*, los plantines son sometidos a un proceso de aclimatación en un invernadero, especialmente adaptado para este fin. Para evitar la deshidratación de los plantines, este proceso se lleva a cabo en un ambiente con elevada humedad relativa (HR = 80%-100%) y baja intensidad de luz. Posteriormente, se llevan a cabo dos etapas de propagación convencional en campo (semilleros Básico y Registrados) antes de que la caña semilla se distribuya a los productores. La implementación del Proyecto Vitroplantas ha mejorado en gran medida el estado sanitario y los rendimientos de caña de azúcar en Tucumán, R. Argentina.

**Palabras clave:** pureza genética, micropropagación, sanidad, cultivo de meristemas.

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## INTRODUCTION

A little more than a decade ago, sugarcane production in Tucumán encountered an important sanitary problem, primarily caused by systemic diseases in seed cane. One of the strategies adopted to solve this problem was seed cane hot water treatment. However, this therapy treatment *per se* did not solve the whole sanitary problem, as it was very effective against bacterial diseases but not against viruses. Therefore, in order to tackle the problem of viral diseases, a meristem culture system was developed which had been successfully applied to other plant species to eradicate viruses and other pathogens (Ashmore, 1997; Ferreira *et al*., 1998; Hoy and Flynn, 2001; Miassar *et al*., 2011; Smith and Drew, 1990). The combined effort of introducing a systematic process to provide healthy seed cane, and of using hot water treatment, healthy donor plants for meristem production, and in vitro apical meristem cultures helped to overcome the sanitary problem of systemic diseases. In addition, the production of high quality seed cane did not only improve sugarcane health status, but also improved important agronomic traits such as sprouting, tillering, and biomass yield (Ramallo and Vázquez de Ramallo, 2001).

In order to properly evaluate and guarantee the health of *in vitro* propagated plant material, it is vital to have access to sensitive diagnostic methods which allow detection of very low levels of pathogens. The Biotechnology Department of Estación Experimental Agroindustrial Obispo Colombres (EEAOC) has optimized molecular diagnostic protocols based on PCR amplification of nucleic acids for each one of the major systemic diseases found in sugarcane (Pan *et al*., 1998 and 1999; Yang and Mirkov, 1997). These protocols are all routinely employed each year to ensure healthy *in vitro* propagated plantlets for future seed cane production. Bacterial species tested for in meristem mother plants and in vitro propagated plantlets include *Leifsonia xyli* subsp. *xyli* and *Xanthomonas albilineans*, causal agents of the two most important diseases in sugarcane: ratoon stunt disease (RSD) and leaf scald, respectively. Furthermore, two viruses responsible for sugarcane mosaic disease, *Sugarcane mosaic virus* (SCMV) and *Sorghum mosaic virus* (SrMV), are tested for.

Since the implementation of the "Vitroplantas" Program, the process of obtaining *in vitro* propagated plantlets in the laboratory has been subjected to constant evaluation and improvements in order to maximize the quality of the final product. An important additional method in 2007 was the introduction of molecular markers in order to evaluate possible genetic changes associated with *in vitro* micropropagation (Larkin and Scowcroft, 1981), generally referred to as somaclonal variations. These genetic changes (mutations) are due to stress conditions experienced by plant cells during the propagation procedure (Phillips *et al*., 1994). Somaclonal variation is one of the most important drawbacks of commercial *in vitro* micropropagation, as all mutations in the genome are transferred to subsequent generations, possibly affecting important agronomic and/or biochemical traits of the crop (Soniya *et al*., 2001). It is therefore of utmost importance to maintain the genetic purity of the commercial variety being propagated, and to ensure that no genetic change has taken place, before continuing propagation of the seed cane in the field (Ahmed *et al*., 2002).

The "Vitroplantas" Program does not only serve as a means to propagate established commercial cultivars, but is also an enormously valuable tool for rapid and massive distribution of new elite varieties produced by the EEAOC Breeding Program.

### Procedures in "Vitroplantas" production

All *in vitro* plantlets are produced using protocols optimized for each variety in order to produce plantlets with excellent vigor. The process is divided into two major phases: (1) meristem culture and micropropagation, which can in turn be divided into five separate stages, and (2) genetic analysis and phytosanitary assessment.

### 1. Generation and micropropagation of plant material

#### 1.1. Stage 0: Preparation of starting plant material

The genotypes or varieties multiplied each year are chosen based on demands from producers in Tucumán and on the recommendations of EEAOC breeders. One important factor when selecting plant material is to widen the diversity of varieties, in order to reduce risks associated with the cultivation of a handful of elite varieties within a region.

Donor plants are introduced into the program by taking one-node cuttings (with one shoot), which are subjected to hot water treatment at 50ºC for 2 h. As mentioned earlier, this treatment effectively controls the bacterial diseases RSD and leaf scald. These donor plants constitute a collection of plants grown under perfect health and nutritional conditions in a special greenhouse, with an anti-aphid net. This system of a donor plant collection is renewed every three years. It was first implemented in 2006 to facilitate work procedures and reduce costs, as it assures high quality starting material, especially in regard to health, by diminishing the production of phenolic compounds causing oxidation of growth medium, and lowering bacterial contamination. The apical meristem is excised from donor plants to initiate the propagation.

#### 1.2. Stage 1: Establishing plant culture

The apical meristem is excised from the apical tip of plants from the collection of donor plants, approximately 30 days after sprouting.

Once the tip has been removed, all expanded and encircling leaves are removed, leaving a cylinder with a diameter of around 0.7 cm and 5.0 cm of length. These

cylinders are washed in water supplemented with detergents, thereafter disinfected with sodium hypochlorite solution, and subsequently rinsed three times in sterile distilled water. The apical meristem (3 mm to 5 mm in length) is obtained by removing all plant material surrounding the uppermost part of the tip. The latter is cut and inserted in an inverted position into a solid growth MS medium (Murashige and Skoog, 1962) with an adequate composition of hormones. Each implanted meristem forms a culture line which is identified with a code, which permits maintaining traceability of the micropropagation process. Cultures are incubated in darkness for seven days at 26ºC in order to diminish phenolic oxidation, and to assure good survival rates of plant material. Cultures are thereafter transferred to a plant growth chamber with a photoperiod of 16 h and a temperature of 26ºC, until formation of shoots. This normally takes around 30 days, but is genotype dependent.

**1.3. Step 2: Multiplication of plant material**

At this stage, massive proliferation of new shoots is induced, using the first shoot obtained in the previous stage as starting material. In order to achieve this, the shoot is transferred to a new growth medium containing higher concentrations of plant hormone cytokinin, which induces the formation of new shoots. Newly formed shoots are subdivided in groups of three to four, and again grown on shoot-inducing media to produce more shoots. In general, each cycle takes around 30 days and is repeated a maximum of six times to minimize the occurrence of somaclonal variations. The stage of multiplication is the most time-consuming part of the whole micropropagation process and it is where the number of plantlets is exponentially increased.

The potential of obtaining shoots from a meristem is very high, but is somewhat dependent on the genotype and number of sub-cultures used. As a consequence of lowering the round of sub-cultures to minimize occurrence of genetic variations, our procedure is yielding a relatively low multiplication rate, which generally generates between 1800 and 2000 plantlets at the end of the multiplication stage.

At this stage, before the second sub-culture of shoots, health is evaluated and only totally healthy plants are used for further multiplication.

**1.4. Stage 3: Root formation**

At the final stage of the multiplication process, and when shoots have developed sufficiently, root formation is induced in a growth medium that lacks plant growth hormones and is supplemented with a high sugar content (4%), with mineral nutrients reduced to 50% of the original concentration of MS medium. This is a process that normally takes around 30 days, which is sufficient to obtain good root formation. Roots need to be well developed so that they can succeed in adapting during the acclimatization of these *in vitro* grown plantlets to *ex vitro* growth conditions.

**1.5. Stage 4: Acclimatization**

The acclimatization stage consists of a gradual adaptation of the plantlets to *ex vitro* growth under controlled growth conditions in a greenhouse. Before transferring plants to the new growth environment, they are removed from the *in vitro* growth jar/tube and all remains of solid growth medium are washed away from roots to avoid microbial infection. Plants are thereafter separated and classified into four individual categories, based on plant size (<3 cm; 3-5 cm; 5-7 cm and >7cm), and finally treated in a Captan fungicide solution (2%0) for 24 h.

The acclimatization process is initiated by transferring the washed and fungicide-treated plants to a disinfected growth substrate consisting of humus, soil and a solid support of perlite (3:2:1 ratio) in the greenhouse. From a physiological point of view, during the process plantlets are changing from an *in vitro* heterotrophic growth manner to a photosynthetic and completely autotrophic growth behavior, which includes water balance regulation with the external environment. These physiological and morphological changes are provoked by *in vitro* growth conditions, which lead to low photosynthetic activity, zero or low regulation of stomata, formation of large intercellular spaces and lack of wax formation, all of which have to be reverted during the acclimatization growth stage, so that these plants are able to survive and grow under field conditions (Denng and Donnelly, 1993).

Acclimatization is performed in an especially conditioned greenhouse with high humidity (RH=80-100%) and low light intensity during the first two weeks, to avoid dehydration. After the initial two weeks, light intensity is gradually increased and humidity slowly lowered (Díaz Romero *et al*., 2005). Under these conditions, this critical procedure stage, which to a large extent determines the commercial viability of the whole process, normally takes around 90 days to complete.

**2. Phytosanitary and genetic purity evaluations**

During the laboratory phase of “Vitroplantas” production, plant material is evaluated continuously using different molecular methods to ensure that it is free from systemic pathogens and genetically identical to parental genotypes.

The phytosanitary evaluation is performed after the first sub-culture of shoots at stage 2, as described previously, while genetic variation is tested by using molecular markers at the acclimatization stage. All these tests are routinely and systematically performed annually to ensure that a high quality product departs from our laboratories, to form the basis for future seed cane to be used in the fields of the province.

**2.1 Phytosanitary diagnostics**

During the first four years of the "Vitroplantas" Program, pathogen detection was done primarily by serological testing, using enzyme-linked immunosorbent assays (ELISA) (Filippone *et al*., 2010). In 2005 it was decided that ELISA would be replaced with more sensitive diagnostic methods based on PCR amplification of DNA and cDNA. Pathogens are checked for routinely in the donor plant collection before meristems are introduced, as well as on *in vitro* plantlets, in the initial part of the multiplication stage. No molecular testing is done on plants in the Basic and Registered field propagation stage, due to logistic difficulties in handling and processing such a high number of samples.

Nucleic acid extraction (Aljanabi *et al*., 1999) from high quality plant material is the first step to perform molecular diagnostics, and different protocols have been optimized for different pathogens. In the case of RSD and leaf scald, the basic protocol for nucleic acid extraction is essentially the one described by Pan *et al*. (1998, 1999) with minor adjustments, and for SCMV and SrMV virus testing, an optimized protocol based on a method first described by Yang and Mirkov (1997) is used.

Since the successful introduction of molecular diagnostics, the incidence of pathogens detected in field seed cane propagation stages has been markedly reduced, in some cases reaching levels of no pathogen detection in the Basic seed cane production stage, and negligible occurrence at the Registration propagation stage.

**2.2 Evaluation of genetic stability**

In order to detect any genetic change during the *in vitro* propagation procedure, at the acclimatization stage genetic profiles of propagated plantlets are compared to the profile of the parental genotype using AFLP markers. A difference in marker profiling between a plant sample and the parental variety confirms a genetic change at some point in the micropropagation process, so all plants belonging to that specific line of multiplication are removed (Perera *et al*., 2010). Genetic analysis to check for somaclonal variation was introduced in 2007. The results from the first two years of studies show a very low frequency of genetic changes (< 1% was detected) using the procedure for "Vitroplantas" production described here.

**Final remarks and conclusions**

The first two campaigns of "Vitroplantas" Project were under the charge of the Phytopathology Department of the EEAOC, but since 2003 all work has been done at the Biotechnology Department, which was founded in 2002.

In Table 1 the total number of plantlets produced for each variety in the "Vitroplantas" Program, throughout all seasons from 2001 to 2011, is shown.

As observed in Table 1, over half a million plantlets have been produced over the 10 years the program has been in operation. Encouragingly, demand from producers is increasing every year, and for 2012, it is forecast that the program will produce over 100,000 plantlets for the first time. Thanks to the "Vitroplantas" Program, almost 70% of all sugarcane planted in Tucumán originates from plantlets produced in this propagation scheme. It is noteworthy that a recent preliminary study of two of the varieties propagated in the program (CP 65-357 and CP 85-384) indicated a clear yield improvement when planting *in vitro* propagated seed cane as compared to planting conventional seed cane (García *et al*., 2011).

**Table 1. Number of seedlings per genotype produced by the "Vitroplantas" Project (Tucumán, Argentina) in the 2001–2011 period.**

| Sugarcane genotypes | Years | | | | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | |
| **LCP 85-384** | 47345 | | 3118 | 12275 | 10423 | 15401 | 12853 | 16326 | 16587 | 15112 | 18600 | 168040 |
| **CP 65-357** | 21547 | | 4190 | | 2443 | 13324 | | | | | | 41504 |
| **TUC 77-42** | 1611 | 3091 | 3347 | 1071 | 7199 | 4960 | 8757 | 9890 | 9579 | 5550 | 4067 | 59122 |
| **LCP 85-376** | 3096 | 3735 | 3201 | | | | | | | | | 10032 |
| **RA 87-2** | 1546 | 4958 | 1522 | | | | | | | | | 8026 |
| **RA 87-3** | 3409 | 5808 | 9293 | 5304 | 28344 | 16682 | 9103 | 3533 | 9016 | | | 81476 |
| **L 75-33** | | | 3223 | | 3612 | | | | | | | 6835 |
| **RA 89-28** | | | | 82 | | | 10030 | 1424 | | | | 11536 |
| **RA 95-37** | | | | 317 | | | 6360 | 6255 | 12718 | 13248 | 18087 | 56985 |
| **RA 97-8** | | | | | | | 6239 | 11360 | 16666 | 13047 | 11671 | 58983 |
| **TUC 95-10** | | | | | | | | | | 24635 | 36706 | 61341 |
| **Elite clones** | | | | 7143 | 2168 | 5078 | 17729 | 6389 | 9720 | | | 48227 |
| **Total** | 78554 | 17592 | 27894 | 26192 | 54189 | 55445 | 71071 | 55177 | 74286 | 71592 | 89131 | **621123** |

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