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'. On average 70 000 sugarcane plantlets of commercial varieties are produced through *in vitro* meristem culture in order to eliminate systemic diseases caused by bacteria and viruses. The sanitation of plant material is achieved through *in vitro* culture of apical meristems from donor plants previously hot-water-treated and grown for 3 years under greenhouse conditions with anti-aphid screens. Systemic diseases are detected 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 parental varieties, molecular markers to quantify and detect somaclonal variation are routinely applied. Thus, the aim of this project is to guarantee healthy and genetically pure plantlets before transfer to the field for seed cane propagation. After propagation and testing, in vitro plantlets undergo an acclimatisation process in a specially adapted greenhouse at the EEAOC. In order to avoid dehydration of plantlets, the process takes place in a greenhouse with very high relative humidity (RH=80–100%) and low light intensity. After acclimatisation, two more stages of conventional field propagation (Basic and Registered), are carried out before the seed cane is finally distributed among sugarcane growers. The implementation of the project 'Vitroplantas' has greatly improved the health and biomass yields of sugarcane production in Tucuman, Argentina.

Keywords: ***genetic purity, micropropagation, plant health, meristem culture, sugarcane***

Introduction

A little more than a decade ago, the sugarcane production in Tucuman encountered an important sanitary problem, primarily caused by systemic diseases in cane seed. One of the strategies adopted to resolve this problem was to introduce hot-water treatment of seed canes. However, this therapy treatment *per se* did not solve the whole sanitary problem, as it is very effective against bacterial diseases but not against viruses.

Therefore, in order to resolve the problem of viral diseases, a meristem culture system was developed which had been successfully applied in 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 including hot water treatment, healthy donor plants for meristem production and apical meristem *in vitro* cultures, helped to overcome the sanitary problem of systemic diseases. In addition, the production of high quality seed cane did not only improve the health state of the sugarcane 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 to 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 section at EEAOC has optimised 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; 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, the Sugarcane Mosaic Virus (SCMV) and *Sorghum Mosaic Virus* (SrMV) are tested for.

Since 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 maximise 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) and generally referred to as somaclonal variations. These genetic changes (mutations) are due to stress conditions experienced by the 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 within the Breeding Program of EEAOC.

Procedures in production of 'Vitroplantas'

All *in vitro* plantlets are produced using protocols optimised for each variety in order to produce plantlets with excellent vigour. The process is divided in two major phases: (1) meristem culture and micropropagation which can be divided into 5 separate stages and (2) genetic analysis and phytosanitary assessment.

Generation and micropropagation of plant material

Stage 0: Preparation of starting plant material

The genotypes or varieties multiplied each year are chosen based on demands from producers in Tucuman and from the recommendations of the breeders at the EEAOC. One important factor when selecting the 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 to the program by taking one-node cuttings (with one shoot), and subject them to hot-water treatment at 50 °C for 2 h. This treatment as mentioned earlier 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 anti-aphid net.

This system of a donor plant collection is renewed every three years. This donor plant collection was implemented in 2006 to help facilitate the work and reduce costs as it assures high quality starting material, especially in the health aspect by diminishing 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.

Stage 1: Establishing the 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 cm of length. These cylinders are washed in water supplemented with detergents and thereafter disinfected using sodium hypochlorite solution and subsequently rinsed three times in sterile distilled water. The apical meristem (3–5 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 7 days at 26 ºC 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 26 ºC until formation of shoots. This normally takes around 30 days but is genotype dependent.

Stage 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 the plant hormone cytokinin which induces the formation of new shoots. Newly formed shoots are subdivided in groups of 3–4 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 6 times to minimise the occurrence of somaclonal variations. The stage of multiplication is the most time-consuming part of the whole micropropagation process and is where the number of plantlets is exponentially increased.

The potential for 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 minimise occurrence of genetic variations, our procedure is yielding a relatively low multiplication rate, which generally generates between 1800–2000 plantlets at the end of the multiplication stage.

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

Stage 3: Root formation

At the final stage of the multiplication process, and when shoots have developed sufficiently, root formation is induced in growth medium lacking plant growth hormones and supplemented with high sugar (4%) and 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. Well-developed roots are fundamental in order to successively be able to adapt or acclimatise these *in vitro* grown plantlets to growth conditions ex vitro.

Stage 4: Acclimatisation

The acclimatisation stage consists of a gradual adaptation of the plantlets to growth ex vitro 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 4 individual categories based on plant size (<3 cm; 3–5 cm; 5–7 cm and >7 cm) and finally treated in a Captan fungicide solution (2%) for 24 h.

The acclimatisation 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. During the process, plantlets are, from a physiological point of view, changing from a heterotrophic growth manner *in vitro* to a photosynthetic and completely autotrophic growth behaviour including regulation of its water balance with the external environment.

These physiological and morphological changes are provoked by the growth conditions *in vitro*, which lead to low photosynthetic activity, no or low regulation of stomata, formation of large intercellular spaces and lack of wax formation, which all have to be reverted during the stage of growth acclimatisation in order to enable these plants to grow and perform under field conditions (Denng and Donnelly, 1993).

Acclimatisation is performed in a specially 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 (Diaz Romero *et al*., 2005). Under these conditions, this critical stage of the procedure, which to a large extent determines the commercial viability of the whole process, normally takes around 90 days to complete.

Phytosanitary and genetic purity evaluations

During the laboratory phase of the production of 'Vitroplantas', plant material is evaluated continuously using different molecular methods to ensure freedom 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 acclimatisation stage. All these tests are routinely and systematically performed annually to ensure that a high quality product is leaving our laboratories, forming the base of future seed canes in the province of Tucuman.

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 to replace ELISA with more sensitive diagnostic methods based on PCR amplification of DNA and cDNA. Checking for pathogens is routinely performed in the donor plant collection before meristems are introduced and 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 high number of samples.

Nucleic acid extraction (Aljanabi *et al*., 1999) from plant material of high quality is the first step to perform molecular

diagnostics, and different protocols have been optimised for different pathogens. In the case of RSD and leaf scald, the basic protocol for nucleic acid extraction is essentially as described by Pan *et al*. (1998, 1999) with minor adjustments and for virus testing of SCMV and SrMV, an optimised 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 the field propagation stages of seed cane 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 stage of propagation.

Evaluation of genetic stability

To be able to detect any genetic change during the *in vitro* propagation procedure, genetic profiles of propagated plantlets, at the acclimatisation stage, using AFLP markers are compared to the profile of the parental genotype. A difference in marker profiling between a plant sample and the parental variety confirm a genetic change at some point in the micropropagation process and 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 2 years of studies show a very low frequency of genetic changes (< 1% was detected) using the procedure for producing Vitroplantas as described here.

Conclusions

The first two campaigns of 'Vitroplantas' were performed by the Phytopathology section of the EEAOC but since 2003 all work is done at the Biotechnology section which was founded in 2002. In Table 1, the total number of plantlets produced in the 'Vitroplantas' program for each variety in all campaigns from 2001 to 2011 is shown.

As observed in Table 1, more than half a million plantlets have been produced during the 10 years the program has been in operation. Encouragingly, demand from producers is increasing every year and for 2012 it is forecast to produce over 100 000 plantlets for the first time. As a result of this program, almost 70% of all sugarcane planted in Tucuman originates from plantlets produced in the propagation program of 'Vitroplantas'. It is noteworthy, that a recent preliminary study including two of the varieties propagated within the program (CP65-357 and LCP85-384) indicate a clear yield improvement when planting *in vitro* propagated cane seed compared to conventional seed cane (García *et al*., 2012).

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Table 1. Number of seedling per genotype produced in the period 2001–2011.

Sugarcane genotypes	Years											Total
	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	
LCP 85-384	47 345		3118	12 275	10 423	15 401	12 853	16 326	16 587	15 112	18 600	168 040
CP 65-357	21 547		4190		2443	13324						41 504
TUC 77-42	1611	3091	3347	1071	7199	4960	8757	9890	9579	5550	4067	59 122
LCP 85-376	3096	3735	3201									10 032
RA 87-2	1546	4958	1522									8026
RA 87-3	3409	5808	9293	5304	28344	16682	9103	3533	9016			81 476
L 75-33			3223		3612							6835
				82			10 030	1424				11 536
RA 95-37				317			6360	6255	12 718	13 248	18 087	56 985
RA 97-8							6239	11 360	16 666	13 047	11 671	58 983
TUC 95-10										24 635	36 706	61 341
Elite clones				7143	2168	5078	17 729	6389	9720			48 227
Total	78 554	17 592	27 894	26 192	54 189	55 445	71 071	55 177	74 286	71 592	89 131	621 123

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