Plant Cell, Tissue and Organ Culture **34**: 217–222, 1993.

Development of an *in vitro* culture technique for conservation of *Saccharum* spp. hybrid germplasm

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Received 10 December 1992; accepted in revised form 17 March 1993

Key words: apical buds, germplasm, meristems, sugarcane

Abstract

An *in vitro* method for the establishment and storage of over 200 *Saccharum* spp. hybrid clones was developed that involved only 1 medium for shoot development and multiplication, and no decontamination procedures. Apical buds, from the leaf axils of developing leaves surrounding the apical meristem, were cultured on medium containing the plant growth regulators 6-benzylaminopurine (BAP) and 6-furfurylaminopurine (kinetin), and regenerated multiple shoots. Shoots transferred to medium containing naphthaleneacetic acid (NAA) developed roots. *In vitro* plants transferred to a medium containing half strength salts and vitamins without plant growth regulators were placed in storage at 18°C. After 12 months of storage plants were transferred to fresh medium and returned to storage. The genetic integrity of clones (based on phenotype assessment) was not affected by the *in vitro* culture method and up to 14 months of low-maintenance storage conditions. These *in vitro* plants will be further tested for genetic stability using biochemical and molecular techniques.

Introduction

A network of Australian Plant Genetic Resource Centres has been established for the conservation of crop and pasture germplasm. The Australian Sugarcane Genetic Resource Centre is located at Meringa Sugar Experiment Station near Cairns, Queensland, Australia, where clones of various *Saccharum* spp. are maintained in the field or in concrete containers.

Maintaining large numbers of sugarcane clones as growing plants requires a large facility and adequate staff and resources. Plants grown under field conditions are also at risk from adverse environmental conditions such as cyclones, and pests and diseases. The use of *in vitro* methods for germplasm preservation, coupled with low nutrient medium and low temperature, will provide an alternative for conservation of delicate or disease-susceptible clones.

In vitro storage of sugarcane plants has been developed at the Sugarcane Breeding Institute, Coimbatore, India and at the French International aid organisation CIRAD in Montpellier, France. At CIRAD, *in vitro* sugarcane plants were established from apical meristem tissue and axillary buds (buds growing in the upper leaf axils of stalks), and maintained at 18°C for up to 2 years before subculturing (Paulet et al. 1991). In India, *in vitro* plants established from apical meristems were maintained at 25°C and subcultured after 6 months (Sreenivasan & Sreenivasan 1985). Sugarcane has been micropropagated for rapid multiplication using axillary buds (Sauvaire & Galzy 1978) and meristem tissue (Hendre et al. 1983; Lee 1987; Grisham & Bourg 1989).

Development of *in vitro* techniques in sugarcane has resulted in more efficient and effective means for international exchange of germplasm. Pest and disease-free plants have been produced

through thermotherapy and chemotherapy treatment of meristem tissue, the mass of plant material transferred is reduced, and the transferred material is contained (Hendre et al. 1975; Waterworth & Kahn 1978; Anon 1982; Wagih 1989). The aim of this study was to develop a low-maintenance, *in vitro* culture technique for the establishment and long term storage of a range of Australian and foreign *Saccharum* spp. hybrid clones in axenic culture.

Materials and methods

Initial experiments involved determining the type of meristematic tissue best suited to culture *in vitro*, and optimising the type and concentration of plant growth regulators for establishing *in vitro* plants. These experiments were carried out with sugarcane (*Saccharum* spp. hybrid) cultivars from Australia (Pindar, Q44, Q63, Q87, Q96, Q110 and Q137), Barbados (B4362) and Canal Point, USA (CP44-101). Tissue for culturing was selected from 6- to 8-month-old plants grown in the field at the BSES Pathology Farm, Brisbane, Queensland, Australia.

Effect of tissue type on in vitro *shoot formation*

Two types of meristematic tissue were selected for subculturing.

- Axillary buds from the upper 1–2 cm portion of stalks were excised by cutting behind and along the base of the bud with a sterilised scalpel blade.
- Apical meristems and surrounding 2 to 3 whorls of developing leaves were transversely cut from the upper 0.5–1 cm of stalk.

The developing leaves surrounding the apical meristems contained axillary buds (0.5 to 1.0 mm diameter) in the leaf axils. These buds are referred to as apical buds. Axillary buds and apical meristems were cultured on 10 ml of solid (0.8% w/v, agar; Agar 750, Langdon Co., Sydney, Australia) MS medium (Murashige & Skoog 1962) with the addition of 5% (v/v) coconut water, 20 g l^{-1} sucrose, 0.2 mg l^{-1} 6-benzylaminopurine (BAP) and 0.1 mg l^{-1} 6-furfurylaminopurine (kinetin), in plastic bottles (2.5 cm diameter, 8 cm in length) sealed with a screw-capped lid (referred to as MS1). Cultures were incubated under diffuse white light with a 12-h photoperiod at 27°C. The number of days for shoots to develop to 1 cm in length was recorded.

Effect of plant growth regulators on establishment of in vitro *plants*

The plant growth regulators tested in this study were selected based on the plant growth regulators used by Sauvaire & Galzy (1978), Hendre et al. (1983), Sreenivasan & Sreenivasan (1985), Lee (1987) and Grisham & Bourg (1989) for micropropagation of sugarcane. Apical buds were cultured on MS medium containing a range of concentrations of BAP and kinetin, or naphthalene acetic acid (NAA). Buds were also pretreated by culturing on MS medium containing gibberellic acid (GA_3) and indolebutyric acid (IBA) for 39 days prior to being transferred to MS1 medium containing 0.2 mg l^{-1} BAP and 0.1 mg l^{-1} kinetin.

When the new shoots that developed from the base of the primary shoot were 8 cm in height, the top 3 cm of leaves were discarded and individual shoots were either transferred to fresh MS1 medium for further multiplication or transferred to MS medium containing 60 g l^{-1} sucrose and 1 mg l^{-1} NAA (referred to as MS2) to induce roots (Wagih 1989).

In vitro *culture and storage of a range of* Saccharum *spp. hybrid clones*

During 1990 and 1991, 200 clones of *Saccharum* spp. from the field collection at Meringa Sugar Experiment Station, originating from Argentina, Australia, Brasil, Cuba, Fiji, India, Indonesia, Japan, Mauritius, Mexico, Philippines, Reunion, South Africa, Taiwan, United States of America and West Indies were cultured. Apical buds from 4 to 7 replicates of each clone were excised and incubated on MS1 medium containing 0.2 mg l^{-1} BAP and 0.1 mg l^{-1} kinetin. Shoots that developed were transferred to MS2 medium to promote root growth.

Plants containing roots 1 to 2 cm in length were transferred to 25 ml of solid (0.8% w/v, agar) MS medium containing half the concen-

tration of MS salts and vitamins, 10 g l^{-1} sucrose and 2.5% (v/v) coconut water (referred to as MS3) in plastic containers (4 cm diameter, 11 cm in length) with screw-capped lids. Four plants of each clone were placed in each of 4 containers and incubated under diffuse white light with a 12-h photoperiod at 18°C (Paulet et al. 1991). After 12 months incubation at 18°C, new plants developed from the base of the primary plant. These plants were separated, the top 5 cm of leaves discarded, the roots trimmed and plants transferred to fresh MS3 medium and reincubated at 18°C.

Phenotype assessment of genetic integrity of clones maintained in storage

In vitro plants of clones B4362, Pindar, Q63, Q110 and Q137 maintained in storage for 14 months were pretreated by incubating at 27°C for 1 week before being transferred to pots and grown in the glasshouse at the Pathology Farm, Brisbane. After 6 months, 4 cuttings of each clone were planted in the field adjacent to cuttings from the source field-grown clones.

Four *in vitro* plants of each of CL65-279, H73-6110, M1819-63 and TS67-74 clones maintained in storage at 18°C for 3, 6 and 12 months were transferred to pots, grown in the glasshouse at Meringa Sugar Experiment Station and then 3 to 5 cuttings of each clone were planted in the field adjacent to cuttings from the source field-grown clones. Plants derived from *in vitro* culture were compared to the source clone for changes in phenotypic characters, such as changes in the shape of the leaf blade and leaf sheath (including auricle, dewlap, ligule, hair groups); stalk internode shape, size and colour; and the bud shape and size.

Results and discussion

Effect of tissue type on in vitro *shoot formation*

Both apical buds and axillary buds for all sugarcane cultivars tested developed shoots within 16 days of culture, whereas apical meristems from the same cultivars developed shoots after 21 days (Table 1). Roots formed on shoots that developed from both axillary and apical buds after 7 to 8 days which was almost half the time as that required for roots to form on shoots developed from apical meristems (Table 1).

Table 1. Effect of tissue type on *in vitro* shoot formation for 6 sugarcane clones.

Tissue type	Shoot formation (days)	Root formation (days)
Axillary buds	16 ± 2 (35)	8 ± 1 (28)
Apical buds	16 ± 2 (6)	7 ± 2 (6)
Apical meristems	21 ± 1 (8)	13 ± 3 (3)

Results are the mean ± SE with number of replicates in parentheses.

There are advantages to the development of *in vitro* plants from apical buds rather than from axillary buds or apical meristems. Two to four apical buds located in the leaf axils in each culture developed into shoots, in contrast to apical meristems where only 1 shoot developed per culture. Contamination with saprophytic microorganisms was less in apical buds (20% to 40%) than in axillary buds (more than 90%; data not shown). Contaminants killed the tissue during culture. Axillary buds are located in the leaf axils of mature and senesced leaves, and are morphologically more developed than apical buds; it is therefore, more likely that the vascular tissue or bud scales of axillary buds will be colonised by saprophytic fungi, yeast and bacteria (Wagih 1989).

Effect of plant growth regulators on establishment of in vitro *plants*

Apical buds cultured on medium containing 0.2 mg l^{-1} BAP and 0.1 mg l^{-1} kinetin developed shoots after 15 days and had the highest number of shoot multiplication, compared to the other tested plant growth regulators (Table 2). *In vitro* plants developed directly from the buds without the formation of callus tissue, and this may be important in maintaining genetic integrity. Formation of callus may lead to tissue culture instability which may result in genetic changes in regenerated plants (Scowcroft 1984). Shoots which developed on medium containing 0.2 mg l^{-1} BAP and 0.1 mg l^{-1} kinetin formed roots within 8 days of transfer to root-inducing medium (MS2). However, shoots that developed

Table 2. Effect of NAA, BAP and kinetin, and a 39 day pretreatment of GA_3 and IBA (after pretreatment the apical buds were transferred to 0.2 mg l^{-1} BAP and 0.1 mg l^{-1} kinetin) on shoot and root formation.

Plant growth regulators (mg l^{-1})	Shoot formation (days)	Number of shoots per bud	Root formation (days)
Control	25 ± 4 (4)	1 ± 1 (4)	10 ± 3 (3)
BAP 0.2	20 ± 2 (6)	10 ± 4 (6)	25 ± 16 (6)
BAP 0.4	17 ± 2 (4)	2 ± 1 (2)	100 (1)
Kinetin 0.1	23 ± 3 (4)	10 ± 4 (4)	31 ± 11 (13)
Kinetin 0.2	13 ± 2 (5)	4 ± 2 (5)	49 ± 15 (8)
BAP 0.2 Kinetin 0.1	15 ± 2 (4)	32 ± 4 (4)	8 ± 1 (17)
NAA 1.0	49 ± 11 (10)	6 ± 4 (7)	34 ± 15 (10)
NAA 2.0	28 ± 2 (9)	6 ± 2 (9)	5 ± 1 (15)
NAA 3.0	42 ± 10 (10)	5 ± 1 (8)	25 ± 13 (10)
GA_3 0.1	54 (2)	2 ± 2 (2)	53 ± 47 (2)
GA_3 0.2	83 ± 18 (2)	1 (1)	no roots (2)
IBA 0.01	83 ± 17 (3)	2 ± 2 (3)	7 (1)
IBA 0.05	57 ± 4 (2)	3 ± 2 (2)	54 ± 46 (2)
GA_3 0.1 IBA 0.01	69 ± 16 (3)	3 ± 1 (2)	100 (2)

Root formation occurred after shoots were transferred to medium containing 1 mg l^{-1} NAA and 6% sucrose. Results are the mean ± SE with number of replicates in parentheses.

on medium containing either 0.2 mg l^{-1} BAP or 0.1 mg l^{-1} kinetin separately formed roots after 25 and 31 days respectively. A pretreatment of GA_3 and/or IBA did not enhance shoot or root formation or shoot multiplication (Table 2). Root formation was faster on shoots transferred to medium containing 1 mg l^{-1} NAA than on medium containing no NAA, where root formation was very slow (data not shown).

In vitro sugarcane plants have previously been established from meristematic tissue cultured on medium containing GA_3 and IBA to promote shoot formation, then transferred to medium containing kinetin and BAP for shoot multiplication (Hendre et al. 1983). Using this technique Hendre et al. (1983) found that shoot growth was more rapid from apical buds than from axillary buds. Lee (1987) and Sreenivasan & Sreenivasan (1985) regenerated sugarcane plants from apical meristems with kinetin and BAP; and GA_3, IBA and kinetin, respectively. At the Sugarcane Breeding Station in Barbados, axillary buds cultured with NAA were used to establish plants *in vitro* (Anon 1982). All of these techniques have the disadvantages of being developed for only one or a limited number of sugarcane clones, and involved lengthy procedures for decontaminating the plant tissue prior to culture. Also, the media used in the establishment of shoots were often different from media used for the multiplication of shoots. This increases handling of *in vitro* tissues which may increase the risk of contamination. In contrast, the *in vitro* culture technique developed in this study was applicable to a wide range of sugarcane cultivars, did not need lengthy decontamination procedures, and involved only 1 medium for shoot development and multiplication.

In vitro *culture and storage of a range of* Saccharum *spp. hybrid clones*

Plants of over 200 Australian and overseas clones were established *in vitro* and stored at 18°C. Generally, 2 to 3 months of culture were required for the multiplication of 16 shoots for each clone and a further 2 to 3 weeks for root induction, before plants were transferred to storage.

Contamination of apical bud tissue occurred in some replicates of nearly all clones cultured, but most apical buds from a clone remained clean, hence decontamination procedures were not necessary and *in vitro* plants were established. Hendre et al. (1985), Sreenivasan & Sreenivasan (1985), Lee (1987) and Wagih (1989) found that a high proportion of meristematic tissue was contaminated by saprophytes, and therefore implemented complex thermotherapy and chemotherapy methods to decontaminate the tissue. In most of these cases, axillary buds were selected for culture and, as discussed previously, this tissue is more likely to be colonised by microorganisms.

Of all the apical buds cultured, only 4% failed to grow. Growth failure occurred in 1 or 2

replicates of a genetically diverse range of clones. This failure to grow may have been due to unknown infectious agents such as viruses or endophytic bacteria, or due to the metabolism of toxic by-products such as polyphenol oxidases produced by the plant tissue. Plant pathogenic viruses can be transmitted through meristematic tissue, albeit for some at a low frequency (Hendre et al. 1975). Bacilliform virus has recently been found to infect sugarcane and can be readily transmitted through tissue culture (B Lockhart pers. comm.). Leifert et al. (1991) showed that certain bacteria remained latent in tissue cultured plants, producing neither visible growth on the medium nor symptoms on the plant tissue; elimination of these bacterial contaminants increased growth rates of the plants. Further research is needed to determine the cause of bud germination failure by assessing these buds for the presence of viruses or endophytic bacteria.

Phenotype assessment of genetic integrity of clones maintained in storage

It is important in the establishment of an *in vitro* culture collection to maintain the genetic integrity of the clones being cultured. Plants of B4362, Pindar, Q63, Q110 and Q137, derived from *in vitro* plants stored for 14 months, and plants of CL 65-279, H73-6110, M1819-63 and TS 67-74 derived from *in vitro* plant stored for 3, 6 and 12 months showed no phenotypic (leaf blade and leaf sheath shape; stalk internode size, shape and colour; and bud shape and size) differences compared to the source field grown clones when grown in the field, with the exception that the *in vitro* plants had more tillers (data not shown). Researchers at CIRAD in France showed that some variation in stalk thickness and tillering occurred in plants that had been in culture at 18°C for up to 9 years prior to propagation in the field. However, this variation disappeared after the first vegetative planting in field trials, and so was assumed to be epigenetic (J-C Glaszmann, pers. comm.). *In vitro* cultured plants established in this study will be further tested for genetic stability using biochemical and molecular techniques. Further work will also assess the viability and genetic stability of *in vitro* cultured plants in extended storage for periods longer than 14 months.

Acknowledgements

We wish to thank Nils Berding for helpful discussions during the project, and Warren Owens and Barry Pearce for collecting plant material and attending to the plants in the glasshouse. This research was supported by BSES and a grant from the Sugar Research and Development Corporation. BSES is not a partner, employee or agent of SRDC and has no authority to legally bind SRDC.

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