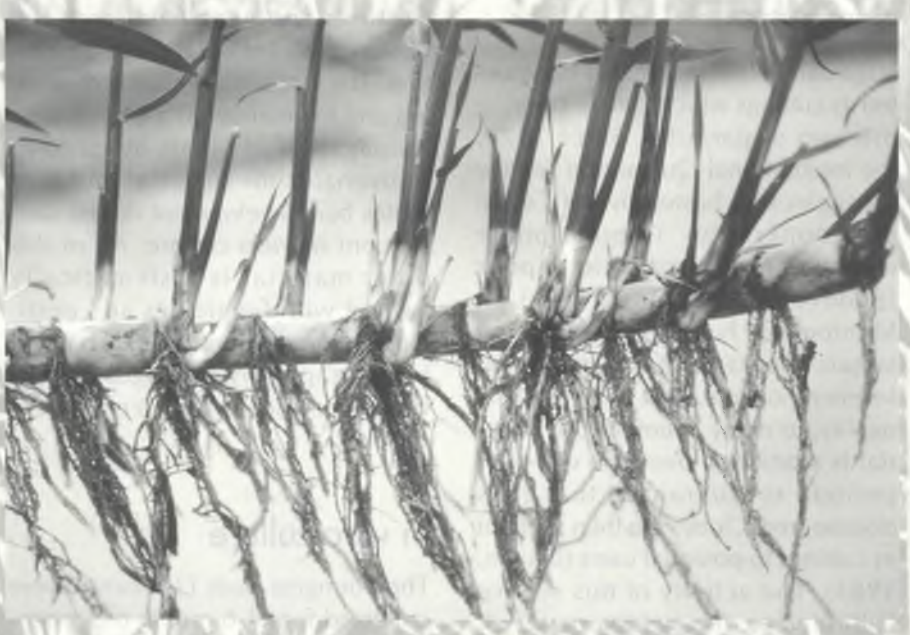
Biotechnological support for varietal extension of sugarcane

Biotechnical advances have benefitted sugarcane varietal extension and exchange. An *in vitro* sugarcane collection has been set up to enable quick access to top agronomic quality and certified healthy plant material.

Sugarcane is multiplied from cuttings and each variety is a clone. Hybridization to create new sugarcane varieties is a particularly long and expensive process. This can be explained on a genetic basis. Sugarcane varieties have a highly complex genome, with 100-130 chromosomes derived from several different ancestral species. Each cross completely destructures the parental traits, and very few of the progeny have any agricultural potential. Evaluating this potential is also complex. The sugarcane growth cycle includes several harvests, with an initial plant stage that occurs a year after the cuttings are planted out. There are several subsequent ratoons, but the traits are not very correlated between stages, especially between the plant stage and successive ratoons. Moreover, performances at the same stage can vary depending on the environment and the year. This means that very complicated assessment procedures are required when breeding for high performance clones.

Different plant breeding stations worldwide produce varieties that are adapted to their regions. These certified materials can be of prime interest as progenitors in other variety improvement programmes. They are sometimes found to be well adapted



Sugarcane axillary bud sprouts.
Photo R. Fauconnier

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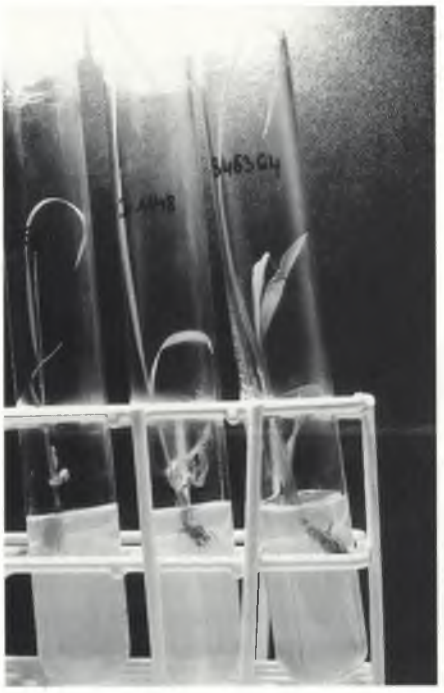


Storage of *in vitro* plantlets at 18 °C.
Photo CIRAD-CA

to areas beyond those where they were originally bred. This means that they can be used for varietal renewal at stations that do not have their own sugarcane breeding programme. Such exchange of difficult-to-breed sugarcane varieties represents a major advance.

Sugarcane plant material is transported as cuttings which can be carrying diseases or parasites. Since 1978, the International Quarantine Service for sugarcane, hosted by the Centre de coopération internationale en recherche agronomique pour le développement (CIRAD) in Montpellier, has been dispatching sugarcane varieties of industrial interest, or material close to this quality, to many countries. All material is monitored over two one-year periods to guarantee that it is disease-free. Clones are then sent out as cuttings to potential users (Baudin, 1984). The activity of this service is limited to dispatching material, i.e. they are not able to keep material after it is released from quarantine due to the limited facilities.

An *in vitro* sugarcane collection, was set up downstream from the Quarantine Service in an attempt to deal with the above-mentioned limitations (Paulet *et al.*, 1991).

In vitro collections already exist for some other crops such as potato and cassava (Rocca *et al.*, 1989). This is an ideal means of preserving potentially useful germplasm without requiring much space, and enables rapid distribution of a broad range of certified healthy plants. This *in vitro* plant material can also be cytopreserved, a useful procedure for very long-term germplasm conservation. In addition, enzyme electrophoresis and other new molecular biology tools are used for rigourous control of the collection.

Production of *in vitro* plantlets

In vitro plantlets are obtained by culture of axillary buds from healthy plants, as described by Sauvaire & Galzy (1978).

Greenhouse maintenance of stock material

Cuttings are taken from certified healthy plants released from quarantine. The cuttings are sprouted, planted and maintained in a greenhouse. Propagation of plants by cuttings rejuvenates the material and promotes bud development during subsequent *in vitro* culture. All of this plant material is systematically treated with fungicides and pesticides alternately on a weekly basis to hinder saprophytic fungus attacks which could jeopardize *in vitro* culture.

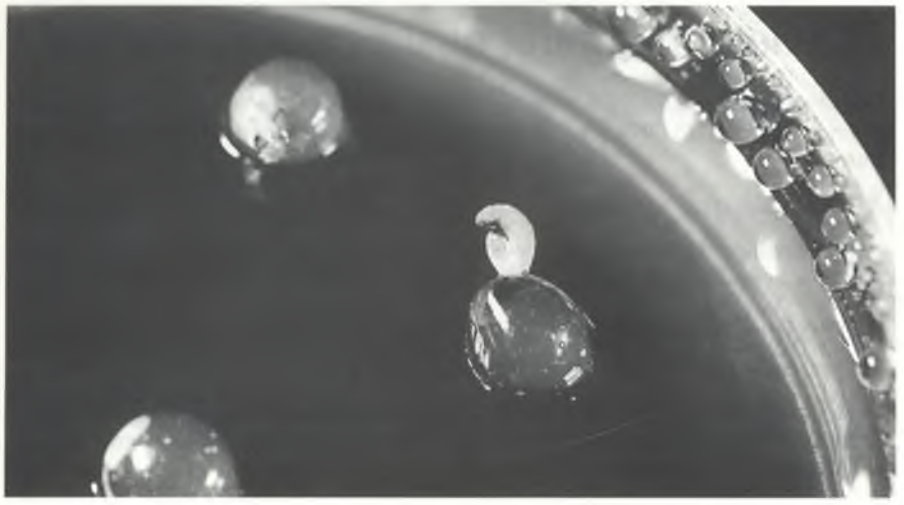
In vitro culture

The youngest buds (3-4/cane) were collected fom 4-6 month old greenhouse-grown sugarcane plants (Montpellier, France). They were then cultured on MS medium (Murashige & Skoog, 1962) in petri dishes at 25°C. They were subcultured separately into culture tubes after 4-6 weeks when new suckers appeared. Once 10 well-formed plantlets were available, they were again subcultured separately into culture tubes and stored at 18°C on diluted MS medium. These *in vitro* conditions decelerate plantlet growth, thus reducing the number of culture manipulations required. The nutrient medium level and leaf withering are monitored regularly to detect clones that should be subcultured first. Subculture intervals vary from 6 months to 2 years, depending on the variety (suckering rates) and depletion of the medium.

Use of *in vitro* plantlets

These *in vitro* plantlets can be used for rapid introduction of new varieties at sugarcane plantations. After a request is filed, cuttings can be made directly or they can undergo rapid micropropagation (Feldmann & Rott, 1991).

Sugarcane bud 15 days after *in vitro* subculture.
Photo CIRAD-CA



Apex sprout 10 days following cryopreservation.
Photo P. Feldmann

Dispatching *in vitro* plantlets

When a given variety is requested, some of the plantlets available at the Montpellier *in vitro* collection are placed at 25°C for multiplication. It then takes 3-6 months to produce about 10 new plantlets by natural suckering. These plantlets are then subcultured onto rooting medium and dispatched. This multiplication process can be continued if the user has a suitable laboratory. The waiting time to receive these *in vitro* plantlets is thus reduced to 6 months, whereas 2-3 years would be required to put the requested varieties through a new quarantine cycle.

Performance of *in vitro* plantlets

When the *in vitro* plantlet is transferred to soil, it is critical to follow the recommended procedure in order to optimize regrowth (cf. procedure for transferring *in vitro* sugarcane plantlets to soil). Planted out sugarcane *in vitro* plantlets have a particularly distinct facies, differing from plants propagated by cuttings (Feldmann & Rott, 1991). This distinctiveness is variety-dependent and generally expressed by increased tillering and decreased cane diameters; yields are thus close to those obtained with cuttings. These differences gradually disappear over the course of field propagation.

Current status of the *in vitro* collection

The CIRAD *in vitro* sugarcane collection in Montpellier (France) was founded in 1982. The collection presently includes 363 varieties, most of which are established and stored at 18°C. Improvements are being investigated to deal with the increased size of the collection.

Varieties in the collection

The collection includes 363 varieties available upon authorization of the corresponding breeding institutions (cf. list of sugarcane varieties stored at the *in vitro* collection).

Genetic stability and cryopreservation technique

It seems that stored whole plant tissues (i.e. without dedifferentiation — buds, plantlets, etc.) can *a priori* be maintained in a genetically stable state on culture medium containing no growth hormones. However, a technique is still needed to reduce *in vitro* plantlet manipulations and especially to limit the need for cell multiplication under artificial conditions. A cryopreservation technique has been developed in experiments with variety Co 6415 (Dereuddre, 1994), whereby apices encapsulated in alginate beads are frozen in liquid nitrogen. The technique was successfully tested on 16 different sugarcane varieties, with regeneration rates of 14-91% (Gonzalez Arnao *et al.*, 1993; Paulet *et al.*, 1993). Cryogenic preservation theoretically permits indefinite storage of plant material.

Controlling genetic variation

To avoid varietal mislabellings, it is essential to be able to detect possible confusions that could occur as a result of the many steps between the time the clone arrives as a cutting and its release as an *in vitro* plantlet. Enzyme electrophoresis can be used to assess clonal identity. A pilot study carried out with 62 sugarcane varieties revealed the presence of 21 polymorphic bands in some of the varieties, while absent in others. Each variety was separately compared with each of the other varieties (i.e. 1 891 comparisons) and only two pairs could not be distinguished; the band patterns obtained for each of the other 58 varieties were unique. By this technique, enzymes are extracted from young fresh leaves cut from an *in vitro* plantlet. Characterization by restriction fragment length polymorphism (RFLP) DNA analysis is more complex but also much more precise; a fingerprint can thus be obtained for each variety. RFLP analysis, in contrast to isoenzymatic techniques, can be performed on dried leaves. This means that clones from the Montpellier *in vitro* collection can be compared at any time with others preserved under tropical field conditions.

Conclusion

Biotechnology is sometimes used to create new unique plant varieties, e.g. genetic transformation. Biotechnological techniques are employed to certify the genetic purity of germplasm conserved in the CIRAD sugarcane collection in Montpellier. Hence, hundreds and even thousands of varieties can be stored in a small space — totally protected from insects, diseases, climatic hazards, with very little risk of genetic drift or accidental between-variety mixing. This collection is of major interest for varietal extension and should prove to be a key component for international sugarcane germplasm conservation.

Genetic mapping of sugarcane varieties

Several steps are required for varietal characterization by restriction fragment length polymorphism (RFLP) DNA analysis (nuclear DNA is considered here, not cytoplasmic DNA):

– DNA extraction;

– DNA digestion, i.e. restriction enzyme digestion of DNA to cleave at well-defined sites, thus forming fragments of different lengths;

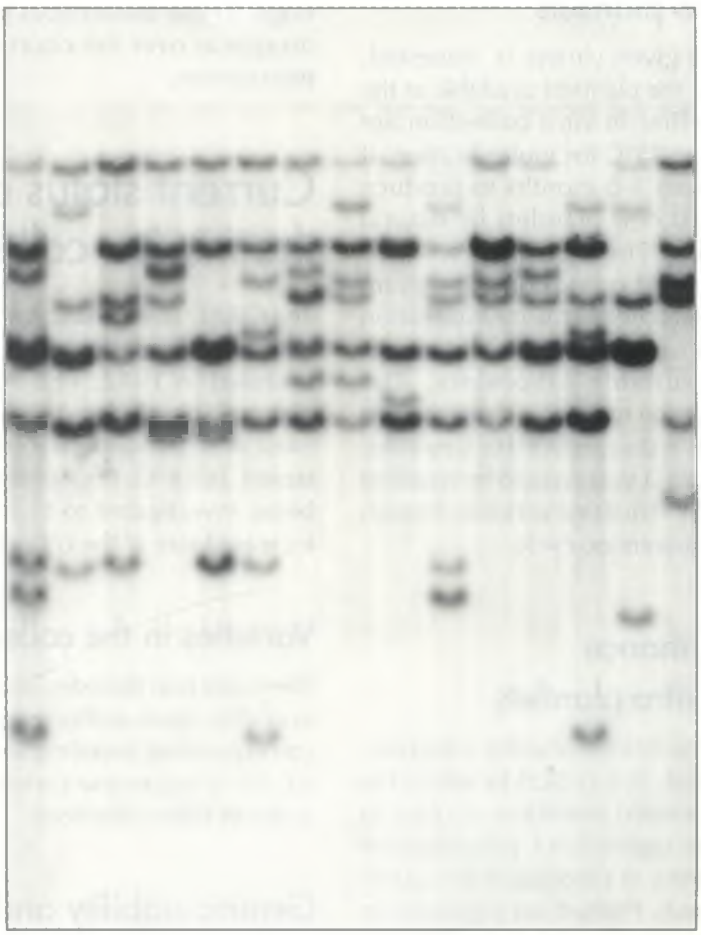
– agarose gel electrophoresis separation of fragments by length;

– transfer of separated fragments onto nylon membranes;

– molecular hybridization with specific DNA probes (pre-labelled with phosphorus 32 for instance): each probe detects and binds to fragments on the membrane that contain complementary sequences;

– recognized fragments are then revealed by placing the membrane in contact with an autoradiographic film which is then printed by the probe radioactivity;

Each clone is thus characterized by a set of bands representing its fingerprint (photo). Many different probes are available. With only five probes, it is possible to distinguish more than 10 000 randomly chosen varieties.



Banding profiles obtained for 15 sugarcane varieties. For each variety, DNA was digested by the Hind III restriction enzyme, fragments were separated by electrophoresis, and hybridization was performed with the UMC39 probe.
Photo J.-C. Glaszmann

Transferring *in vitro* sugarcane plantlets to soil

Storage of *in vitro* plantlets

The *in vitro* plantlets are stored on rooting medium in sterile sealed plastic culture tubes. After subculturing, they are kept at 25-30°C for 3-4 weeks before being dispatched.

Removal from sterile conditions

This can be done once the rooted *in vitro* plantlets have 5-6 roots that are 2-3 cm long. Uncap the culture tubes, carefully remove the plants, rinse the roots in water, clip off the leaves to reduce evaporation and stimulate bud sprouting.

Fungicide treatment

Dip the plantlets in a solution with Benlate (1 g/l formulated product) or Aliette (1 g/l formulated product).

Dipping the plantlets in a fungicide solution.

Transplanting

In vitro plantlets are transferred to Jiffy 7 (compressed peat pellets). First, soak the peat pellets for 10 min with the above described fungicide solution. Pierce the pellet and slip the roots into the hole. Close the hole and pile the peat up around the plantlet.

Transplanting plantlets in Jiffy 7.

Rooted *in vitro* plantlets ready for planting in soil

Soil planting conditions

Place the ready-to-plant *in vitro* plantlets out of the wind and sun. Cover the Jiffy 7 pellets with clear plastic to hinder drying and leave them covered until 2-3 new leaves are produced. The plantlets can be misted if any withering is noted. Water the plantlets daily, with a leaf-type fertilizer supplement (e.g. Mairol, 2 g/l formulated product) after 2-3 weeks. Spray the plantlets weekly with Bayleton 5 (1 g/l formulated product) to control fungal attacks.

Field planting

This can be done after 4-6 weeks if the plantlets are growing vigourously. Dip the Jiffy 7 pellet and plantlet in Bayleton (1 g/l), prepare the planting site, dig holes large enough to plant the peat pellets. Do not damage the planting block or roots. Place the Jiffy 7 pellets in the soil directly.

Plantlets in Jiffy 7 under a minigreenhouse.

Greenhouse plantlets, 6-8 weeks old, ready for planting.

Clip the leaves slightly to reduce drying. If the plantlets are not very vigourous after 6 weeks in Jiffy 7, transfer them to pots with more substrate to supplement the depleted Jiffy 7. Treat the plantlets with a systemic insecticide (Curater).

The transfer to soil should be carried out when the soil temperature is above 18°C. High atmospheric humidity is favourable. The plantlets should be misted periodically, even if the fields are irrigated.

Field planting.

Photos P. Feldmann

Sugarcane varieties in the *in vitro* collection

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B37172	Co312	CP52-43	CP72-356	L60-25	Phil56226	RB705007	Tuc68-18
B4362	Co331	CP52-48	CP72-370	L62-96	Phil58260	RB705146	Tuc68-19
B46364	Co419	CP57-603	CP72-1312	L65-69	Phil6553	RB705375	Tuc69-117
B47258	Co421	CP59-73	CP73-351	L66-97	Phil6559	RB735220	Tuc71-5
B49119	Co449	CP60-01	CP73-1547	L72-85	Phil6607	RB735275	Tuc72-16
B51129	Co462	CP62-258	CP74-383	LF63-863	POJ2878	RB748022	Tuc72-23
B52107	Co527	CP63-306	CP74-2005	LF66-2918	PR1007	RD7410	Tuc74-1
B60125	Co740	CP63-588	CP77-414	M31/45	PR1016	RP148-70	Tuc74-6
B63118	Co775	CP65-357	CRA6026	M569/69	PR61632	S17	Tuc74-10
B63119	Co785	CP66-315	EAK7076	N11	R397	SIP58-136	Tuc74-24
B64277	Co842	CP66-346	FR80234	N12	R469	SP70-1005	Tuc74-26
C334-64	Co1001	CP67-412	FR80236	N14	R472	SP70-1078	Tuc74-34
C323-68	Co1148	CP67-413	FR80412	N15	R526	SP70-1081	Tuc74-46
CB36-24	Co1157	CP670	IAC4865	N16	R566	SP70-1284	Tuc75-1
CB40-13	Co1177	CP68-350	IAC51205	N17	R567	SP70-1423	Tuc75-2
CB41-76	Co1202	CP68-1022	IAC52150	N18	R568	SP70-1478	Tuc75-3
CB45-155	Co1230	CP68-1026	IAC58480	N19	R570	SP70-3225	UCW5465
CB46-47	Co62175	CP68-1067	IAC64257	N52219	R572	SP70-3370	WI82777
CB47-15	Co6304	CP68-1154	Ja59-03	N53216	R70367	SP71-799	
CB47-355	Co6415	CP69-1059	Ja64-11	NA56-79	R7417	SP71-6163	
CB49-260	Co6806	CP70-1133	Ja64-15	NCo310	R75631	SP72-4928	
CB53-98	CoS443	CP72-353	Ja64-20	NCo334	RB7096	SP75-179	
CB56-126	CoS510	CP72-355	KWT57-423	NCo376	RB70194	Tuc67-27	
B6504	B7695	B7882	B79118	H56-278	M657/66	MY64-26	Q126
B6623	B76102	B78130	B79130	H56-4848	M3035/66	NI1	Q127
B69379	B76113	B78178	B79226	H57-5174	M695/69	NIF3	Q129
B69566	B76132	B78224	B8007	H59-3775	MEX64-1214	NIN2	Q130
B69758	B76146	B78237	B8008	H62-4671	MEX65-1424	Q75	Q134
B70442	B76181	B78242	B8066	H65-7052	MEX66-1247	Q84	Q135
B70462	B76196	B78244	B8093	H66-4927	MEX68-200	Q90	Q137
B70482	B76247	B78245	B80276	H68-1158	MEX69-290	Q95	Q138
B70520	B76398	B78249	B80361	H68-2235	MEX70-485	Q96	RK65-37
B70531	B7784	B78266	B80689	H69-8235	Mol45-03	Q102	SES14
B70532	B7795	B78299	BJ7013	H69-9103	MQ72-1175	Q103	SES231
B72191	B77123	B78358	F146	H70-144	MQ72-4005	Q108	US56-15-8
B74254	B77126	B78360	F151	H72-8597	MQ72-5006	Q109	Galoa
B74359	B77392	B78366	F156	H73-6110	MQ72-5089	Q110	Kaba
B74477	B77415	B78436	F160	H75-8776	MQ76-23	Q111	Mali
B75300	B77565	B78482	F161	H77-6694	MQ76-53	Q113	Mana
B75412	B77639	B78560	F167	H78-7234	MY53-53	Q114	Ono
B75519	B77740	B78604	F175	HJ57-41	MY53-173	Q115	Tabongo
B75524	B7802	B78628	F176	M574/62	MY54-62	Q117	Triton
B75527	B7814	B78697	F178	M237/62	MY54-129	Q120	Trojan
B75532	B7852	B78700	H37-1933	M2173/63	MY55-14	Q121	Uba Naguin
B7639	B7877	B7997	H50-7209	M376/64	MY57-15	Q124	

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Abstract... Resumen... Résumé

F. PAULET, J.-C. GLASZMANN – **Biotechnological support for varietal extension of sugarcane.**

Biotechnological advances have benefitted sugarcane varietal extension and exchange. An *in vitro* sugarcane collection has now been set up as a supplement to the "International Quarantine Service" organized by CIRAD in Montpellier (France). *In vitro* sucker regeneration and latency conservation of the resulting plantlets has been developed. Almost 400 varieties or clones (10 specimens of each) have now been stored. This *in vitro* collection does not take much space and enables distribution of a broad ronge of healthy plants rapidly. This *in vitro* plant material can also be cryopreserved, a useful procedure for genetic resource conservation. Enzyme electrophoresis and other molecular biology tools (RFLP) are used to control the collection, thus guaranteeing its reliability.

Key words: *Saccharum*, sugarcane, clone, *in vitro* culture, molecular biology, enzyme electrophoresis, cryopreservation, *in vitro* collection, quarantine.

F. PAULET, J.-C. GLASZMANN – **Las biotecnologías como ayuda a la difusión de variedades de caña de azúcar.**

La diffusión y los intercambios de variedades de caña de azúcar sacan provecho hoy de los progresos realizados en biotecnología. Como complemento del servicio de "cuarentena internacional" organizado por el CIRAD en Montpellier, se ha emprendido la constitución de una colección de caña de azúcar *in vitro*. Se ha llevado a cabo la regeneración *in vitro* de yemas axilares y la conservación en letargo de las plántulas obtenidas. Actualmente se tienen almacenadas cerca de 400 variedades (o clones), con 10 muestras por variedad. Esta "vitroteca" ocupa un espacio reducido y permite suministrar rápidamente plantas sanas para una amplia gama de material. Esta forma *in vitro* también posibilita la aplicación de la crioconservación, que es útil para la preservación de recursos genéticos. La utilización de la electroforesis de enzimas y de las nuevas herramientas de la biología molecular (RFLP) refuerza el control y la fiabilidad de la colección.

Palabras clave: *Saccharum*, caña de azúcar, clon, cultivo *in vitro*, biología molecular, electroforesis enzimática, crioconservación, vitroteca, cuarentena.

F. PAULET, J.-C. GLASZMANN – **Les biotechnologies en soutien à la diffusion variétale chez la canne à sucre.**

Diffusion et échanges variétaux chez la canne à sucre bénéficient aujourd'hui des progrès en biotechnologie. En complément du service de « quarantaine internationale » organisé par le CIRAD à Montpellier, il a été entrepris de constituer une collection de canne à sucre *in vitro*. La régénération *in vitro* de bourgeons axillaires et la conservation en vie ralentie des plantules obtenues ont été mises au point. Près de 400 variétés (ou clones) sont maintenant stockées à raison de 10 échantillons chacune. Cette « vitrothèque » occupe un espace réduit et permet de fournir rapidement des plants sains pour une large gamme de matériel. Cette forme *in vitro* rend possible également l'application de la cryoconservation, utile pour la préservation des ressources génétiques. Le recours à l'électrophorèse d'enzymes et oux nouveaux outils de la biologie moléculaire (RFLP) vient renforcer le contrôle et la fiabilité de la collection.

Mots-clés : *Saccharum*, canne à sucre, clone, culture *in vitro*, biologie moléculaire, électrophorèse enzymatique, cryoconservation, vitrothèque, quarantaine.