In vitro manipulations and strategies for the improvement of sugarcane cultivars in South Africa

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SA Sugarcane Research Institute

• 500 employees in 5 Resource centres
• 6 research farms
• 50 scientists
• 15 postgraduate students + 20 interns
Sugar industry in SA

- ranked in top 15 producers globally
- 423,000 ha sugarcane planted
- 35,000 growers, 80% small-scale account for 11% of the crop
- sugarcane crush: 18.9 million tons
- sugar production: 2.2 million tons
- total average industry income: R 8 billion p.a
- job opportunities: 77,000
In vitro applications in:

1. Micropropagation
2. Virus elimination
3. Genetic engineering
4. Mutagenic breeding
5. Germplasm conservation

Routes of sugarcane regeneration

**Indirect morphogenesis**
- Indirect shoot formation
- Indirect somatic embryogenesis

**Direct morphogenesis**
- Direct shoot formation
- Direct somatic embryogenesis

- Shoot meristem culture

**Conventional vegetative propagation using setts**
1. Micropropagation

Conventional vegetative propagation is routine in sugarcane

Problems associated with this:
• Spread of disease
• Slow rate of propagation
• Affects adoption rate of new cultivars

Limitations of conventional vegetative propagation make *in vitro* propagation an attractive alternative

At SASRI two different routes of morphogenesis have been used: somatic embryogenesis and meristem culture
Micropropagation: somatic embryogenesis

Collection of tops from field

Immature leaf roll cultured

RITA® vessel

Embryo germination

Embryo production
Micropropagation: meristem culture

- Bud germination or field collection of stalk apices
- Shoot multiplication
- Meristem excision
- Shoot establishment
1. Micropropagation

NovaCane®:
• disease-free tissue culture plants generated in laboratory and planted in field for seedcane
• registered as a Trademark by SASRI

• The technology has been tested on several commercial cultivars
  N12, N16, N19, N25, N31, N32, N36, N40, N41, N46, N49, N50

• The multiplication rate after 5 months varies between genotype:
  800-3,500 plants/meristem
  500-10,000 plants/30 leaf discs (somatic embryogenesis)

• Two commercial labs, DuRoi (Letsitele) and Dube AgriLab (Durban) are using NovaCane® to supply plants to sugarcane growers for seedcane purposes
2. *In vitro* culture and virus elimination

- SASRI Quarantine - facilitates import of foreign germplasm and disease-indexes to ensure free of causal agents.

- Traditional screening process involves molecular disease-indexing and vegetative growth and takes 2-3 years prior to release of germplasm.

- Thermotherapy and meristem culture can shorten this by half and can clean up germplasm infected with viruses such as SCYLV and SCMV.

2. *In vitro* culture and virus elimination

Table showing incidence of viruses in plants tested by molecular diagnosis after bud thermotherapy, meristem excision and culture (n=10)

<table>
<thead>
<tr>
<th>Meristem size (mm)</th>
<th>After 14 weeks <em>in vitro</em></th>
<th>After 9 months (glasshouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SCMV</td>
<td>SCYLV</td>
</tr>
<tr>
<td>0.5 -1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1 - 2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2 - 10</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
3. Genetic engineering

*In vitro* aspects critical for genetic transformation:

- selection and generation of target material
- process of regeneration
- response to selection agents
- genotypic response
- novel application of suspension cultures for evaluation of transgene expression

3. Genetic engineering

- Complex polyploid and aneuploid genome
- No fertile pollen under natural conditions in SA
- Limited gene pool for desirable trait exploitation
- Unpredictability of obtaining elite progeny
- Certain traits are negatively associated e.g. smut and eldana
3. Genetic engineering

- GM proof of concept
  - Herbicide tolerance
  - Insect resistance (Bt)
  - Mosaic virus
  - NUE
  - Sucrose metabolism
  - Drought tolerance

3. Genetic engineering

GM proof of concept

- Herbicide tolerance
- Insect resistance (Bt)
- mosaic virus
- NUE
- Drought tolerance

R&D – proof of concept
‘get it to the field’

vs

Commercialisation
The ‘whole deal’

3. Genetic engineering

<table>
<thead>
<tr>
<th>Trait (gene)</th>
<th>Stage of assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herbicide tolerance</td>
<td>Field trials: commercial cultivars transformed and tested over several ratoons</td>
</tr>
<tr>
<td>- Glyphosate (<em>Agrobacterium</em> CP4 gene)</td>
<td></td>
</tr>
<tr>
<td>- Glufosinate ammonium (<em>Streptomyces viridochromogenes pat</em> gene)</td>
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3. Genetic engineering

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<th>Trait (gene)</th>
<th>Stage of assessment</th>
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<tr>
<td>Eldana resistance</td>
<td>Greenhouse pot trials</td>
</tr>
<tr>
<td><em>(Bacillus thuringiensis Cry1Ac gene)</em></td>
<td></td>
</tr>
</tbody>
</table>

**Comparison of eldana larvae per stalk in transgenic and wildtype lines**

## 3. Genetic engineering

<table>
<thead>
<tr>
<th>Trait (gene)</th>
<th>Stage of assessment</th>
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<tbody>
<tr>
<td>Sugarcane mosaic virus resistance (antisense SCMV potyvirus coat protein)</td>
<td>Field trials over 2 ratoons</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Line designation and variety</th>
<th>Mosaic resistance rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic NCo310 lines</td>
<td></td>
</tr>
<tr>
<td>TG76</td>
<td>5</td>
</tr>
<tr>
<td>TG77</td>
<td>2</td>
</tr>
<tr>
<td>TG78</td>
<td>5</td>
</tr>
<tr>
<td>TG79</td>
<td>4</td>
</tr>
<tr>
<td>TG86</td>
<td>4</td>
</tr>
<tr>
<td>TG87</td>
<td>4</td>
</tr>
<tr>
<td>TG88</td>
<td>3</td>
</tr>
<tr>
<td>TG89</td>
<td>4</td>
</tr>
<tr>
<td>TG91</td>
<td>6</td>
</tr>
<tr>
<td>TG92</td>
<td>4</td>
</tr>
<tr>
<td>TG93</td>
<td>4</td>
</tr>
<tr>
<td>Wild type NCo310</td>
<td>9</td>
</tr>
<tr>
<td>NCo376 (HS)</td>
<td>9</td>
</tr>
<tr>
<td>N19 (S)</td>
<td>9</td>
</tr>
<tr>
<td>N12 (I)</td>
<td>4</td>
</tr>
<tr>
<td>N16 (I)</td>
<td>5</td>
</tr>
<tr>
<td>N36 (I)</td>
<td>4</td>
</tr>
<tr>
<td>N21 (R)</td>
<td>3</td>
</tr>
<tr>
<td>N27 (R)</td>
<td>2</td>
</tr>
</tbody>
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## 3. Genetic engineering

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<tr>
<td>*Sucrose metabolism and modified cell wall composition for ethanol production (collaboration with IPB, University of Stellenbosch) [neutral invertase, pyrophosphate:fructose 6-phosphate 1-phosphotransferase (PFP), sucrose synthase, UDP glucose dehydrogenase]</td>
<td>Glasshouse and selected lines in field trials</td>
</tr>
<tr>
<td>Drought tolerance (DREB transcription factors)</td>
<td>Laboratory</td>
</tr>
<tr>
<td>N use efficiency (alanine aminotransferase – Arcadia Biosciences)</td>
<td>Laboratory and glasshouse pot trials underway</td>
</tr>
</tbody>
</table>

3. Genetic engineering

Major restriction is access to intellectual property (IP) i.e. gene constructs

• Extremely high licencing costs
• Relatively small size SA sugar industry compared with e.g. Brazil

The role of the sugar Industry in progressing GM sugarcane

• Implement decision-making process on GM trait to be commercialised
• Enter commercial agreements with IP owners of desired trait
• Allocate financial resources for royalties and biosafety dossiers
• Overcome logistic and infrastructural challenges associated with sugar production from GM and non-GM sugarcane
• Establish agricultural and environmental stewardship programmes

International GM sugarcane landscape

**USA**
- GM sugarbeet deregulated i.e. sugar derived from GM plants soon on world market
- GM sugarcane field trials and regulatory dossier (mosaic virus and herbicide tolerance)

**Brazil**
- Monsanto and Syngenta – conventional breeding, transgenesis and micropropagation
- CTC collaborating with BASF, Bayer and Dow Agroscience
- Field trials – 2nd generation ethanol, weed and insect control

**South Africa**
- GM sugarcane field trials
- License from Arcadia Biosciences, USA – improved N use efficiency

**Argentina**
- Research

**India**
- Research

**China**
- Research

**Japan**
- Research

**Indonesia**
- Drought tolerant sugarcane passed through biosafety committee
- For local market only

**Australia**
- GM sugarcane field trials
- Collaborating with DuPont, Syngenta for commercial release – weed control and ‘Sugarbooster’ (isomaltulose)
4. *In vitro* mutagenesis

Somaclonal variation
- auxin, UV, chemical mutagens
- potential for commercial application

- Approach involves exposure of embryogenic callus to the chemical mutagens ethyl methanesulfonate (EMS) and/or 5-Azacytidine

**Herbicide tolerance**: to imazapyr (Arsenal), conferred by single point mutation in enzyme acetolactate synthase (ALS)

**Eldana + Fusarium spp. tolerance**:
- (a) Plant tolerance to Fusarium
- (b) ‘biological’ control of eldana
4. *In vitro* mutagenesis - herbicide

Leaf appearance 6 weeks after herbicide application

<table>
<thead>
<tr>
<th>Mut1</th>
<th>Mut2</th>
<th>Mut3</th>
<th>Mut4</th>
<th>Mut5</th>
<th>Mut6</th>
<th>Mut7</th>
<th>N12</th>
</tr>
</thead>
</table>

Plot A
unsprayed

Plot B
imazapyr applied at 312 g a.i. ha\(^{-1}\)

Plot C
imazapyr applied at 625 g a.i. ha\(^{-1}\)

Rutherford et al. (2014) *J Hort Sci and Biotech* 89:1-16
4. *In vitro* mutagenesis - herbicide

Germination (as a % of the N12 control) 3 weeks after planting of 3-budded setts in untreated and treated (imazapyr) plots.
4. *In vitro* mutagenesis - *eldana*

Putative tolerant plants: 2 months after acclimation

Lesion severity ratings: 2 months after inoculation by stabbing stems with *F. sacchari* PNG40-colonised toothpicks

Confirming identity of isolates using Inter-Simple Sequence Repeat (ISSR) analyses

Re-isolation of *F. sacchari* PNG40 on *Fusarium* semi-selective medium

5. Germplasm conservation

Cryopreservation and Minimal Growth

Slow growth = restricting growth *in vitro* by
- reduction in temperature and nutrients
- addition of osmotically-active agents
- absence of growth regulators

Advantages:
- international germplasm exchange
- germplasm conservation
- management of *in vitro* material e.g. transgenic samples

Aim: to hold material *in vitro* at three stages

- embryos
- shoot meristems
- germinated plantlets
5. Minimal growth

- 24 °C for 8 months
- 18 °C for 12 months
5. Minimal growth $18^\circ C$

Collaborative projects

1. International Consortium for Sugarcane Biotechnology (ICSB)

Member countries (Argentina, Australia, Brazil, Colombia, Ecuador, France/Reunion, Guatemala, India, Mauritius, Philippines, South Africa, USA – Florida, Louisiana, Texas, Hawaii, West Indies)

Range of projects – mapping, marker assisted selection, chloroplast transformation, germplasm introgression

2. Sugarcane genome sequencing initiative (SUGESI)

Consortium (Australia, Brazil, France, South Africa, USA)
Concluding remarks

• In vitro culture integral to several applications for sugarcane improvement
• Focus – to manipulate conditions to decrease labour + time input

Sett germination in vitro

Holding plantlets in water for up to 2 weeks prior to planting out in glasshouse
Acknowledgements
Thank you