AN OBJECTIVE METHOD FOR COUNTING CHROMOSOMES IN SUGARCANE ROOT MERISTEMS

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ABSTRACT
A root-tip squash technique for the study of sugarcane chromosomes is described. It involves the germination of cane cuttings in a humid atmosphere and the cultivation of roots in nutrient solution under controlled conditions in a special growth chamber. Selected roots are pre-treated in an aqueous solution of cycloheximide and 8-hydroxyquinoline, fixed in Farmer’s fixative, treated with pectinase and Feulgen stain and squashed, using a special press, in iron-aceto-haematoxylin. An accurate and objective chromosome counting method is described.

INTRODUCTION
Price \(^6,8\) developed a leaf squash technique which enabled him to count sugarcane chromosomes on a routine basis. Several other techniques have been described, e.g. Nair and Ratnambal, \(^6\) Stevenson, \(^10\) Jagathesan and Sreenivasan. \(^9\) A root-tip squash method was described by Sisodia \(^11\) and Jagathesan and Ratnambal. \(^4\) None of these techniques has been widely adopted outside the laboratory where it was originally developed. This tends to indicate that a substantial part of each procedure is dependent on the skill of a particular cytologist. Realisation of this situation and an urgent need for a reliable technique for counting chromosomes in large populations of experimental sugarcane varieties led us to develop our own procedure. After experimenting for some time with leaf squashes using the methods of Price, \(^8\) we turned to the root-tip method of Sisodia \(^13\) which has the advantages of ease of manipulation and the availability of more experimental material.

At about this time, our attention was drawn to the possibility of using cycloheximide \(^16\) as a pre-treatment agent. We eventually used this reagent in combination with 8-hydroxyquinoline. \(^12\) This pre-treatment procedure has proved to be most effective (See Figs. 1 and 2). We are now using the method for large scale investigations on sugarcane chromosome numbers and already have results for over 900 clones.

DESCRIPTION OF THE METHODS USED

Many separate steps are involved before it is possible to determine the number of chromosomes characteristic of a sugarcane clone. The process starts with obtaining suitable material and ends with a computer analysis of the results. The various steps are discussed below.

Surface sterilization of cane cuttings
The cane cuttings are treated in 5% water solution of calcium hypochlorite for 15 minutes, then washed for 15 minutes in tap water. Finally they are
FIGURE 1. Prophase chromosomes super-contracted by cycloheximide: NCo 310 (2n = 112) root tip meristem.


FIGURES 3 to 6. These illustrate consecutive steps in the chromosome counting procedure. The demonstration cell (approx 108 chromosomes) comes from a root tip of sugarcane, variety Pindar.

FIGURE 3. Photograph of cell no. 15230 ready to be counted.

FIGURE 4. Chromosomes in ambiguous clusters are marked after interpretation of the original slide under 1600 x magnification.

FIGURE 5. Chromosome complement divided into fields to facilitate counting.

FIGURE 6. Chromosome complement counted with an electronic colony counter.
soaked for another 15 minutes in a 0.1% solution of the commercial fungicide Benlate (Du Pont Co).

**Sealing with wax**

The setts are air-dried and the cut ends sealed with hot paraffin wax.

**Germination**

a) Entire potted plants or stalks up to 1 m long are placed in a dark humidity chamber where small target sprayers disperse pre-heated tap water into a fine fog. The sprayers are oriented in such a way that direct impact by high velocity water droplets is avoided. Temperature is maintained at 33°C.

b) Short, single-eye cuttings are placed in special trays about 1 cm above an aerated nutrient solution. (We use the commercial complete nutrient mixture Aquasol, manufactured by Hortico Ltd, Melbourne, Australia). The trays are covered with a sheet of transparent plastic in the growth chamber, again at 33°C but with 8 hours of light per day. The light supplied by three 40W fluorescent tubes is sufficient to direct the growth of roots towards the nutrient solution, which is changed automatically every 3 to 6 hours.

Alternatively, the cultivation trays may contain quartz pebbles 3–5 mm in diameter which are periodically wetted by nutrient solution. Whatever method is used, the roots grow to 30–80 mm in length within about 90 hours.

**Pre-treatment**

Root tips 10-20 mm long are collected with forceps and placed in small glass cylinders with fine nylon mesh on both sides. The cylinders are submerged in aerated pre-treatment solution. During the 4.5 hour pre-treatment period the temperature is allowed to drop from 33°C to about 20°C.

Pre-treatment solution is prepared as follows: dissolve 250 mg of 8-hydroxyquinoline (BDH Lab Chem, Eng) in a litre of distilled water at 70°C, agitate for 2 hours, cool to 35°C and add 70 mg of cycloheximide (Actidione, Upjohn & Co, USA). 8-hydroxyquinoline is stable for several days but cycloheximide should be added just before use.

**Fixation**

The pre-treatment solution is replaced by Farmer’s fixative (acetic alcohol, 1:3) kept at the same temperature overnight.

**Storage**

Excised root-tips are stored in glass containers in 70% ethanol. It is possible to store the material for at least 6 months at 4°C without noticeable deterioration in quality.

**Washing**

Root-tips are placed in PVC staining containers within a large petri dish. The alcohol is gradually replaced by water and the root-tips washed in running water for at least 30 minutes. (The PVC containers are made from 15 mm lengths of plastic tubing (diameter 25 mm) with stainless steel mesh heat-sealed to the bottom).
Hydrolysis

The material is hydrolysed in 1N HCl for 25 minutes at 60°C. The correct time varies between 20 and 40 minutes according to the size of the root-tip. Larger root-tips require longer periods.

Feulgen stain

The root-tips are stained in 1% Schiff's reagent for at least 30 minutes and washed in running water for another 30 minutes.

Pectinase treatment

A 4% solution of pectinase is used. The length of the treatment again varies between 15 and 40 minutes at 25°C according to the size of the root. The usual time is 20 minutes. Material which has been freshly fixed requires a longer pectinase digestion than material which has been stored in alcohol for several months. (Koch-Light pectinase is used with satisfactory results. It is dissolved in acetic buffer pH 3.6).

Freezing

The material is frozen in 45% acetic acid until squashes are made. It may be stored at −35°C for up to 3 days.

Iron impregnation

The samples, still in plastic containers, are transferred to iron-acetic mordant solution for about 5 minutes. (Mordant solution is made by dissolving 2.41 g of ferric ammonium sulphate in 500 ml of water and then adding glacial acetic acid to 1 000 ml). The mordant solution is used either undiluted (with relatively fresh haematoxylin) or diluted by 45% acetic acid up to 100-fold (if properly aged stain is used)²,³.

Squashing

Two or 3 root-tips are squashed on the micro-slide in acetic haematoxylin² with the aluminium handle of a dissecting needle. Large pieces of tissue are removed with a fine nylon thread fitted on a wire bow. The coverslip is positioned by holding it by one corner with a piece of blotting paper and the cells are spread by gently tapping the coverslip with a needle. The coverslip is pressed gently with another piece of blotting paper to remove excessive stain.

Haematoxylin stain is made by dissolving 2 g of haematoxylin in 100 ml of 45% acetic acid. The solution should be aged for at least 2 months before use.² If fresh solution must be used, more iron acetate is required in the mordant fluid.

Heating and pressing

The slide is heated to 78°C on a hot plate. The hot micro-slide is transferred to the press and the pressure is slowly increased to 6 kg/cm². A preliminary examination of the slides can be made at this stage.

Sealing of slides

The edges of the coverslip are cleaned with a fine brush moistened with xylene and sealed with Eukitt (Carl Zeiss). The mounting medium solidifies over several hours with constant pressure maintained on the coverslip by a spring-type clothes peg.

Storage of slides

The slides may be stored in the deep freeze, if necessary for several months.
Cell interpretation

The cell is relocated in the microscope field and viewed at 1600×. A photograph of the cell is placed by the side of the microscope. The intensity of light in the room and in the microscope are adjusted so that it is easy to compare and study both images simultaneously. Fig. 3 shows a photograph of a cell of average quality at prophase ready to be interpreted. This photograph is typical since it includes:

a) unambiguous chromosomes, i.e. chromosomes which can be recognised clearly from the photograph and counted without any need to refer to the original cell under the microscope.

b) ambiguous chromosomes, i.e. those which may be clustered or overlapping in groups of 2 or more and which need further examination under the microscope in order to determine more accurately the number which the group comprises. Results of the microscopic examination of ambiguous chromosomes are recorded as red marks, one for each chromosome, by a sharp felt pen in the appropriate position on the photograph (see Fig. 4). These red marks thus represent our interpretation of the number of chromosomes in the ambiguous cluster.

Chromosome count

In order to facilitate counting, arbitrary fields are delineated on the photograph using a blue pen (Fig. 5). The photograph is placed under thin transparent plastic and both the number of interpreted chromosomes and the total number of chromosomes are counted. The procedure is facilitated by using a bacteriological colony counter equipped with a felt pen connected to a miniature pressure switch. When the pen is depressed, a count is registered and the pen leaves a blue mark (Fig. 6) on the covering plastic sheet over the chromosome counted. The final count is verified by repeating this step.

Cell quality classification

Fly-leaves attached to each photograph are folded back so that neither the counts obtained nor the identity codes are visible. The cells are then classified according to their visual appearance on 5 characteristics, i.e. degree of chromosome spreading, degree of chromosome contraction, mitotic phase, chromosome morphology and cell integrity rating. All cells are classified first for chromosome spreading, then contraction, etc. Results of these classifications are recorded on each photograph in a numerical form according to the scheme outlined in Table 1.

Data processing

Data relevant to each chromosome count are entered into a computer on a paper tape. A special Fortran programme then calculates accuracy limits for each count, establishes the most likely chromosome number characteristic of each clone and compares it with the expected chromosome number. Principles of the numerical methods have been outlined in Sugarcane Breeder's Newsletter.16

DISCUSSION

Chromosome counts made directly under the microscope are tedious and prone to errors. Even the human chromosome complement, with chromosomes
at least 3 times as large as those of sugarcane and far less numerous, was misinterpreted for more than 2 decades.\textsuperscript{13} In sugarcane the existence of chromosome mosaics makes counting even more difficult.

High quality squash preparations are pre-requisite to any chromosome counting technique. Our studies have revealed quite clearly that the success of any particular preparation is already largely determined during the growth of the roots. The rate of cell division in \textit{Saccharum} is significantly lower than in many other genera and is extremely sensitive to fluctuation of temperature and the availability of oxygen, water and nutrients. It is thus necessary to maintain a high degree of control over cultivation conditions.

It was found inconvenient to handle Sisodia's\textsuperscript{11} soil mixture in our laboratory and sugarcane cuttings are thus germinated in air of controlled humidity and temperature. The mitotic rate is further increased and chromosome spreading improved if the root tips, after an initial period in moist air, are cultivated in an aerated nutrient solution. Our present growth chamber includes provision for changing temperatures, solutions and photoperiods in any sequence according to programmes prepared on a paper tape.

The problem of infection of the roots by fungi and bacteria was solved by surface sterilisation of the cuttings with calcium hypochlorite and by an adequate rate of change of nutrient solution. Further improvement in root growth was achieved by placing the cane sets on beds of quartz pebbles periodically moistened by nutrient solutions.

A range of possible pre-treatment agents was tried but gave equivocal results. Chemicals such as alpha-brom naphthalene and 8-hydroxyquinoline have a low solubility in water and apparently this is associated with slow penetration into active meristematic tissue. This results in substantial reduction of the mitotic rate and thus a reduction in the number of metaphase plates suitable for chromosome counts.

Colchicine, on the other hand, penetrates rapidly into living tissues and accumulates cells at the metaphase stage. However, the relatively high concentrations commonly used (0.1 \%-0.5\%) make the treatment rather costly. In contrast, cycloheximide is active at concentrations as low as 5 ppm and, furthermore, its action is immediate. The mechanism of action of this reagent is widely known and therefore is outlined here only very briefly. Cycloheximide is a powerful inhibitor of protein synthesis (\textit{Rose}\textsuperscript{9}) and aborts mitosis especially at prophase, early metaphase and late interphase. Prophase and metaphase chromosomes are contracted abnormally. Counts of chromosomes in cycloheximide-affected prophase are facilitated by the fact that the supercontracted chromosomes are still contained within the nuclear membrane and therefore tend to be very evenly spaced (Fig. 1).

In the traditional methods, chromosomes are usually counted at metaphase when it is difficult to be certain that the chromosome complement is still entire (Fig. 2). This uncertainty creates a serious problem in clones with large numbers of chromosomes. In contrast, the chromosome complements at prophase are usually intact and an occasional loss of chromosomes is in most cases easily detected. (Compare Figs. 1 and 2).

The reagent 8-hydroxyquinoline acts slowly but it reveals fine details of chromosome morphology, presumably by interfering with chromatid separation at late metaphase. The slow initial action of this reagent can be circumvented
by combining it with cycloheximide which apparently retards the cycle at prometaphase and early metaphase until the 8-hydroxyquinoline becomes effective. The large number of cells both at prophase and metaphase facilitates the search for cells with well-spread chromosomes.

The duration of pectinase treatment was found to be very critical and furthermore the treatment is not entirely terminated by washing in water before transfer to the mordant (45% acetic acid). It was found that freezing the root samples in mordant terminated enzymatic action and improved chromosome spreading.

Aceto-carmine, aceto-orcein, toluidine blue, Giemsa stain and propionic-iron-haematoxylin were studied as possible differential stains. A quantitative response to the concentration of iron acetate in the mordant solution was the major factor which led to our selection of acetic-iron-haematoxylin for this purpose.

In order to eliminate sliding of coverslips, we constructed a special pneumatic press which allowed a slowly increasing pressure to be applied to the preparation.

The development of our new cytological technique was constantly monitored by the system of cell classification outlined in Table 1. The effects of each treatment or modification of the procedure on the quality of squash preparations could thus be evaluated more objectively.


<table>
<thead>
<tr>
<th>Rating</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
<th>4th</th>
<th>5th</th>
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<tbody>
<tr>
<td></td>
<td>Chromosome spreading</td>
<td>Mitotic phase</td>
<td>Degree of chromosome contraction</td>
<td>Chromosome morphology</td>
<td>Cell integrity rating. Chromosome complement</td>
</tr>
<tr>
<td>1</td>
<td>not sufficient</td>
<td>prophase</td>
<td>long chromosomes</td>
<td>not visible</td>
<td>may not be entire</td>
</tr>
<tr>
<td>2</td>
<td>medium</td>
<td>metaphase</td>
<td>medium chromosomes</td>
<td>visible on some chromosomes</td>
<td>probably entire</td>
</tr>
<tr>
<td>3</td>
<td>good</td>
<td>anaphase and telophase</td>
<td>short chromosomes</td>
<td>clearly visible on all chromosomes</td>
<td>certainly entire</td>
</tr>
<tr>
<td>4</td>
<td>short chromosomes, chromatids begin to separate</td>
<td></td>
<td></td>
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A critical examination of counting techniques employed for other genera revealed that none was suitable for routine counts in sugarcane and we have therefore developed a new procedure involving photography.

Subsequent detection of chromosome mosaics in sugarcane drew our attention to the much-neglected problem of accuracy of counts. Because
subjective bias in counting sugarcane chromosomes was a major problem, we have attempted to develop a statistical method for the separation of random fluctuations from the real differences in chromosome numbers. The method uses probabilistic calculations for establishing accuracy limits for each chromosome count. These limits are then utilised for distinguishing between somatically stable and unstable clones.16

We believe that, in order to make accurate and unbiased counts, it is necessary that the counts should be made without recourse to any expected chromosome number, without the knowledge of previous counts made in a particular clone, and that the identity of the cell should not be known during the counting procedure.

Once a chromosome count is finished, we regard it not necessarily as an unequivocally true count but as the best final estimate of the chromosome number for that particular cell. The accuracy of the count depends on the cytological quality of the cell. This can be simply expressed as the number of "interpreted" chromosomes per 100 chromosomes counted, where "interpretation" means, as we have seen, the necessity to resolve ambiguous clusters under the microscope. If no "interpretation" is needed and if all chromosomes are clearly visible in the photograph, the accuracy of that count is absolute.

The most important feature of our technique is that the counting itself is separated from the analysis of the results. The calculation of accuracy limits for each count helps to prevent reading into the data more reliability than they merit.

Modern sugarcane breeding tends towards the use of exotic interspecific crosses, mutation methods and manipulation during tissue culture. To follow chromosome changes associated with these procedures, it is of paramount importance to have accurate, reliable and unbiased counting procedures available. We believe that the methods outlined on this paper are at least a step in this direction.

ACKNOWLEDGMENTS

The authors are grateful to Dr D. Adamson and Dr K. T. Glassiou for drawing attention to cycloheximide as an anti-mitotic agent, to Mr R. Shannon for the photography and to CSR Co Ltd for permission to publish this paper.

REFERENCES

PLANT BREEDING


UN METODO OBJETIVO PARA EFECTUAR RECUENTOS CROMOSOMICOS EN MERISTEMAS RADICULARES DE CAÑA DE AZUCAR

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RESUMEN

Se describe una técnica para el estudio cromosómico en apices radiculares de caña de azúcar mediante el aplastamiento. El método comprende la germinación (brotación) de estacas de caña en una atmósfera húmeda y el cultivo de raíces en soluciones nutritivas bajo condiciones controladas en una cámara de crecimiento especial. Raíces seleccionadas son sometidas a pretratamiento en una solución acuosa de cicloheximida y 8-hidroxiquinolina, fijadas en fijador Farmer, tratadas con pectinasa y Feulgen y luego aplastadas en una prensa especial, en Fe-aceto-hematoxilina. Se describe asimismo un método seguro y objetivo para el recuento de cromosomas.