

THE ELIMINATION OF THE VIRUS DISEASE, STREAK, BY THERMOTHERAPY AND TISSUE CULTURING

By G. ROTH

South African Sugar Association Experiment Station

Abstract

In an attempt to eliminate virus diseases in cane, very small portions of meristematic tissue were cultured. Mortality was high and elimination of disease was not achieved. Thermo and chemotherapeutic treatment of whole buds was then attempted, followed by culturing of the remaining living tissues. Better survival rates were achieved and heat treatment standards were established for the complete elimination of visual symptoms of the virus disease "Streak". The treatment selected was immersion of bud tissues in hot water at 59°C for ten minutes. Subsequent culturing yielded plants which, when established in the field, were free from the symptoms of "streak" exhibited by the parent material.

Introduction

Commercial sugarcane is very often found to be infected with virus diseases. Ratoon stunting and mosaic are those most commonly found in South Africa, while two others, streak and chlorotic streak are found occasionally. Virus diseases have a debilitating effect on the crop, but resultant losses vary, according to management and growing conditions. In the case of RSD, losses range from 10 to 100%, and for mosaic up to 30%, although small areas may suffer a much higher rate of loss.

Virus diseases of sugarcane often remain unrecognized by growers and, as a result, are an important cause of industry-wide yield loss. This loss can very largely be combated by ensuring that commercial seed is freed from recognizable maladies. There remains, however, a possibility that unknown or symptomless virus infections exist, and these could be a cause of the phenomenon known as yield decline.

It has been shown with a number of vegetatively propagated crops, that running-out or deterioration of varieties can be overcome, temporarily at least, by rearing stocks from cultured apical meristems^{1, 4, 6, 7, 8, 9}. The possibility of emulating this with sugarcane is attractive, particularly if it means the prospect of restoring the vigour of such well known cane varieties as Co.281 and N:Co.310. The fruits of success could be substantial, provided arrangements existed to safeguard production standards of commercial seed cane.

Successes achieved in eliminating virus infection vary according to the nature of the virus complex concerned. Chemicals cannot be expected to control virus infections as those toxic to the virus will inevitably kill the plant also. Heat treatment is an effective means of achieving control, but Kassanis and Posnette (1961)⁵ have shown that only about 50% of known plant viruses can be eliminated

using heat. The remainder, if they are to be removed, must be tackled by culturing meristem tissue to which infection has not already spread.

Attempts to culture sugarcane tissues with a view to freeing them from virus infection, were started at Mount Edgecombe in 1966. The techniques employed and the goals attained, are outlined herein.

Materials and methods

The variety Uba, infected by the virus disease streak, was selected for study because symptoms of the disease appear on the green leaves as soon as they can be seen. This provides a relatively easy and rapid measure of success or failure of the various treatments.

Samples used as basic material for these studies were derived from the top 20 to 25 cm of cane stalks. To ensure the elimination of surface contaminants, the stalk samples were first washed in soapy water, then rinsed in sterile water, disinfected for 30 minutes in a 5% solution of calcium chloride, washed several times in sterilized water and finally washed for five minutes in 70% alcohol. Following this surface sterilization, the samples were wrapped in sterilized, unglazed, open textured paper and transferred to the culture room which had been sterilized using ultra-violet light and disinfectants. The leaf sheaths were then removed, one at a time, the exposed bud swabbed with alcohol and then excised with minute sterilized stainless steel scalpels.

For the initial trials, meristem tissue samples excised from the buds, were of three size ranges, namely 0.2 to 1.0 mm; 1 to 2 mm; and 2 to 5 mm. Following excision, they were successively immersed for 10 minute intervals in "Aretan", 0.2% HCl, sterile water and again in sterile water. They were then placed on nutrient media in test tubes of different sizes. One hundred tissue samples were used in each size range. For the later, modified trials, whole bud samples were used.

Culture media

The media employed — based on those of White and Heller, as described by White (1963)¹⁰ — were prepared in seven stock solutions. Best results were attained with a White-Heller medium supplemented with 15 to 20% coconut milk, 2 ml/litre of a stock solution, 1 mg/litre of both gibberellic acid and n-naphthalene acetic acid, and 0.1 mg/litre of kine-tin. The stock solution was prepared by dissolving, in 100 ml of distilled water, 50 mg of nicotine amide, 50 mg of arginine, 10 mg aneurine, and 10 mg pyridoxine.

Treatments

In the first set of trials, culturing of meristem tissues was attempted using the material and culture media described. The effects of various chemical additives were examined and in some cases meristem tips were floated for different periods of time in a range of sterile liquid media, before culturing. In later trials, tissues were subjected to heat treatment before culturing. These included exposure for different intervals of time to hot air within a temperature range of 57 to 67°C and immersion in hot water between 50 and 59°C.

Results

Initial trials

The only satisfactory culture medium was a modified White and Heller solution employing supplements of gibberellic acid, n-naphthalene acetic acid and kinetin. On this medium, the tissues of the smallest units, which initially were 0.2 to 1 mm in size, differentiated 15 to 20 months after implantation, but only when they had been transferred several times to fresh culture media. The largest meristem units of 2-5 mm, developed within 12 to 15 months to the stage when the first symptoms of virus infection, if this were present, might be observed.

Of 1,200 meristem tips, comprising 400 of each size group, only 76 developed to the point where tissue differentiation occurred, and the bulk of these were derived from the large tissue units. The remainder died or were destroyed as a result of contamination by micro-organisms. Only two out of the 76 units which grew, failed to show symptoms of the virus disease streak while still in the test tubes. These two eventually developed the symptoms when they had been transferred to pots.

Modified trials

In view of the unsatisfactory outcome of the initial trials, it was decided that the techniques employed should be modified. Bud tissues to be used were taken from the tops of stalks as previously, but buds from the maturing sections of the stalk, were also used.

The stalk sections were surface sterilized as for the first trial but while still whole, they were subjected to various heat treatments. Temperature exposures were four hours at 50°C; two hours at 51°C; one hour at 52°C; 30 minutes at 53°C, 54°C, 55°C and 56°C; 15 minutes at 57°C; 10 minutes at 58°C; and 10 minutes at 59°C. The tissues excised from the stalks were all approximately 10 x 6 mm in size, and consisted of a notch containing a bud cut from the stalk sample. They were all treated with "Aretan", 0.2% HCl and washed in sterile water as for the first trial, but batches were then subjected to different chemical dips. These included 0.2 to 0.5% HCl, 0.1 to 0.5% HNO₃, various concentrations of Cu SO₄, and 0.02 to 0.2% HgCl₂. Finally they were rinsed with sterile water and transferred under aseptic conditions to a nutrient medium in test tubes. Fifty meristem samples were used for each combination of temperature and chemical treatment.

Examination of treated tissues showed that heat

treatment had killed most of the cells in the bud and that only a few within the meristem itself remained alive. This surrounding envelope of dead tissue served to protect the small core of living cells and it also absorbed and transmitted nutrient from the agar medium to the living cells. The cultures in their test tubes were placed in darkness in an incubator at a temperature of 30°C and within a few days the living tissue started to grow. The first visible symptoms of growth were noted two weeks after implantation, and in some samples the first green tissues appeared at six to eight weeks, provided the cultures were exposed to daylight for not less than one week beforehand. Differentiation of tissues to form roots started between eight and 16 weeks after placement of the tissues on nutrient agar. At this stage, cultures which failed to display vigorous growth were destroyed.

Examination of the cultures as the tissues differentiated and developed, revealed that regardless of chemical treatment, symptoms of streak had not been eliminated where heat treatments were less than 57°C. At this temperature there was some evidence of abatement of infection, but greater success was achieved in cultures which had been subjected to heat treatment at 58°C. Virtually all the samples treated at 59°C were free from symptoms of streak. Furthermore, despite the high temperature involved, 50% of the cultures were grown successfully on the culture media.

In addition to the hot water treatments, tests were carried out with hot air. The stick samples, before excision of the buds were placed in a ventilated drying oven for six hours at various temperatures ranging from 57°C to 67°C. The excised tissues were then subjected to the same chemical disinfection as the hot water treated samples and placed in cultures under aseptic conditions immediately following heat treatment. Despite this, not one of the samples, when it developed, was free from the symptoms of streak disease.

Plantlets grown from cultures subjected to thermo-therapeutic treatment, and freed from visual symptoms of streak, were removed from their test tubes when the second leaf and a few roots had formed. They were then planted in good, prepared compost in pots and protected by plastic bags to maintain 95 to 100% atmospheric humidity around the foliage. When they were established and growing well, the plants were hardened off and planted out in the field. These plants remained free from any symptoms of streak, and, when used as seed material, produced plants which were also free from visible signs of the disease. No genetic change or harmful effect was seen despite the treatment they had undergone.

The early development of meristem tissues in culture

For anatomical studies, dormant buds were used as a standard to provide comparisons with cultures of meristematic tissues derived from terminal and lateral growing points. These bud tissues, and the cultured tissues subjected to thermo-therapeutic treatments, were sectioned for examination at intervals of 48 hours. The tissues were prepared for

sectioning by the butyl alcohol method of paraffin infiltration. They were stained successfully employing the technique described by Boke (1939)², which involves the use of Delafield's hematoxylin, then counter stained with safranin for three to four minutes. An alternative method, which also proved successful, was that described by Johansen (1940)³. It involves the use of Heidenhain's hematoxylin, followed by counter staining with safranin, which colours the chromosomes of the nucleus blue-black to purple and the cellulose walls a light purple.

Forty eight hours after hot water treatment, meristems of treated apical and lateral buds were sectioned. One such section is shown in Fig. 7, and in this the nucleus and cells within the living tissues are starting to divide. This stage is followed rapidly by reproduction and swelling of the surviving tissue, and this can be seen in Fig. 1 and 3. The rapid growth, due to formation of new cells, seems to take place uniformly throughout the tissue from three to five weeks after implantation (Fig. 8 to 10). Differentiation to create leaf primordia occurs in the embryonic tissue near the apex. These primordia develop initially as a rounded protuberance on the side of the apical meristem. True differentiation and the production of the initial vascular bundles follows, as illustrated in Fig. 8 and 11. At this very early stage of development the meristematic tissue extends into the young leaf initials and thus provides for the creation of such leaf structures as the epidermis, palisade parenchyma, and spongy mesophyll. When development is more advanced and partial differentiation of the five to seven leaf initials has occurred, rapid cell divisions occur in the sub epidermal layers, indicating the development of root primordia.

From this study it can be stated that under the conditions described, the cultured tissue when it has developed to about 10 mm in length, contains between six and nine partially differentiated leaf initials which envelop the growing point and the initials of the lateral buds.

Discussion and conclusions

The preliminary trials were carried out to examine the problems of tissue culturing, and particularly to shed light on the types of tissue that should be employed, their treatment, the relative merits of culture media, and the influence of tissue size on growth and development. Mortality was high and, regardless of treatment and size of tissue, none of the cultures reared produced plants free from

symptoms of streak. It was concluded that tissue culturing alone, even of very small meristematic tissue samples, could not prevent a carry over of infection with streak.

Thermo and chemo therapeutic treatments were then examined in conjunction with tissue culturing. Heat and chemical treatments were applied to stalk samples, following which bud tissues were excised, surface sterilized, and finally cultured under aseptic conditions. Heat treatment proved to be the sole effective means of eliminating streak and the most effective treatment was immersion in water maintained at 59°C for 10 minutes. Plants reared from cultures subjected to such treatment were free from visual symptoms of streak. Furthermore, these symptoms have not reappeared over a period of 18 months, even in plants which were derived by propagation from the treated parents. There is good reason therefore, to believe that the infection has in fact been destroyed. The vigour of the plants derived from cultured tissues has yet to be compared in field trials with normal material of the same variety. It is therefore too early to assess the practical significance of the technique. By virtue of its technical success, however, it has opened up a line of study which may be of considerable potential importance.

References

1. Belkengren, R. O. and Miller, P. W. (1962). Culture of apical meristems of *Fragaria vesca* strawberry plants as a method of excluding latent A-virus. *Plant Dis. Repr.* 46: 119-122.
2. Boke, H. H. (1939). Delafield's hematoxylin and safranin for staining meristematic tissue. *Stain Tech.* 14: 129-131.
3. Johansen, D. E. (1940). *Plant microtechnique*. 1st ed. McGraw Hill, New York.
4. Kassanis, B. (1957). The use of tissue cultures to produce virus-free clones from infected potato varieties. *Ann. appl. Biol.* 45: 422-427.
5. Kassanis, B. and Posnette, A. F. (1961). The chemotherapy of virus-infected plants. *Recent Adv. Bot.* 1 (Int. bot. Congr. Montreal, 1959): 557-653.
6. Morel, G. (1960). Producing virus-free Cymbidiums. *Bull. Amer. Orchid Soc.* 29: 495-497.
7. Morel, G. and Martin, C. (1952). Guérison de dahlias atteints d'une maladie à virus. *C.R. Acad. Sci., Paris* 235: 1324-1325.
8. Morel, G. and Muller, J. F. (1964). La culture in vitro du méristème apical de la pomme de terre. *C.R. Acad. Sci. Paris* 258: 5250-5252.
9. Quak, F. (1957). Meristeeencultuur gecombineerde met warmtebehandeling voor het verkrijgen van virusvrije anjerplanten. *T. Pl.—ziekten* 63: 13-14.
10. White, P. R. (1963). *The cultivation of animal and plant cells*. 2nd ed. The Ronald Press Company, New York.

Discussion

Mr. du Toit (in the chair): It is possible to eliminate part of ratoon stunting disease in a variety by heat treating to 50.5°C but, with Co281 for instance, it has not been possible to restore it to its previous vigour. Is there some other virus that is not killed at 50.5°C?

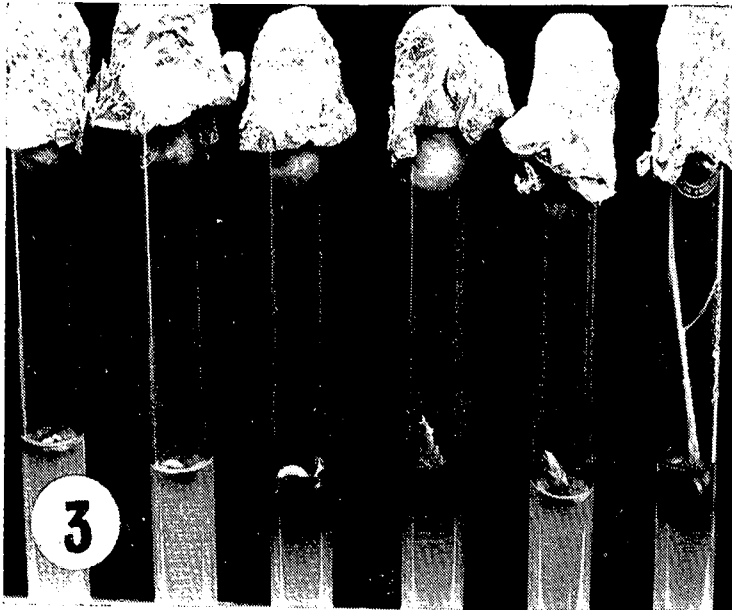
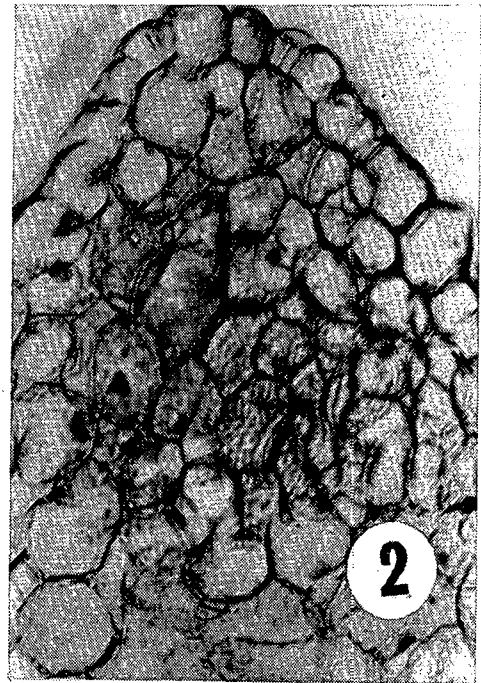
Dr. Roth has worked with small tissues of plant at higher temperatures and by going as high as 59°C has eliminated Streak. Probably other viruses were also eliminated and it now has to be seen if the plant can be grown and restored to its original vigour. He must be congratulated on his work and on having grown a sugarcane plant in a test tube.

Even if Co281 was restored it could not compete

with N:Co310 and N:Co376 but we do not know if there is anything hindering the last two mentioned varieties from giving greater potential. It is now up to Dr. Roth to further his work in order to try and find out if there has been a factor limiting the growth of modern varieties.

Mr. Whitehead: The technique of tissue culturing, as employed by Dr. Roth, opens up the prospect of producing material free from a whole range of diseases; in fact of producing stock from which we can develop nucleus seed for commercial nurseries.

Dr. Roth: If we could increase the potential of our varieties by 20% to 50% by using completely healthy seed it would prove of enormous economic advantage to the sugar industry.



(1) Stages of development of whole bud cultures which have been subjected to thermo- and chemo-therapeutic treatment.

(2) A bud meristem, excised from the surrounding bud tissue at the time of implantation on agar.

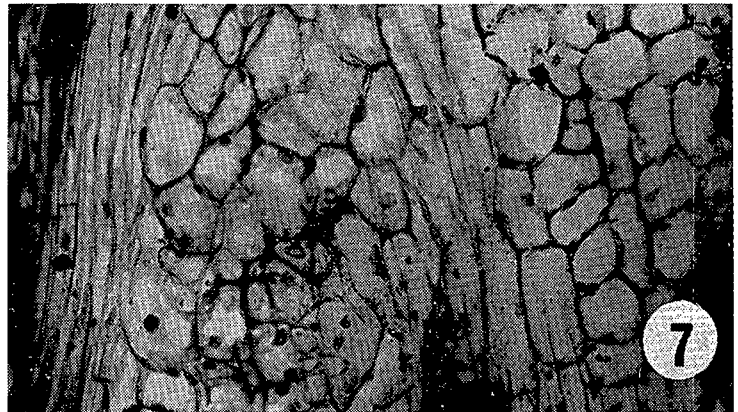
(3) Successive stages in the development of small tissue units, following differentiation of meristematic tissue.

(4) Meristem cultures reared on a nutrient medium supplemented, from left to right, with increasing quantities of 2,4-D and N.A.A. Note the excessive growth of callus tissue.

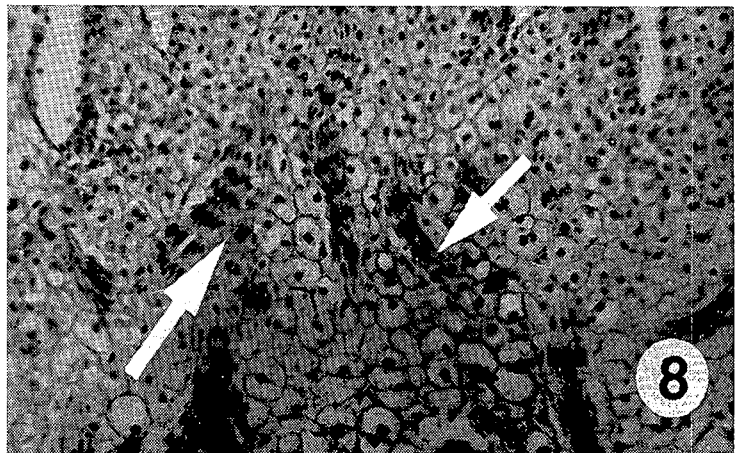
(5) Plants of the variety UBA, raised in culture from heat-treated buds and then established in pots. These plants display no symptoms of the virus disease streak.



(6) Bud section in which the surviving meristem is surrounded by tissues which have been killed by heat.

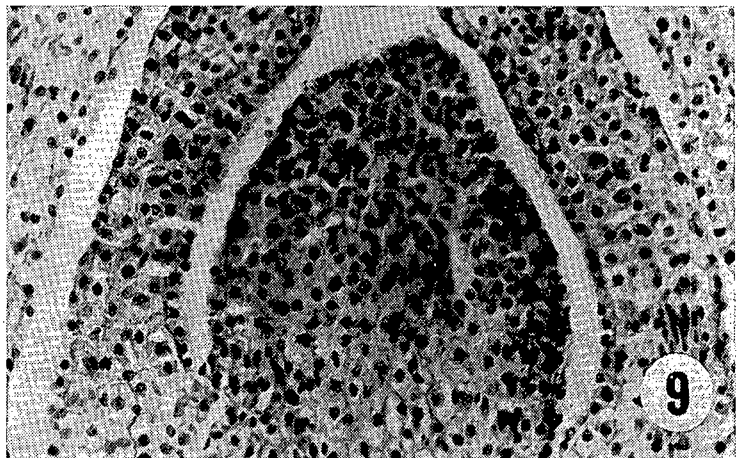


(7) Close-up of living meristematic tissue surrounded by dead cells.

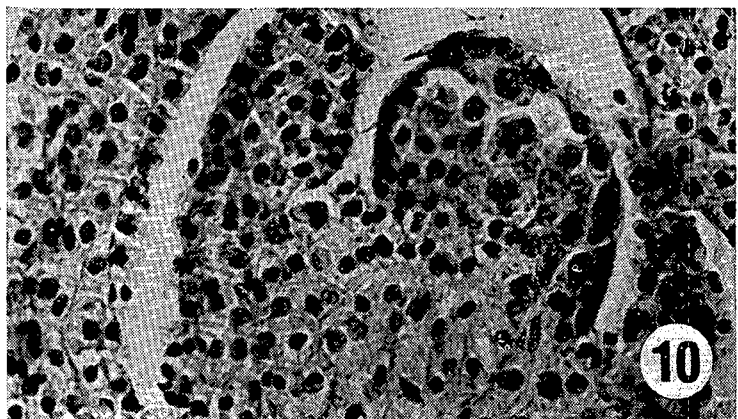


(8) L.S. of heat-treated bud tissue, four weeks after implantation on a culture medium. Note the developing tracheids (arrowed).

(9) L.S. showing meristem surrounded by developing leaf initials. Note the concentration of nuclei in the meristem.



(10) L.S. of meristem, showing the separation of a new leaf initial.



(11) L.S. of cultured tissue, 4 weeks after implantation on its sterile culture medium. Note the enveloping leaf initials and the isolated tracheids.

