MICROPROPAGATION AND ISOZYME STUDIES ON INTERCLONAL AND SOMACLONAL VARIATIONS IN *IN VITRO* REGENERATED SUGAR CANE (*Saccharum officinarum*)

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BY

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DECLARATION.

a). This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION.

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ABBREVIATIONS USED.

AA........Acrylamide.

AP........Ammonium peroxidisulphate.

BAP.......Benzylaminopurine

BIS.......N,N'-Methylene-bis-acrylamide.

2,4-D.....2,4-Dichlorophenoxyacetic acid.

DMAPN.....3-Dimethylamino-propionitrile.

DDH_2O.....Double distilled water.

EDTA......Ethylene diamine tetra-acetic acid.

GA_3.......Gibberellic acid.

Glycine...Aminoacetic acid.

HCL.......Hydrochloric acid.

IAA.......Indole-3-acetic acid.

IBA.......Indole butyric acid.

Kinetin...6-Furfurylaminopurine.

MS........Murashige and Skoog's (1962) medium.

NAA.......a-Naphthaleneacetic acid.

PAA.......Polyacrylamide.

PAGE......Polyacrylamide gel electrophoresis.

PoroPAGE..PAGE in porosity gradients.

TEMED.....N,N,N',N'-Tetramethyl-ethylenediamine.

TRIS......Tris(hydroxymethyl)-aminomethane.

ABSTRACT.

An *in vitro* protocol for sugar cane micropropagation was developed through callus induction and plant regeneration of ten commercial sugar cane clones in Kenya. The explants were prepared from young shoot tips and the surrounding 2-3 whorls obtained from 6-8 week-old young plants grown in wooden boxes outside the laboratory. The shoot tips were transversely cut into small sections 2-2.5 mm wide and about 4 mm long and surface sterilized in 0.5 % sodium hypochloride solution for five minutes and then rinsed in sterile distilled water. These were then aseptically cultured into MS medium in combination with 0, 1.5, 3.0 and 4.5 mg/l 2,4-D and then solidified with 10 g/l agar in universal bottles. Callus induction was realised within one week of incubation in growth chambers illuminated with fluorescent lighting of 16 - hour photoperiod at 25 °C.

Analysis of variance (ANOVA) for calli fresh weights obtained at the four concentrations of 2,4-D indicated that they were significantly different ($p = 0.5$) with a concentration of 3.0 mg/l giving the best results for callus induction.

Caulogenesis was obtained by transfering the callus to the above MS medium without 2,4-D but supplimented with 10 % coconut water. After two weeks, shoot regeneration from callus was evident arising from compact calli with green islands. Shoot multiplication of the regenerants was obtained by supplementing the culture medium with 2 mg/l BAP whereas rhizogenesis was achieved by supplementing the culture medium with 2 mg/l IBA. Enzymatic browning was controlled by introducing 5 g/l of activated charcoal to the medium. The presence of activated charcoal also led to a faster rhizogenesis and rootlets could be noticed even after only three days of incubation. Plantlets were transfered into a soil/white pellets growing medium and successfully acclimated to the greenhouse conditions.

Isozyme variation was used to identify the biochemical markers of potential utility in sugar cane genetics and breeding. Peroxidase and esterase isozyme patterns from leaf extracts of the *in vitro* regenerated plants were analysed using porosity gradient gel electrophoresis (PoroPAGE). Electrophoretic polymorphism identified all the clones analysed. Assays of isozymes revealed that whereas both enzyme systems could be reliably used to identify the clones based on basic bands, peroxidase isozymes proved to be the most appropriate in detecting somaclonal variation. Upto 20 % somaclonal variation was detected with over 15 % being detected by peroxidase patterns alone.

Apart from the increased band intensity and clarity, there was an increase in the number of bands in established plants in the greenhouse when compared with *in vitro* plantlets in the laboratory. No variation was observed in individual established plants in the greenhouse at various growth stages. Morphological variations in tillering abilities and errectness were noticed in three-month old somaclones. No variations were observed in plants resulting from multiplication of individual plantlets.

1.0 INTRODUCTION.

Sugar cane is a perennial plant belonging to the grass family (Graminae). Its cultivated varieties today are mainly derived from hybridisation of the noble cane (*Saccharum officinarum)* with the cultivated species *S. sinense* and *S. barberi*. Like potato, sugar cane is a polyploid and all new sugar cane varieties are now raised from seeds produced in crosses where parentage is controlled. In cultivation, however, varieties are propagated vegetatively and are clones rather than varieties in strict sense (Stevenson, 1965). The breeding of the crop has been largely through clonal selection and propagation.

Among East African countries, Kenya leads in sugar cane production with 111,000 hectares under cane (Anon, 1988a). In Kenya, the crop is mainly produced from three provinces: Nyanza (56,000 ha); Western (55,000 ha) and Coast (6,300 ha) (Anon, 1990a, 1990b). Currently the output from Coast Province has stagnated due to the collapse of Ramisi Associated Sugar Company and production is now limited to the Mtwapa Research Station where breeding work is done.

Sugar cane growing areas in Kenya are termed as Sugar Belts, with the main ones being the Nyando Sugar Belt (NSB) and the Western Sugar Belt (WSB). In these belts, sugar cane is grown either by factory - owned nuclear estates or by outgrowers located within 16 to 21 km on the precincts of the sugar cane factories (Anon, 1991b). Fifty - eight percent of the outgrowers are small scale rural farmers. Improvement in sugar cane production at farm level would therefore directly benefit the small scale farmers.

Yields obtained from cane fields vary from zone to zone (or factory to factory) and also from one year to the other. The average yields, however, range from 60 - 100 tonnes/ha (Appendix 1).

The greatest performance of the sugar industry during the last decade was achieved in 1989, when sugar production was highest at 442,000 tonnes. The production has since dropped to 433,000 tonnes in 1991 and further down to 370,000 tonnes in 1992 (KSA, 1993; Appendix 2). Sugar consumption on the other hand has been increasing steadily from 1986 and was highest in 1992 when the amount consumed was 552,000 tonnes. This implies that from 1985, Kenya has had to import white sugar to suppliment its local production so as to meet the local demand.

Economically, sugar cane is the third most significant cash crop in value terms after tea and coffee. The cane industry is a source of Government revenue in terms of taxes, rates, levies and dividends to parastatal companies. It also saves the country foreign exchange which would have otherwise been used to import it. For example, in 1991, Kenya produced 440,000 tonnes, thus saving the country $420 million in foreign exchange (Anon, 1991a). Expressed on the basis of foreign exchange saved, sugar cane contributed 6 % of Kenya's Gross Domestic Product in 1991. Despite this contribution to Kenya's economy, the supply does not yet meet local demand as already stated. For example, in 1987, imported sugar constituted 50 % of Kenya's total food imports, while in 1992, Kenya imported over 100,000 tonnes of white sugar (costing about $70 million in foreign exchange). With the recurrent devaluation of the Kenyan currency, importation of sugar cannot be relied on as a long - term solution to the country's sugar deficit. Thus, the sugar

cane industry's objective of import substitution through improved cane production at farm level should be a feasible solution to this problem.

1.1. CONSTRAINTS TO INCREASED SUGAR CANE PRODUCTION.

Whereas the demand for sugar has continuously increased over the years, cane farmers have continued to face certain constraints which have limited increased production. Notable among these constraints are:

a) Unavailability of high yielding and high quality varieties.

b) Susceptibility of the current grown clones to diseases such as ratoon stunting disease, smuts, mosaic virus, yellow wilt and leaf spots.

c) Susceptibility to pests such as white scale (*Aucalapsis legalensis*), white grub (*Schizonycha spp)* and termites *(Pseudocanthoterms militaris)*.

d) Poor cane management at farm level.

e) Non payment or late payment of farmers.

It was to circumvent these limitations that the Sugar cane Breeding Programme was incepted in Kenya.

1.2. SUGAR CANE BREEDING.

Unavailability of high yielding sugar cane clones is still a major constraint to the cane industry in Kenya (Anon, 1990a). Three of the major cane clones cultivated in Kenya (CO 421, CO 617 and CO 331) are all introductions. Research to develop new varieties has been successfully carried out and has resulted in the release of new varieties which are more productive than the standard clone CO 421 (KARI,1991). The drawback, however, has been the slow distribution of these clones to farmers for commercial production. To obtain mature setts from seedlings requires atleast 18 months and the number of setts available depends on the tillering ability of the clone itself. This implies that limited amount of planting material can be availed to farmers within a short period of time. This conventional method of vegetative multiplication of propagules also encourages the spread of diseases.

Procurement of disease free as well as resistant varieties also takes a considerable length of time. Developing and releasing a new variety by conventional approach currently takes 12 - 14 years (Breaux, 1972). Criticisms of the sugar cane research programme are based on this slow process and yet still insist that the locally produced and selected varieties are bound to perform better than the varieties acquired through introductions.

Another aspect of cane research is variety identification. Currently, morphological markers are used to differentiate the various commercial varieties (Nyangau and Jagathesan, 1990). This method is quite deficient because morphological traits may vary environmentally, succumb to the adverse effects even within the same environment and are only assessable at the maturity stage of the plant. An alternative approach to variety

identification is to use biochemical markers which are quite constant and are detectable even at the early stages of plant growth.

1.3. OBJECTIVES OF THE RESEARCH STUDY.

Advancement in sugar cane improvement based on the conventional method of breeding is limited due to: a) longevity of the crop cycle; b) inadequate genetic variability; c) and slowness by which a newly developed variety is multiplied and supplied to farmers. It is because of these reasons that this research study was carried out with the aim of augmenting the conventional approach currently in use with the newly developed biotechnology techniques. The objectives of the study were in line with the overall objectives of the sugar cane breeding programme in Kenya and were therefore as follows:

1. To develop and adapt micropropagation techniques for large scale *in vitro* multiplication of the commercial sugar cane clones in Kenya. This involved the determination of the most appropriate protocol for sugar cane tissue culture.
2. To use isoenzyme analysis to achieve two purposes.
    - (a) As an alternative means to quickly and reliably identify the commercial sugar cane clones grown in Kenya (Interclonal variation).
    - (b) To test the existence and extend of somaclonal variation within the regenerants obtained from tissue culture (Somaclonal variation).

2.0. LITERATURE REVIEW.

Sugar cane is one of the few plants that store their carbohydrate reserve in form of sucrose. It is grown throughout the tropics and provides more than half of the world's sugar; the remainder being supplied by sugarbeet which is a root crop grown in temperate areas (Acland, 1989). The primary goal of most sugar cane breeding programs is to develop cultivars with improved sucrose yields as well as increased resistance to pests and diseases.

2.1 HISTORY OF SUGAR CANE.

Sugar cane is among the earliest crops to be domesticated. It was cultivated in India as early as 400 BC and even in ancient times, it was processed into sugar by methods not greatly different from those still used today in primitive mills (Ochse et al., 1961). Cane production and the art of sugar making was spread early from India to China as well as to Arabia. From there it was carried to Mediterranean where a prosperous sugar industry developed. Southern Europe supplied the world market with sugar until sugar cane was introduced to Madeira and the Azores about 1420. It thrived so well in these Islands that the industry in Southern Europe could not compete with it and practically disappeared within a short time.

Later on it spread to other areas like Cuba, Puerto Rico, Mexico, Peru and Brazil. The next major event in the sugar cane history occurred 300 years later when Captain Blight transported varieties of *S. officinarum* L. from Tahiti to Jamaica in 1791. This species of

sugar cane seems to have come from South Pacific with New Guinea as the probable focal point. Its varieties spread to the new world with the main ones being Bourbon, Lahiana, Cana Blanca and Otaheite. These varieties proved so successful wherever they were planted.

Production of these varieties decreased and later disappeared because of low yields, diseases and pests. For example, root diseases wiped out Otaheite cane in Mauritius in 1840 and in Puerto Rico in 1872 and also EK 28 cane in Java in 1920. Sugar cane mosaic virus disease also spread in Puerto Rico in 1915, in Argentina in 1920 and in Louisiana in 1926. Argentina imported POJ 36 and POJ 213 to replace them but were also wiped out in 1940 by smuts caused by *Ustilago scitaminea*. This led to development of other new varieties to replace those that were wiped out by diseases.

2.2.0. CYTOGENETICS OF SUGAR CANE.

The sugar cane, known botanically by its generic name of *Saccharum* is a member of Graminae family in the tribe Andropogoneae. The genus has several species, both wild and cultivated. The most notable ones are described below.

2.2.1. *Saccharum officinarum*.

Early descriptions referred to this group as the original sugar cane varieties but were termed `noble canes' by the Dutch in Java about 1920 due to their splendid appearance, bright colours, large size and good quality in comparison with the others. There is little doubt that the place of origin of the noble canes was in South Pacific Islands (Stevenson, 1965). The *S. officinarum* has several clones. Artschwager and Brandes (1958) listed some 441 clones worldwide based on their anatomical and morphological characters (Clements,

1980). In general, *S. officinarum* clones produce moderately tall and variously coloured thick stalks low in fibre content but with very sweet juices. Curiously, all the officinarums have 2n=80 chromosomes. Sucrose production from cane was first done in India whereas cane production was known since 400 B.C. in Mauritius. The noble cane `Otaheite' was the only one grown from the beginning of cane industry between 1737 to 1782. This clone reached French Indies in 1787 and became a standard variety in much of the new world including Hawaii, where it was planted at Lahaina (Maiu) in 1854 and became known locally as the `Lahiana' variety (Clements, 1980). It flourished in the Islands for many years until it succumbed to the so-called Lahiana disease.

2.2.2. *Saccharum spontaneum.*

This is the most outspread species of Saccharum with the greatest capacity for adaptability to drought, cold, disease and poor growing conditions hence its germplasm has been used as a source of resistance to these conditions. Although the clones of *Saccharum spontaneum* are so numerous and so varied, ranging from miniature plants no more than 18 inches in height to large vigorous types which exceed 20 ft, they have many morphological features in common, and they form a natural group which is easily distinguished from the noble canes. Generally, they are much more fibrous, more pithy, more rhizomatous and contain less sugar than officinarums. They have profuse suckering and vary enormously in their appearances from short bushy plants growing in clumps to very tall spreading plants. Their chromosome numbers vary alot from 2n=54 to 2n=128, most of them being multiples of 4, 8 and 10. Chromosome numbers of plants found in Coimbatore, India, some collections have as low as 40 chromosomes with multiple

distribution as follows: 4 (20 clones), 8 (10 clones) and 10 (8 clones); (Clements, 1980).

2.2.3. *Saccharum robustum* Jeswiet Grassl.

This is another very important wild species of cane indigenous only to New Guinea and the adjacent islands of Melanesia. This species was discovered by Jeswiet in 1928 during a collection expedition when he suspected the existence of another *Saccharum* species, since he considered it impossible for *S. officinarum* to have been derived directly from *S. spontaneum.* Its nearness to home of *S. officinarum* and its susceptibility to similar diseases strongly suggests that officinarum is a development of robustum rather than of spontaneum. In its natural habitat, *S. robustum* is extremely vigorous forming compact tufts and a larger stem diameter always greatest near the nodes and with swollen growth ring. The stems are hard, woody, pithy in the interior and sometimes with a hollow centre and with little juice. Its chromosome count is basically of two kinds, 2n=60 and 2n=80. Its clones have been used in several countries with varying success.

2.2.4. *Saccharum barberi.*

Initial sugar production in Northern India and Eastern Pakistan was dependent, for centuries, on the indigenous sub-tropical forms of cane which were evolved there and had been grown since earliest recorded times. The canes which were widely distributed in those regions portrayed various clonal variations and differences were even expressed by the same variety at different environments. Barber (1918) undertook a taxonomic study and grouped *S. barberi* canes into five groups namely: Chunnee (2n=91), Mungo (2n=82), Saretha (2n=92), Nargori (2n=89-91) and Kansar (2n=92)(Ref. Tempany and Christ, 1958). The canes are believed to have arisen from *S. spontaneum* by mutation and selection, since many diagnostic features are traceable directly to forms of this species.

Javan breeders obtained several useful varieties directly from crosses between Chunee, a Saretha clone and the noble clones Black Cheribon and Striped Preanger. These include POJ 136, POJ 213, and POJ 234 which cultivated extensively in Louisiana, Argentina, Taiwan and India and have been used successfully in breeding programs. POJ 213 is an ancestor of many clones of the POJ, CO and B series currently in cultivation.

2.2.5. *Saccharum shinense* Hassk.

This species is found in the Northern parts of the normal Saccharum areas and has been used for sugar production in northern India, China, Formosa, Southern Japan, the Philippines and Hawaii. Infact, Vavilov (1952) suspected it to have originated in `The Chinese center of origin of cultivated plants'. Uba cane, now regarded as perhaps the most typical of the sinense clones has been grown commercially in various parts of the world and is resistant to sugar cane mosaic disease but it has poor juice quality and undesirable

milling qualities. Though it is semi-sterile, the Uba cane has been successfully used in Barbados where it has been crossed with other breeding lines to give the B series of canes.

Saccharum sinense Hassk has chromosome numbers 2n=116, 117, or 118 and responds to drought by a slow down of longitudinal growth but not a pinching out in diameter at the top. When the rains come, the plant renews its normal growth but in other varieties the plant top breaks off because of narrowing of the stems and only suckers re-establish a stand.

2.2.6. *Saccharum edule* Hassk.

This is the sixth generally recognised species of sugar cane although it could easily be placed within the other groups. The distinction is that its inflorescence fails to become paniculate but becomes like cauliflower and it is also used as human food. Its clones must have arisen as mutations and since they depend upon vegetative means of reproduction, they must have remained fairly unchanged except for the possibility of further vegetative mutations. In 1967, Grassl (1969) established that this sterile species is an intergeneric hybrid resulting from a cross between *Miscanthus floridus* and *S. robustum*.(Ref. tempany and Christ, 1958)

From all the above elucidated *Saccharum* species, several hybrids have been developed and used commercially for sugar production. Such hybrids have been basically maintained through vegetative propagation.

2.3.0. SUGAR CANE IMPROVEMENT.

Sugar cane is one crop which plant breeding techniques have produced remarkable

results in both yield quality and quantity. It is an example of a monocotyledonous crop in which selective breeding from seed has been successfully combined with vegetative propagation (Tempany and Crist, 1958). The breeding of this graminaceous crop has for a long time been through conventional breeding approach and only recently has this approach been complemented with *in vitro* techniques in some breeding programs.

2.3.1. Breeding and selection through conventional approach.

Although it had been known that many cane varieties flowered, it was thought that the seed was infertile until the discovery in 1889 by Harrison and Bovell in Barbados and independently by Soltwedel in Java that some varieties produced fertile seeds from which new seeds could be raised. Morris and Stockdale (1906) demonstrated the possibility of raising hybrids, and many hybrids of known parentage were raised though later experiences showed the existence of a narrow genetic base (Tempany and Christ, 1958). This meant that further improvement could only come from related species. Jeswiet in Java and Baber and Venkatraman at Coimbatore in India revealed the possibility of interspecific hybridisation of *S. officinarum* with wild species *S. spontaneum* and *S. barberi*(Ochse *et al.*,1961). In India, first attempts were to improve the quality of the drought and cold hardy but thin stemmed *S. barberi* and *S. spontaneum* varieties by crossing with *S. officinarum.* In Java, original breeding program was directed towards development of varieties resistant to sereh disease through hybridisation of the susceptible *S. officinarum* with resistant *S. barberi* and *S. sinense*. The results were unfruitful here, however, but became successful when *S. spontaneum* was introduced to the program (Ochse et al., 1961).

Bremer (1922) crossed *S. officinarum* (2n=80) and *S. spontaneum* (2n=112) and

obtained a hybrid with 2n=136 and resistant to mosaic disease of sugar cane `Gelestreprenziekte' and sereh. The unexpected chromosomal number was associated with the doubling of chromosomes in the maternal parent; either at the time of fertilization or in the zygote directly after fertilization (Vavilov, 1950). This doubling of chromosomes occurs when *S. officinarum* is crossed with a distantly related species. The first backcross to the maternal parent resulted in a plant with 2n=148 chromosomes, 120 of which were from *S. officinarum* and 28 from *S. spontaneum*. This was again immune to both diseases. A further backcross yielded plants with 2n=114 chromosomes, 100 from the maternal line and the rest from the paternal line. The plants were resistant to mosaic and immune to sereh. The plants were then propagated vegetatively.

Generally, the noble canes provide the thick barrel and high sucrose content whereas the wild parent provides resistance to drought, diseases and general vigour. At least two, but usually three generations of nobilization are necessary before good results are obtained. This was how the widespread clone POJ 2878 (Proefstation Oest Java) obtained. It is a classic example of a clone obtained from a combination of both wild and cultivated species of sugar cane, and became a world standard for many years. The figure below illustrates its parentage.

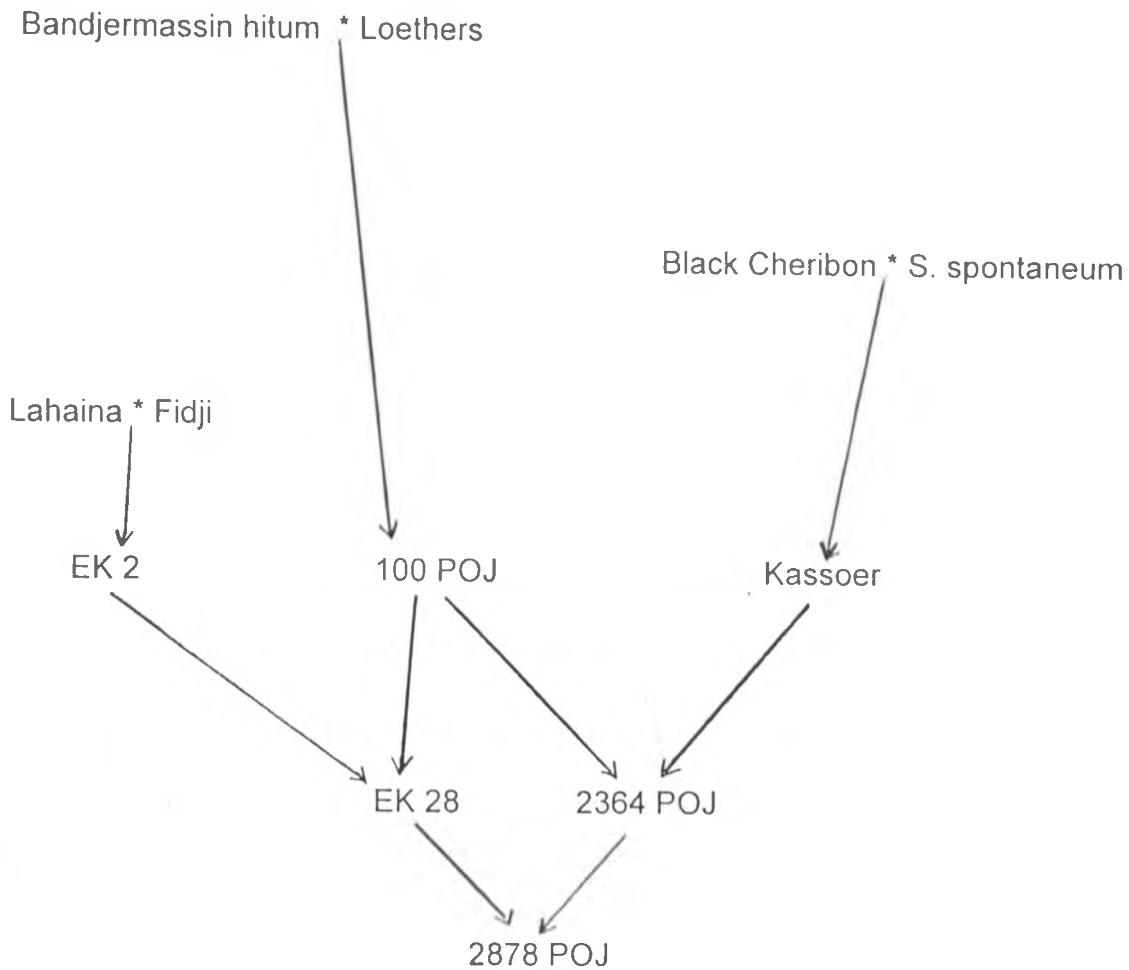

Fig. 1. A pedigree of POJ 2878 sugar cane. (Ochse et al., 1961).

Thus with the appropriate combination of chromosomes and with the aid of backcrossing, it was possible to solve the problem of creating a highly productive and immune variety of sugar cane.

Sugar cane research has since then taken various dimensions in various parts of the tropical and subtropical world. Research has resulted in production of clones with both high sucrose content and resistance to diseases. All the clones produced and released have a specific designation denoting the origin. The clones produced at the world famous Indian

station at Coimbatoire produce the `CO' series. Hawaii produced clones have `H' whereas Brazil have `CB'. Other designations are: Canal Point, Florida,USA (CP); Australia (Q and Trojan); British Guiana (D); Clewiston (CL); Loiusiana (L); Puerto Rico (PR); South Africa (N and N:CO); Taiwan (F); and Venezuela (V).

2.3.2. Improvement through *in vitro* techniques.

The principal basis of screening of excellent genotypes in a plant breeding program is to increase genetic variability. In addition to broading the base of genetic materials for use in current breeding techniques, it is a plant breeder's earnest desire to find new ways of creating genetic variation. The conventional method of sugar cane breeding since its initiation has been chiefly intergeneric and interspecific hybridisation. However, the genetic variability created by gene segregation or combination resulting from sexual crossing may not be sufficient to allow improvement in characteristics of importance (Liu *et al.*, 1972). Other problems facing plant breeding programs are those of low generation turnover and limited population size. These problems can be solved by such advances as tissue culture (Welsh, 1981).

It has been found that several plants can be regenerated by *in vitro* techniques, among them being tobacco, sugar cane, barley, oats, coffee, wheat, corn, sugar beet, cassava and potato (Scowcroft, 1977). Other plants reported to have been raised *in vitro* are interspecific hybrids which would otherwise abort in the mother plant. Obukosia (1985) raised such an interspecific hybrid in beans through embryo culture, whereas Tabaeizadeh et al. (1986) raised somatic interspecific hybrids in graminae through protoplast culture. Tissue culture techniques have been used for crop improvement for a long time in form of

micropropagation. Many commercial laboratories now use tissue culture of shoot tips to propagate a wide range of ornamentals and some crop plants (Evans, 1988). For sugar cane, Liu (1984) gives an extensive review of achievements obtained through *in vitro* techniques, for micropropation and induction and selection of variants for specific objectives.

Sugar cane was among the first crop species to be regenerated *in vitro* (Heinz and Mee, 1969; 1971). The Taiwan Sugar Research Institute has been carrying out a program for application of tissue culture techniques in sugar cane improvement since 1970. The most striking feature of this approach is the tremendous degree of variation observed among regenerated plants, much of which was found to be stable through tiller propagation. Characters affected were general morphology, yield components and disease resistance (Heinz and Mee, 1971; Liu and Chen, 1976). Considerable work, especially on selecting variants resistant to Fiji virus and eyespot fungus has been undertaken in Fiji (Krishnamurthi, 1974) and Australia (Larkin and Scowcroft, 1981). Other research institutes in Florida (Lyrene, 1976; Vasil *et al.*,1979); The Philippines (Lat and Lantin, 1976); Brazil (Evans *et al.*, 1980) and France (Saurvaire and Galzy, 1980) have also initiated work in sugar cane cell and tissue culture. Propagants from variant somaclones exhibiting enhanced yield and disease resistance are already being used for commercial sugar production, and the approach of generating and capturing somaclonal variation for sugar cane improvement will probably be the method of choice for a long time to come (Orton, 1984).

In vitro media: Callus induction and subsequent shoot and root differentiation has been the

centre of research for a long time. This has been through trials ranging from suspension cultures (Nickell and Maretzki, 1969) to the solid media being used by most research stations. In all these, however, the basic media commonly used is that devised by Murashige and Skoog (1962) with modifications mainly on mineral, sucrose and hormonal compositions. Tremendous results have so far been achieved and scientists are still researching on the best media to suit the objectives of their research.

Explant source: Various plant tissues have been used for plantlet regeneration. The tissues which have been succesfully used include sub apical meristems (Heinz and Mee, 1971; Lat and Lantin, 1976); young leaves (Chen and Liu, 1974; Krishnamurthi, 1976, Evans *et al.*, 1980); suspension cells (Maretzki and Nickell, 1973; Chen and Shih, 1983) and most recently apical meristems and buds (Taylor and Dukic, 1993). All these plant parts give good results with varying degree of success.

Regenerated plants: As indicated earlier, a wide range of morphological variants have been reported among regenerants. By studying the morphological characters of eight populations of somaclones, Liu and Chen (1976a) found that the most conspicuous change occurred in auricle length, which accounted for 8.6 % when regenerants were compared with donor clones. Dewlap shape was 6.5 %, hair group 6.2 % and top leaf carriage 1.9 %. Similar observations had also been reported by Heinz and Mee (1971), who also observed tremendous variation in chromosome numbers when compared with the donor plant.

Isoenzyme analysis on regenerants has also indicated some degree of variation. Thom and Maretzki (1970) using polyacrylamide gel electrophoresis were able to study the genetic constitution of regenerated plants

2.4.0. THE USES OF *IN VITRO* TECHNIQUES.

Plant tissue culture appears to be a rich and novel tool now available to the plant breeder in terms of quick method of creating genetic variability.

In addition to creating variability, the plant tissue culture method provides the following advantages in comparison to the conventional breeding program:

1. A large number of plants can be produced in a short period of time hence a high generation turnover.
2. Seedlings can be derived from non flowering clones.
3. Artificial induction procedures are facilitated by the easy handling of small quantities of materials treated in the laboratory and conducting of all induction treatments under controlled conditions (Liu, 1971).
4. Seedlings can be regenerated all year round without restrictions to a certain period of time in the year.
5. Allows for a more effective and efficient means of international exchange of germplasm.
6. Allows for germplasm preservation for a long period of time under low nutrient medium and low temperature (Taylor and Dukic, 1993).

2.5.0. SUGAR CANE RESEARCH IN KENYA.

The current sugar cane research in Kenya is carried out by the National Sugar cane Research Centre (NSRC) at Kibos, under the auspices of the Kenya Agricultural Research Institute (KARI) in conjunction with the Kenya Sugar Authority (KSA). The NSRC co-ordinates the sugar research nationwide and has under it, a regional research centre at Mtwapa in the Coast and other sub-stations at Opapo in South Nyanza and at Mumias.

The breeding programme is based at Mtwapa centre because of its low altitude and warm climate hence sugar cane can grow faster and is able to flower (arrow) readily in that environment. The other sub-stations are used for field trials and agronomic research. Sugar Company Estates have also research departments which collaborate with the Kibos research centre for further field trials.

Currently, the sources of genetic material include Clones CO 421, CO 331, POJ 2878, N:CO 376, N:CO 293 and B 41227 among others. A good number of these clones are also under cultivation in sugar cane producing regions in Kenya. With continuous research, four new varieties have recently been released, namely EAK 71-402, EAK 70-97, EAK 69-47 and CO 945 (KARI, 1991). Another clone, EAK 71-183 is also in final stages of its release. The production of these clones has taken well over 10 years of commitment in research by the institute.

The specific objectives of the sugar cane research programme in Kenya include:

i) Introduction of sugar cane varieties from other growing countries and evaluating the performance and adaptability of such varieties under Kenyan conditions. If they meet the test, they are recommended to farmers for commercial production.

ii) Evaluating the performance of old and new varieties (introduced and bred locally) so as to ascertain their resistance to the prevailing pests and diseases like smut disease. Evaluation is also done for yield per hectare and sucrose content with the aim of assessing the commercial and economic viability.

iii) Carrying out the sugar cane breeding through hybridization with the view of coming up with suitable high yielding, disease resistant and early maturing varieties.

iv) Carrying out the agronomic research on the cane production with a view of coming up with suitable optimal recommendations to farmers on cane production.

Thus in summary, the NSRC objectives are on breeding, agronomy, plant pathology, entomology and sugar technology (KSA,1992).

2.5.1. Uses of sugar cane in Kenya.

As already indicated, sugar cane is the third most significant cash crop economically. The country would therefore benefit alot in terms of foreign exchange savings if self - sufficiency in cane production is achieved. This implies that a closer attention should be placed on cane research for better quality cane.

Improved cane production would also benefit Kenya as a nation because sugar cane is a versatile crop with diverse uses. The main products of sugar cane processing are white sugar and jaggery. In Kenya, raw sugar is processed into white sugar, of which 95 % is consumed by households while 3 - 5 % is utilised in industries. At industrial level, sugar is used as a sweetener in food and drink industries like bakeries, confectioneries, soft drink industries and pineapple canning industries. Important by-products of sugar industry are:

a) Molasses. This contributes to 3.5 % of the cane ground and Kenya exports half

of the quantity produced. It is used in the production of alcohol and also used locally as an animal feed supplement.

b) Bagasse. This is burned to produce electricity and fuel for powering the industries. It is also supplied to the paper factory at Webuye as a raw material.

c) Filter cake. This is returned to the land as a phosphatic fertilizer.

Jaggery is another product of sugar cane obtained in an alternative pathway in the processing. The good quality jaggery is used as a sweetening material in cooking and in sweet meats whereas the poor quality black jaggery is used for illicit distillation of the Nubian gin.

2.5.2. Problems facing sugar cane research.

The problems facing the sugar cane research are varied and include:

a) Inadequate funding,

b) inadequate qualified staff,

c) high staff turnover,

d) poorly equiped laboratories,

e) and inadequate and poorly maintained vehicles thus hindering mobility.

2.6.0. IDENTIFICATION OF CANE VARIETIES.

A major requirement for any successful breeding program is to be able to identify and characterize the available germplasm in the program. This will not only give an indication of the variation existing but will also be a pointer to any possible combination to obtain maximum heterozygosity or homozygosity, as the case may be. Characterization of the

germplasm has ranged from simple botanical and agricultural characteristics to very detailed and precise identification at cellular level. And for sugar cane, both these systems have been used with varying success. Yet it should be noted that for the purposes of comparing varieties, a unified system of rating and recording their various characteristics is needful.

2.6.1. Agricultural and botanical identification.

This is the most widely used method for cane identification. Basically, the method involves the use of such characteristics as stool habit, tillering, top arrowing, colour and the many botanical traits already discussed. Agricultural characteristics used include yield in tons/ha, tons of estimated recoverable sugar per hectare, disease rating and fibre content (KARI, 1991). Most of the botanical characteristics are based on those devised by Daniel and Horley (1971). Disease rating is based on those stipulated by Hutchinson (1967) whereas diagrammatic illustrations and plant descriptions are those used by Artschwager (1939). All these are with modifications to suit the aims of specific cane programs. In Kenya, the above descriptions are generally used (Nyangau & Jagathesan, 1990).

2.6.2. Identification through isoenzyme variation.

Agricultural and botanical characteristics (morphological markers) have been used for clonal identification for a long time but these characteristics are subject to extreme variation due to environmental influence. Electrophoretic techniques have been and are increasingly being used in genetic research for assessment of pathways, determination of genetic relationships and identification of genomes, species and cultivars in crop plants (Bassiri and Adams, 1978). The most important advantage of biochemical markers (isozyme

analysis) over morphological markers is that isozyme patterns specific to cultivars are little affected by differences in environmental factors (Lee and Ronalds, 1967). This is because proteins are direct expressions of genetic make up of plants. Thus the electrophoretic pattern in a given plant is genetically controlled and is consistent for a given tissue and at a particular stage of development.

Electrophoretic patterns, mainly from starch gels and polyacrylamide gels have been used in varietal identification for a long time. They have been successfully used for such crops as wheat (Menke *et al.*, 1973); barley (Kahler & Allard, 1970; Fedak, 1974); rice (Gupta & Malik, 1980); broad beans (Bassiri & Rouhani, 1976) and pyrethrum (Tuikong, 1982). In most of these identifications, esterases and peroxidases gave the most clear indication between cultivars. These procedures have been used also to identify resistant and susceptible lines in crop plants.

Okiror *et al.*(1982) found differences in common bean resistance to anthracnose. In sugar cane, electrophoretic techniques, particularly, polyacrylamide gels have been used with great success. Several enzyme systems of upto 18 enzymes have been attempted to identify the various *Saccharum* species (Feldmann, 1985). These biochemical genetic markers have been successfully used also by various researchers for clonal studies (Barreto *et al.*, 1982; Heinz *et al.*, 1971; Liu *et al.*, 1974; Gonzalez *et al.*, 1982; Zawazaki *et al.*, 1989; Desen, 1989). Dwivedi *et al* (1989) used the glutamine synthetase isozymes to study non-shoot forming (non-chlorophyllous) and shoot forming (chlorophyllous) of the sugar cane clone CO 740 and were able to detect two activity bands. Daniels *et al.* (1989) used ß-amylase chemotaxonomic markers in polyacrylamide gels to differentiate various

sugar cane species. By staining of peroxidase isozymes, clear differences have also been reported between the calli induced from two different clones: JA 60-5 and C 87-51 (Gonzalez *et al.*, 1986). Isozyme variations have also been used to identify some somaclonal variants from cane tissue culture. Rodriquez *et al.* (1986) found out that two out of 42 plants derived from tissue culture were different from the rest in their peroxidase banding. Whereas the others displayed 15 bands in similar positions, one displayed 13 and the other only 12 bands. Somaclonal variants have also been recently reported in an intergeneric hybrid of Saccharum (Nagai *et al.*, 1991). And in all the enzyme systems examined, only peroxidases and esterases have been sufficient enough to give required results, especially for varietal identification.

3.0. MATERIALS AND METHODS.

This study, as already indicated involved a two stage research: i) *In vitro* plantlet regeneration and ii) the use of isozyme techniques for identification of the clones and somaclones.

3.1.0. PLANT MATERIALS.

Ten commercially grown sugar cane clones, obtained from The National Sugar Research Station, Kibos, were used for this study. The setts, consisting of three nodes were obtained from fully mature cane stalks and planted in wooden boxes containing a mixture of sand and red nitosols in equal proportions. These boxes were kept outside the laboratory in an open environment and regularly watered. The young emerging shoots formed the source of the experimental material. Below is a short description of the specific clones sampled for this research.

3.1.1. EAK 70-97.

This clone is one of the newly released sugar cane clones. Its parentage is CO 1007 (Melting Pot) and CO 421. It is an erect clone with medium tillering. The cane stalks are cylindrical with a diameter of 3-4 cm and a characteristic greenish yellow colour and pinkish near the nodes. It generally exceeds the standard clone CO 421 in yield.

3.1.2. CO 421.

This has been the most important clone in Kenya for several years. It is an early introduction to East Africa and has been used as a standard clone for comparison with other varieties. It has a medium tillering ability and a high yielding potential. The leaves encircle the stem loosely and has a fair resistance to smuts. The stem is slightly slender, 2-3 cm in diameter and greenish yellow in colour.

3.1.3. CO 1148.

It is one of the commercial sugar cane clones grown in Western Kenya. It has a medium tillering ability and the stems are greenish yellow in colour. It has a medium yield potential and disease resistance.

3.1.4. CO 645.

Another commercial sugar cane clone with a high tillering ability. It has erect stems, 3-4 cm in diameter. These stems are greenish yellow with brownish nodes. It has medium resistance to diseases, especially smuts.

3.1.5. CO 617.

It also is a commercial clone with medium tillering. Has greenish yellow stems with short internodes. It is generally grown in waterlogged soils and performs better than the standard clone CO 421.

3.1.6. CO 331.

This clone gives moderate yields and performs better than the others in infertile soils. It has a fair smut resistance and a short maturing peak. It has a high tillering ability in good soils. The stems are slender, 2-3cm, and yellowish green in colour.

3.1.7. EAK 71-183.

A new clone developed by NSRC and is under final trials. It is high yielding and has fair resistance to smuts. Contains brownish green stems with short internodes. It has a medium tillering ability.

3.1.8. EAK 71-402.

Another East Africa - Kenya release. Its parentage is PR 1000 X CP 26-291. It exceeds the standard variety in both the main and the ratoon yields. It is moderately resistant to smuts but resistant to mosaic virus. It has a high tillering ability. The internode is cylindrical in diameter, about 4cm, and is light yellow in colour.

3.1.9. EAK 69-47.

It is a new release from the cane breeding program in Kenya. Its parentage is CO 798 MP and CO 421. It is a good yielder, moderately resistant to smuts but resistant to mosaic virus. It has a good tillering ability and vigorous growth. The stems are thick and roundish, 3.5 - 4 cm in diameter, and are yellowish green in colour. It performs better than the standard clone in most areas.

3.1.10. CO 945.

A new release with the parentage POJ 2878 X CO 617. It is a midium maturing variety but with good yields. It is resistant to both smuts and mosaic. It is a poor germinator and the stem is 3 cm in diameter and is greenish yellow with a pink tinge. The yields are generally better than the standard clone.

3.2.0. *IN VITRO* EXPERIMENTS.

3.2.1. Preliminary experiments.

The initial experiments involved callus induction in all the ten clones sampled. Their ability to generate callus was used as a basis for selection of a few clones to be used for further *in vitro* studies. In this experiment, replicated trials at two levels of 2,4-Dichlorophenoxy acetic acid (2,4-D) of 1.5 and 3.0 ppm were carried out. The selection of these levels were based on those used by Liu *et al.* (1972) and Tabaeizadeh *et al.* (1986).

Young shoot meristems and the surrounding 2-3 whorls of developing leaves formed the plant material source for the experiment. These young shoots were obtained from 6-8 week - old young plants growing in the wooden boxes. With the help of a sharp sterilised scalpel blades, the meristems were transversely cut into small sections of 2-2.5 mm in diameter and about 4 mm long. The portions were then surface sterilised by immersing in 0.5 % sodium hypochlorite solution for 5 minutes and then rinsed thoroughly in sterile distilled water. They were then aseptically explanted, using sterile forceps, into the culture bottles containing a modified MS (Murashige & Skoog, 1962) medium supplemented with 30 g/l sucrose and the mentioned levels of 2,4-D. All these activities were carried out on a working lamina flow bench sterilised with 70 % alcohol.

Universal bottles measuring 2.8 x 8.5 cm (and a capacity of 30 mls) were used for callus induction medium, each containing about 10 ml of the medium solidified with 1 % w/v agar. The medium solution had initially been prepared in a flask and the pH adjusted to 5.7. Agar was then added and boiled to allow it dissolve before finally dispensing into the culture bottles. The bottles were then autoclaved at 121 °C and 1.1 kg/cm^2 for 15 minutes

and then left overnight at room temperature to solidify. All the treatments were then placed in growth chambers at about 25 °C and exposed to a 16-hour photoperiod supplied by fluorescent tubes. Four replicates were incubated for each treatment. The culture bottles were distributed at random in the growth chamber as a split plot design.

Callus formation was observed after one week of incubation. Cultivar effects on callus induction were then noted. Callus morphology and colour were also noted. After 8 weeks of cultivation, the calli formed had their fresh weights and diameters measured and recorded.

Attempts were also made to regenerate shoots from the calli by transferring them into media without 2,4-D but supplemented with between 10-15 % coconut milk.

3.2.2. Detailed experiments.

Based on their performance in the preliminary trials, six clones (CO 6415, CO 617, CO 331, EAK 71-183, EAK 71-402 and EAK 69-47) were selected for further analysis. The detailed experiments here involved the optimisation of the type and concentration of growth regulators required for the establishment of the *in vitro* plants. It also involved determining the tissue type, sterilisation solution and period as well as tissue orientation on the explantation medium.

Effect of tissue type and orientation on the culture medium. Several portions of leaves at various stages of development were used and also the young shoot tips. These were obtained and treated as already indicated in the preliminary trials. Two orientations were also tested on the culture medium: horizontal and vertical explant orientation. The horizontal orientation was achieved by simply letting the explant lie horizontally on the

medium, whereas the vertical orientation involved fixing the explant on the medium in a manner as would appear on the growing plant. Observations were made as incubation progressed.

Effect of sterilisation solution and period on callus induction. This was carried out by setting four time intervals of 2.5, 5.0, 7.5 and 10.0 minutes as time of sterilisation on 0.5 % sodium hypochlorite solution. The small young shoot explants were soaked in the sterilant for the specificied time and then rinsed three times in sterile distilled water. Effects resulting from this as observed in the culture medium were noted and used to select the optimum sterilisation period.

Effect of 2,4-D on callus induction. The effects of two factors: clones and hormone 2,4-D on callus induction were investigated. The six clones (CO 645, CO 617, CO331, EAK 71-183, EAK 71-402 and EAK 69-47) and four levels of 2,4-D (0, 1.5, 3.0 and 4.5 mg/l) were used. Each of the six selected clones of cane was subjected to each of the four levels of 2,4-D. This was done in a basic MS medium with no other hormones added, but supplemented with 30 g/l sucrose and solidified with 1 % agar. The explants were cultured and incubated in a growth chamber under a split plot design. The growth chamber was supplied with a 16-hour photoperiod from fluorescent white light tubes. Treatments were replicated five times, each with a reserve replicate to overcome extensive missing data due to possible contamination.

Calli formation was realised after one week of incubation. Callus fresh weight was measured on a weekly basis for six weeks consecutively. The weights were measured with an electronic balance under sterile conditions. The calli were immediately returned to

the same culture media each time after measurement for further growth. The exercise was done on a weekly basis upto six weeks of incubation when the growth increases appeared to be decreasing. The data obtained were used to determine the optimum level of 2,4-D and also the clonal response to various treatments.

Effect of other growth regulators on organogenesis. Organogenesis was induced in the above calli as a second step after callus induction. A set of other young calli were also induced and then used for organogenesis within a period of 3-4 weeks. Shoot formation was achieved by supplementing the MS medium with 10% coconut water and without 2,4-D. The coconut water liquid was filter sterilized using 0.45 micrometre filter and the filtrate dispensed to already autoclaved medium before solidification. The medium was swirled severally and then aseptically dispensed in 20 ml aliquots to sterile scott tissue culture bottles. These bottles were of two sizes, 5.2 x 10.5 cm and 6.5 x 14 cm , in diameter and height respectively and had threaded necks. Once the media had fully solidified, the calli were transferred into them and then cultivated in growth chambers.

Within a period of two to three weeks, some green dots (leaf primordia) could be visibly noticed. These gradually developed and became shoots by the end of the fourth week. The formed shoots could, however, only elongate without forming any roots at all. The developed shoots were then individually as was possible transferred to a multiplication medium supplemented with 6-Benzylaminopurine (BAP) at two levels of 1 and 2 ppm. Shoot responses to these levels were also noted.

Rhizogenesis was induced on well developed shoots. This was achieved by culturing the shoots in another medium supplemented with 2 ppm Indole butyric acid (IBA). It was

possible to initiate roots in this medium within a period of 1-2 weeks. Later experiments also indicated that both multiplication and rooting could be efficiently achieved in a combined medium of 1 and 2 ppm; BAP and IBA respectively.

Oxidative browning: This became a serious problem during subculturing and especially so in the shoot initiation and multiplication stages. This resulted from the apparently wounded parts of the shoots. The brown exudate from the plantlets would diffuse into the medium and was detrimental to further development of the explants, which would become necrotic and eventually die. This was however avoided by addition of 3-4 g/l of activated charcoal. The charcoal also seemed to have a positive effect on root development.

3.2.3. Transplanting plantlets into soil.

When the rooted plantlets were about 8-10 cm high, they were removed from the culture bottles and their rootlets thoroughly washed. This was to ensure that no portion of the agar nutrient medium was carried along as it would form a source of microbial infection and endanger plantlet survival during the hardening off. The washed plantlets were then transplanted to small pots containing a mixture of white pellets and soil in equal proportions. They were then watered and covered with transparent plastic containers to maintain high moisture content. The potted plants were then kept in a greenhouse and watered regularly. Plastic covers were removed within a period of 4-7 days when the plants were established. The young plants were maintained in the greenhouse and formed a source of material for isozyme analysis.

Isozyme analysis was determined on plantlets at various stages of growth, from the young shoots in culture bottles to one to three-month-old plants in the greenhouse. These

were all used, as required, for isoenzyme analysis.

3.3.0. ANALYSIS OF *IN VITRO* EXPERIMENTS.

Two methods were used to analyse the results obtained from *in vitro* plant regeneration.

3.3.1. Relative Growth Rates (RGR).

The relative growth rates (RGR) were calculated based on the formula used by O'Dowd *et al* (1993) and indicates the growth of established calli in a 48 - day growing period.

$$RGR = \frac{3(W_2^{\frac{1}{3}} - W_1^{\frac{1}{3}})}{t_2 - t_1} \; mg/day$$

Where W_1 = Initial callus mass.

W_2 = Final callus mass.

t = Time in days.

$t_2 - t_1$ = 48 days.

Analysis of variance was carried out for the relative growth rate values to determine any significant clonal variations.

3.3.2. Effect of 2,4-D on callus induction.

Fresh weights and diameters of calli were used as parameters to indicate the influence of 2,4-D on the sugar cane clones. Analysis of variance was carried out based on Steel and Torrie (1980) and Singh and Chaudhary (1985). The following model was used for analysis:

$$Y = m + b_i + c_j + h_k + (ch)_{jk} + E_{ijk}$$

Where: b_i is the effect of ith block.

c_j is the effect of jth clone.

h_k is the effect of kth dose of 2,4-D.

$(ch)_{jk}$ is the interaction effect.

E_{ijk} is the error term.

Routine maintenance of established callus cultures was achieved by subculturing into fresh media every 4-5 weeks.

3.3.3. Estimation of the number of regenerants.

The mean number of plantlets obtained from each induced callus was noted for the seven clones considered. ANOVA was also carried out to determine the degree of existing clonal variations.

A similar procedure was also applied to estimate the number of plantlets obtainable when a single regenerant was subcultured in a multiplication medium. This was referred to as *in vitro* tillering and was used to estimate the output of sugar cane micropropagation.

3.4.0. ELECTROPHORESIS IN POROSITY GRADIENT GELS (PoroPAGE).

Polyacrylamide gel electrophoresis was used to analyse the sugar cane clones and somaclones. Specifically, the porosity gradient polyacrylamide gel electrophoresis (PoroPAGE) as stipulated by Stegemann et al. (1988) was employed throughout the analysis. A gradient gel of 10 - 28% polyacrylamide as used by Standke and Rühl (1990) was found to be appropriate and gave clear bands for the enzyme systems analyzed.

3.4.1. Equipments and solutions used.

Some of the equipments used included the following.

(1) Mono - phor apparatus with connections to the power supply and a cooling unit.

(2) Slot former for 24 samples.

(3) Glass plates measuring 250x150x4 mm, with one having fixed spacers in both sides of about 1.5 mm in thickness each.

(4) A gradient mixer with two fixed cylinders.

(5) A magnetic stirrer and stirring bar.

(6) A peristaltic pump to regulate the flow rate.

(7) Connecting tubings of about 1 mm in diameter.

The solutions used and their preparation were as follows.

(1) The electrophoresis buffer: The buffer used was Tris/Boric acid buffer, pH 8.9. The stock solution, C7, was made from 242.0g Trizma base, 18.4g Boric acid and made to a volume of 2000 ml with double distilled water. 300 ml of this solution added to 2100 ml water gave the final electrophoresis buffer.

(2) Solution A: 10% polyacrylamide. This was made from 9.5g Acrylamide, 0.50g

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Bisacrylamide, 1.0g sucrose, 0.03 ml DMAPN, 12.5 ml of solution C7 and then made to 100 ml with double distilled water.

(3) Solution B: 28% Polyacrylamide. This was made from 19.00g Acrylamide, 1.0g Bisacrylamide, 7.0g sucrose, 0.005 ml DMAPN, 12.5 ml of C7 and then filled to 100 ml with water.

(4) Top layer solution. The solution contained 2.5 ml of C7, 0.5 ml of 10% Ammonium persulphate, 0.5 ml of 1% Amido black marker, 0.006 ml DMAPN and finally made to 20 ml with water.

(5) Stacking gel: 8% Polyacrylamide. The stock solution for this gel contained 7.5g Acrylamide, 0.5g Bisacrylamide, 12.5 ml C7 and then made to 100 ml with water. The final tracking gel solution was obtained from 15 ml of the stock solution, 0.06 ml of DMAPN and 0.4 ml of 10% ammonium persulphate, added just before use.

The above solutions were all stored in the refrigerator except for the ammonium persulphate and the top layer solutions which were normally prepared afresh each day when needed.

3.4.2. Preparation of the porosity gradient gel.

The apparatus were first fixed in place. The thoroughly cleaned glass plates with a spacer of about 1.5 mm were held together using plastic clamp and the holding screws tightened. This gave an approximate internal volume of 150x230x1.5 mm between the glass plates. This was then positioned upright and clamped on the removable part of the mono - phor apparatus. The lower part of the glass plates were smeared with petroleum jelly (vaseline) before tightening it on the rubber portion of the apparatus. This was done

to avoid any leakage of the gel solution. Connections were then made from the gradient mixer placed on a magnetic stirrer to the peristaltic pump and to the fixed glass plates using the small capillary tubings. The capillary tube connection to the glass plates was ensured that it was placed in the middle of the plates to allow uniform spread of the gel to both ends.

An aliquot of 24 ml solution B was then pipetted into the cylinder of the gradient mixer connected to the effluent capillary tubing, and the magnetic stirrer bar put into it. The tap connection to the other cylinder was carefully released to allow the solution expel air in it and then quickly shut as soon as a drop of the solution appeared in the other cylinder. An equivalent volume of solution A was also dispensed into this cylinder. The magnetic stirrer was then set to about 300 Upm and allowed to stir solution B. Between 0.17 and 0.18 ml of 10% AP was pipetted to both cylinders and stirred shortly using a glass rod. The peristaltic pump was set at a peristaltic speed of 750 and then switched on as the fluid connection of the gradient mixer cylinders was simultaneously released. This allowed for a continuous flow between the cylinders and also to the glass plates through the peristaltic pump. This continued for around 20 minutes and all the gel solution was finally dispensed into the glass plates and rose to a height of about 12.5 cm on the plates. The pump speed was then reduced to 300 and the top layer solution was then fed onto the gel through a disconnected capillary tubing between the mixer and the peristaltic pump. The solution was allowed to rise to a height of 5 mm above the gel solution. The gel was left to polymerise and this occurred between 1 - 2 hours. The layer solution was then carefully removed with the help of blotting paper. After this, the stacking gel solution was dispensed to fill the glass

plates completely and the slot former carefully inserted into the plates. This again was allowed to polymerise for about 30 minutes. Meanwhile, as the polymerisation was going on, the mono - phor apparatus was loaded with the running buffer to the required level and this required about 5 litres of the buffer. Also during this time, the samples the samples were being prepared in readiness for the loading.

Once polymerisation of the stacking gel was completed, the comb was removed and the slots filled with the running buffer. The gel plates were unclamped, turned upside down and clamped again on the hollow rubber attachment of the electrode. The whole apparatus was then carefully dipped into the buffer, ensuring that no air bubbles were trapped at the base of the gel immersed into the buffer. The top of this apparatus formed the electrode chamber. A small quantity of the running buffer was pippetted once again into the electrode chamber until it was overflowing. Then was the system ready for loading the samples.

3.4.3. Sample preparation for electrophoresis.

Extraction buffer. Extraction buffer consisted of 20g of trizma base in 30 ml of 2M HCL adjusted to a pH of 8.0 and made upto 100 ml with water.

Extraction procedure. Young fresh leaves were obtained either from the plants in the greenhouse or from the plantlets in the tissue culture maintenance room. A leaf sample weighing 0.25g was ground on an ice-cooled glass plate containing tiny wells where 0.75 ml of the extraction buffer was also added. The sample was crushed using a glass rod for about 5 minutes per sample and then transferred into centrifugal tubes and stored for about one hour in the refrigerator. Later trials also showed that extraction could be equally achieved by crushing the samples directly in the tubes containing the 0.75 ml aliquot of the

buffer. The samples were centrifuged at 1500 rpm for 30 min at about 6 °C. The homogenate was pippeted into smaller plastic containers and stored in a frozen state (-20 °C) until when required for electrophoresis. The final loading samples had 0.005 ml of Amido Black marker added to 0.15 ml of the enzyme extract.

3.4.4. Loading the gel.

Sample extracts of between 10 and 30 microlitres were loaded into the wells using micropippetes attached to microsyringes. Preliminary trials revealed that 30 microlitres was the most appropriate and was adapted in subsequent experiments. Each sample was loaded using a fresh pippete no avoid contamination. Once all the slots had been loaded, the electrode chamber was carefully flooded with one litre of the running buffer.

3.4.5. Electrophoresis.

The power pack supply unit was set at a constant voltage of 300 V and a current of 96 mA. The samples were then electrophoresed at 300 V and an initial current of between 36 and 44 mA. The gel was run overnight at 5-8 °C for 16 hours. After the 16-hour period, the gel was stopped and the current had dropped to between 20 and 24 mA.

The gel-containing upper electrode was carefully removed, the buffer in it poured to the lower container and then placed in an upright position. The glass plates containing the gel were carefully disconnected an the plates separated using a strong spatula placed between them. The end of the gel was marked by clipping of the lower edge of the right hand corner of the slab. It was then carefully transferred into the staining solution by holding the glass plate with the gel downward over the solution and then detaching one corner of the gel by a spatula until the whole gel slid into the tray..

3.4.6. Staining of the gels.

Gels were stained to locate areas of isozyme activity. Each enzyme system was stained using a different technique.

Areas of esterase activity were detected using the staining method described by Stegemann et al. (1988). The method employs 1-Napthylacetate as a substrate and Fast Blue RR salt as the dye coupler. The staining buffer consisted of 200 ml of 0.15 M phosphate, pH 7.0. This solution was obtained by mixing 60 ml of stock I and 90 ml of stock II with 100 ml of water. (Stock I contained 0.3 M Disodium hydrogen phosphate made from 26.7g $Na_2HPO_4.2H_2O$ in 500 ml water, whereas stock II contained 0.3 M Sodium-dihydrogen phosphate made from 23.4g of $NaH_2PO_4.2H_2O$). The gel was swayed in this solution in a tray for 10 minutes. Without much exposure to light, 40mg of the substrate was dissolved in 2 ml acetone and 100mg of Fast Blue RR salt was dissolved in 5 ml water and filtered. Its filtrate and the substrate solution were then simultaneously added into the tray, agitated and then placed on a swaying machine. After a few minutes, bands could be noticed and developed fully within one hour. The reaction was stopped and the bands fixed using a destaining solution obtained by mixing 20 ml acetic acid, 120 ml methanol and 280 ml water. The bands were schematically drawn and the gel photographed.

Peroxidases were stained using the benzidine method, as described by Stegemann et al.(1988). 100mg of 3,3'-dimethoxybenzidine (O-dianisidine dihydrochloride) was dissolved in 60ml methanol and 0.3 M Monosodium phosphate (Stock II above). The gel was then gently agitated in this solution for 10 minutes at room temperature and then 0.3 ml of 1 % hydrogen peroxide was added. Bands become visible after a few minutes and

were clear enough after 30 minutes of staining. The gels were then photographed and schematic drawings made.

3.4.7. Photography of the gels.

During photography, the gels were carefully placed in a light table (N° 160) and transilluminated. The light table is equipped with double coated fluorescent bulbs of 20 watts each and the homogeneity of the light area (35cm x 65cm) is achieved by translucent glass and light scattering plexiglass. ISO 50/18° PANF DX film was used for photography with photos being taken at about 50 cm vertically above the gels, using a zoom camera. The lens aperture was set at 22 and three exposure times of 1 second, 0.5 seconds and 0.25 seconds were used.

4.0. RESULTS.

The results obtained from the research study are presented in three sections, namely *in vitro* callus initiation, organogenesis and the establishment of the plantlets into the soil.

4.1.0. *IN VITRO* CALLUS INITIATION.

4.1.1. Callus growth and characteristics.

Table 4.1 shows the calli characteristics and their relative growth rates (RGRs). The callus characteristics generally varied from clone to clone with the dominating callus appearances being creamy, greenish-yellow and brownish. A callus maintained in a single medium for over six weeks changed its colour, for example, clone CO 617 changed colour from creamy to reddish-brown. Callus texture also showed clonal variation and ranged from loosely held callus to well developed compact callus.

Relative growth rates varied from clone to clone and at different treatment levels of 2,4-D. Clone EAK 70-97 had the slowest callus initiation and also portrayed the least relative growth rate of 0.264. Clones EAK 69-47 and CO 6415 which were the most prolific callus-inducing clones also recorded the highest RGR values of 0.375 and 0.349 respectively. These values were significantly different ($p = 0.05$) from most of the other clones. Similar trends were observed at both levels of 2,4-D.

Table 4.1. Mean relative growth rates (RGR) in mg/day and callus characteristics of ten clones cultured in MS medium at two levels of 2,4-D concentration.

CLONE	RGR at 1.500 mg/day 2,4-D	RGR mg/l at 3.00 mg/day 2,4-D	Callus characteristics
EAK 70-97 (1)	0.264a	0.268c	Brownish callus with pink serrated tips and loosely held. Slow growth
CO 421 (2)	0.239a	0.304c	Greenish white callus with brown dots and quite compact.
CO 1148 (3)	0.295ab	0.376d	Greenish callus with serrated tips. Quite large and compact
CO 6415 (4)	0.349b	0.389d	Fast callus induction to form large brown compact callus.
CO 617 (5)	0.329b	0.338cd	Creamy white callus with white loosely held and large tips
CO 331 (6)	0.324ab	0.346d	Greenish yellow callus, compact and fast formed
EAK 71-183 (7)	0.271a	0.302c	Creamy white callus with brownish tips. Medium and compact callus formation.
EAK 71-402 (8)	0.265a	0.304c	Whitish brown callus with smooth round tips and quite compact
EAK 69-47 (9)	0.375b	0.392d	Brownish callus with pinkish brown serrated tips and small greenish bud primordia
CO 945 (10)	0.268a	0.288c	Small brownish compact callus with brownish round tips

Values followed by a common letter(s) in each column do not differ significantly at $p = 0.05$ based on the Duncan's Multiple Range Test (DMRT).

4.1.2. Clonal response to 2,4-D treatment.

There were clonal variations in response to the auxin concentration used as indicated in RGRs, callus fresh weights and diameters (Tables 4.1, 4.2 and Fig. 1). A basal medium supplemented with 3.00 mg/l 2,4-D resulted in significantly higher RGRs for the clones used than the other 2,4-D treatments. This was true for all clones except for EAK 70-97 and CO 617 which had numerically higher RGR values at the higher auxin concentration but not significantly different from values at lower auxin concentration ($p = 0.05$).

Tables of analysis of variance (Appendix 3) indicate that the F values were highly significant ($p = 0.01$) for treatments clones and their interactions.

Table 4.2. Effects of 2,4-D levels and clones on the mean callus fresh weights and diameters of 10 clones after 8 weeks of *in vitro* culture.

	Levels of 2,4-D (mg/l)		Levels of 2,4-D (mg/l)	
	1.50	3.00	1.50	3.00
Clone	Mean fresh wt (mg)		Mean diameter (mm)	
1. EAK 70-97	462.00b	502.00a	9.93b	10.57a
2. CO 421	378.25a	569.25c	8.07a	13.38c
3. CO 1148	542.75d	848.25g	11.68d	18.20f
4. CO 6415	730.75f	894.25h	16.13f	19.17g
5. CO 617	658.06e	717.00e	13.89e	15.27e
6. CO 331	653.00de	743.75f	13.77e	15.52e
7. EAK 71-183	478.00c	581.00d	10.42c	12.60d
8. EAK 71-402	461.75b	584.25d	9.80b	12.73d
9. EAK 69-47	856.00g	944.22i	18.23g	20.07h
10. CO 945	473.79bc	519.25b	10.23bc	11.30b

Values followed by a common letter in each column do not differ significantly at $p = 0.05$. All values in each row differ significantly for both weights and diameters, according to Duncan's Multiple Range Test (DMRT) with four replicates.

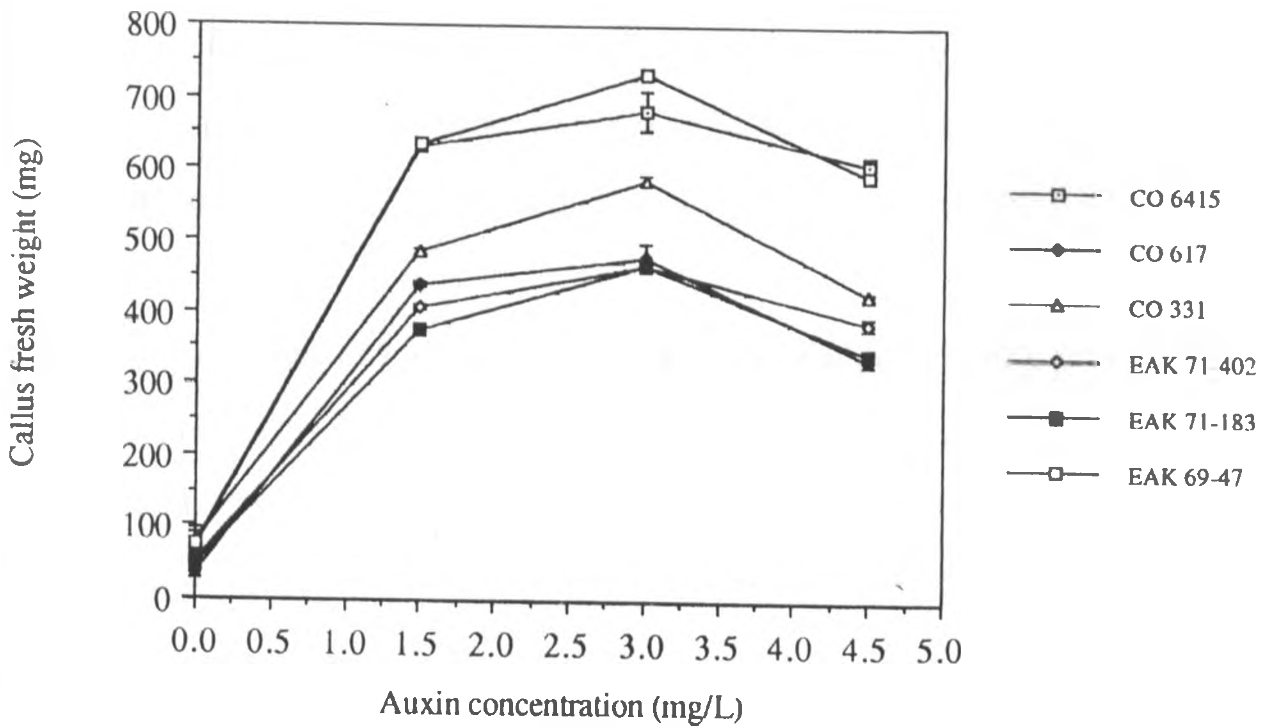

Figure 1. Effect of 2,4-D concentration on callus growth after six weeks in culture.

The fresh weight and callus diameter means were also significantly different ($p = 0.05$) among some clones. These values were , however, all different between the two levels of 2,4-D concentration (Table 4.2). Clone EAK 69-47 recorded the highest values for both the callus fresh weights and diameters at the two treatment levels of 2,4-D. These were also significantly different from the other clones. There was also a general trend that the heavier the callus fresh weight, the greater the diameter. Clones EAK 70-97 and CO 421 had significantly lower values of mean callus fresh weight and diameter when compared to other clones.

4.1.3. The influence of 2,4-D levels on callus fresh weights.

A summary of the results of the detailed experiments involving six clones subjected to four treatment levels of 2,4-D is shown in Table 4.3. The ANOVA for each level of 2,4-D indicated that the differences in clonal response to hormone treatments were highly significant (Appendices 4 & 5).

The 2,4-D effects were not significantly different after the first week in culture but become highly significant ($p = 0.01$) in subsequent weeks in culture. The absence of 2,4-D did not induce any callus at all but the explants generally remained green with slight increase in fresh weights due to tissue expansion. Again a treatment of 3.00 mg/l of 2,4-D gave the highest fresh weight with an increase in each subsequent week.

Table 4.3. The effect of 2,4-D on mean callus fresh weights (mg) for the six clones over a period of six weeks.

LEVEL OF 2,4-D (mg/l)	PERIOD OF CULTIVATION (WEEKS). 1	2	3	4	5	6
0.00	40.87a	45.53a	52.87a	60.03a	65.03a	61.44a
1.50	41.43a	66.97b	110.97b	211.43b	363.04b	494.38b
3.00	42.83a	74.93c	138.03c	258.67c	429.43c	568.74c
4.50	38.57a	60.74d	103.33d	194.47d	340.33d	462.91d

Values followed by a common letter in each column do not differ significantly at $p = 0.05$ based on Duncan's Multiple Range Test (DMRT) and five replicates.

Table 4.2 further illustrates that the clones EAK CO 6415 and EAK 69-47 had the highest and significantly different response to auxin concentration when compared with other clones after six weeks in culture. This trend of the clonal response to auxin treatment was similar within the six-week period in culture (Table 4.3 and Fig. 1). At 2,4-D concentration of 4.5 mg/l, all the clones showed a reduced response in mean callus fresh weight. The fresh callus weights at this level were significantly lower than the weights observed at 3.00 mg/l. There was an increase in fresh weights of callus over the six weeks in culture (Fig. 2). Clones CO 6415 and EAK 69-47 had the greatest increase while clone EAK 71-402 had the least.

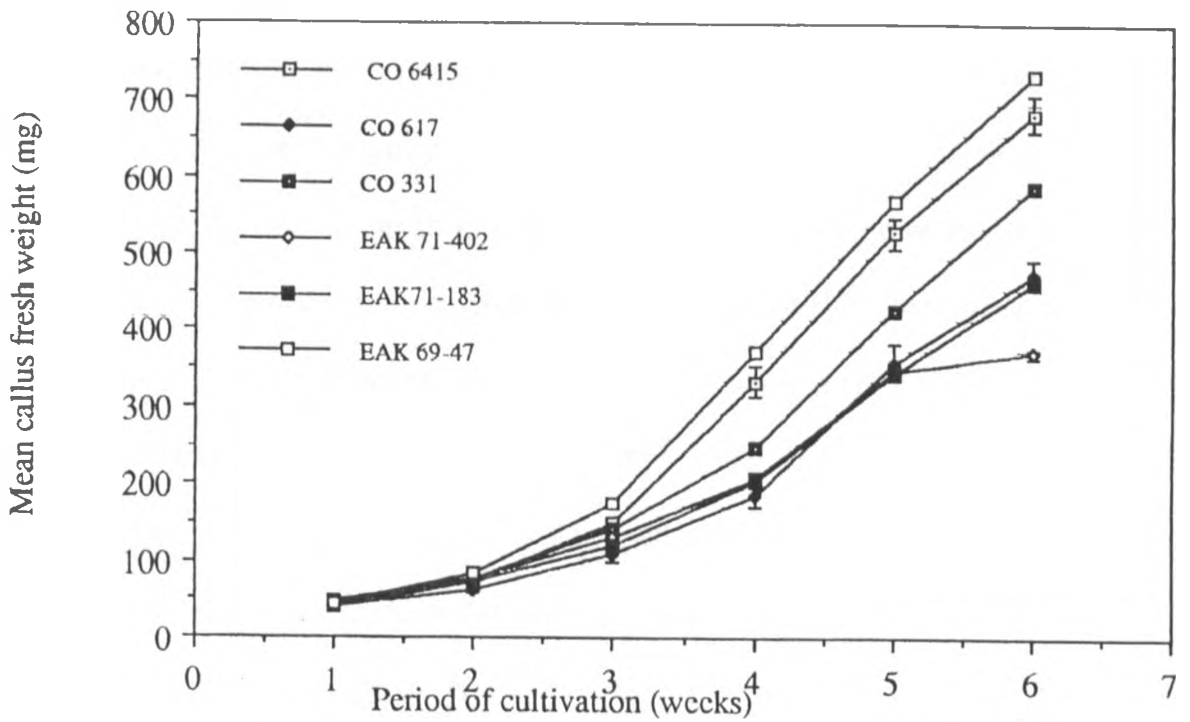

Figure 2. Mean callus growth (fresh weight) as observed in a six-week culture period.

4.2.0. ORGANOGENESIS.

Fully established calli became organogenic when subcultured into MS medium supplemented with different hormonal types and concentrations. Organogenesis occurred in two stages, namely, shoot initiation (caulogenesis) and root initiation (rhizogenesis).

4.2.1. Shoot Initiation (Caulogenesis).

Shoot development was achieved in Murashige and Skoog (MS) basal medium supplemented with 15 % coconut. It was possible to regenerate shoots in all the ten clones (Plates 1, 2 & 6). Distinct differences in shoot initiation were also observed among clones in the initiation medium. Some clones (e.g. EAK 70-97, CO 421 and CO 1148) were quite slow in shoot initiation. Plate 1 shows clone EAK 70-97 which was quite hard to induce shoot regeneration whereas Plate 2 shows clone CO 6415 which was very prolific so that caulogenesis could be obtained in a callus induction medium.

Abnormalities were also noted during shoot initiation. In one of the clone CO 1148 calli, there was no shoot initiation but the callus only formed aerial roots (Plate 3). These hairy roots continued to elongate even after several times of subculturing into fresh medium.

Clone EAK 69-47 was the most responsive to hormonal concentration and formed well established shoots. The shoots resulted from subculturing in three different media concentrations (Plates 4, 5, 6 and 7).

Plate 1. Initiation of shoot development in clone EAK 70-97 callus, after four weeks of growth in MS medium with 15% coconut water.

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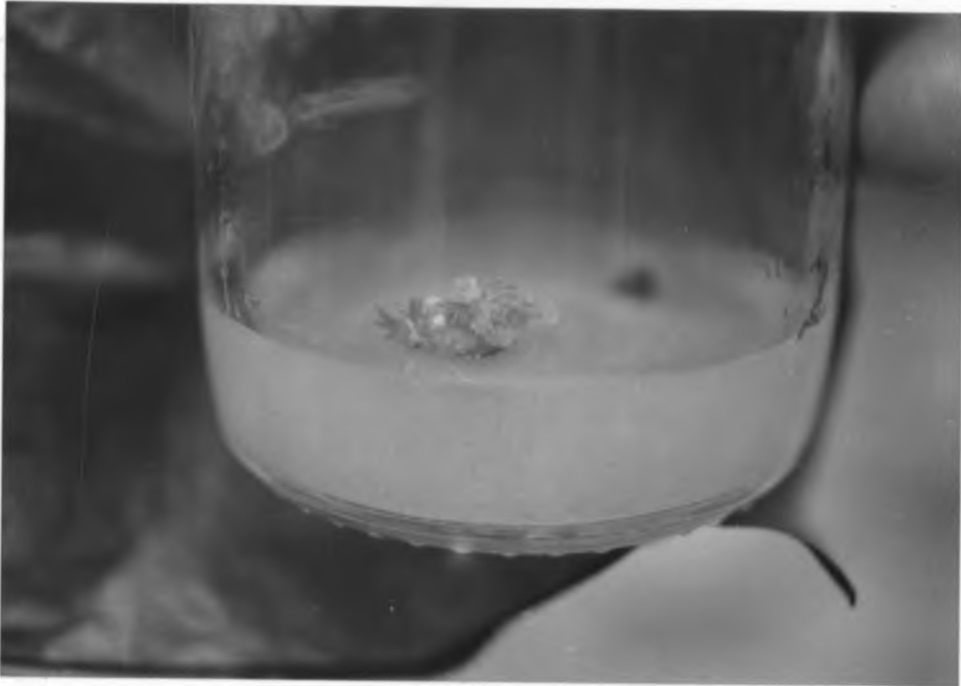


Plate 2. Induction of shoot development from clone CO 6415 callus, five weeks after explantation in medium with 3.00 mg/l 2,4-D.

Plate 3. Abnormal growth of clone C0 1148, five weeks after transfer into media with 15% coconut water. The callus developed aerial roots but not shoots.

The stages that were observed in sugar cane regeneration *in vitro*, shoot development and plantlet transfer and growth in the soil is shown in *Plates* 4-9.

Plate 4. An explant showing expansion, three days after inoculation

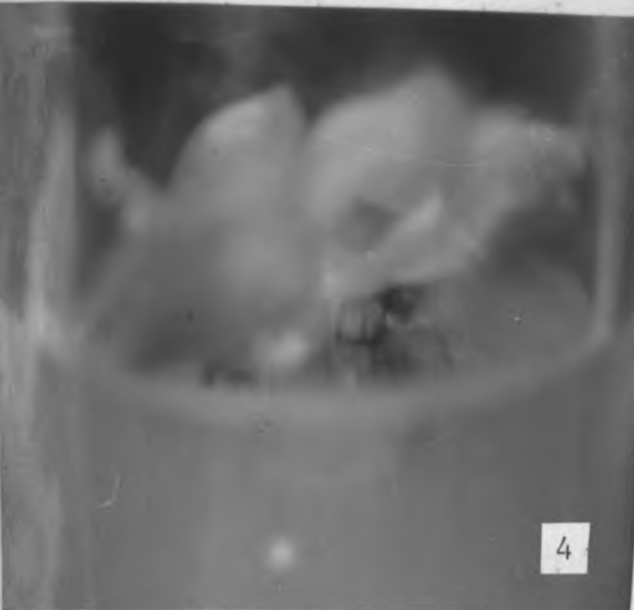
Plate 5. A well developed organogenic callus in a callus induction medium (MS supplemented with 3.00mg/l 2,4-D)

Plate 6. The organogenic callus regenerates shoots in a shoot initiation medium (MS supplemented with 15% coconut water).

Plate 7. Well developed shoot cultured in a root initiation medium (MS with 2.00mg/l IBA) for rhizogenesis to occur.

Plate 8. Young plantlets being established in soil/pellets medium in the greenhouse.

Plate 9. Three-month old sugar cane plants showing extensive tillering.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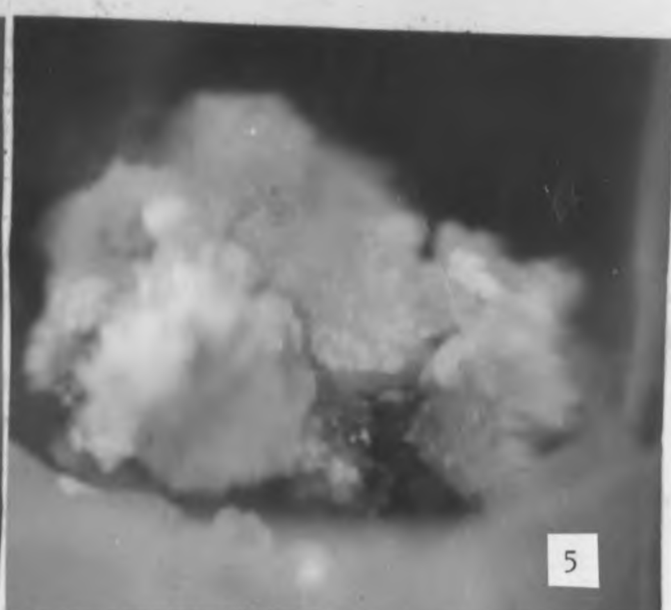
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4.2.2. Shoot Multiplication and Rhizogenesis.

The two levels of BAP at 1.00 and 2.00 mg/l used led to multiplication of the plantlets. A basal MS medium supplemented with 2.00 mg/l of BAP gave a generally higher number of plantlets but not significantly different. Clonal differences were observed also as some prolific clones formed roots in this medium. Most clones, however remained quite recalcitrant in root development in the multiplication medium.

Rhizogenesis occurred in a MS medium containing 2.00 mg/l IBA and clonal differences were again observed in root development (Plate 10). Root initiation occurred within two weeks after subculturing into the initiation medium. Plate 10 shows young plantlets from two clones showing differences in *in vitro* shoot and root development. Clone EAK 71-402 (8) formed slender and upright shoots with long roots while clone CO 331 (6) formed strong short shoots and dense roots. Differences were also evident in shoot development in the root initiation medium (Plate 11). Well rooted plantlets easily established when transplanted into small pots in the greenhouse (Plates 7, 8 & 9).

Chlorotic mutants (albinos) were some of the phenotypic variants observed among the regenerants. These mutants were mainly expressed in clones that were so vigorous in callus growth and shoot regeneration (Plate 12). The mutants could not be established in the greenhouse but were maintained in the multiplication medium.

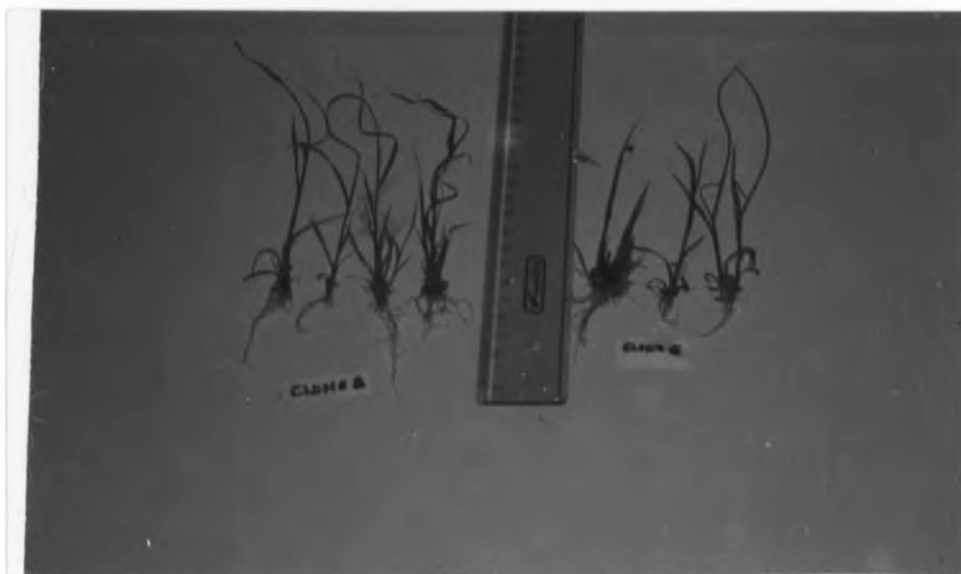

Plate 10. Clone EAK 71-402 (8) and Clone CO 331 (6) plantlets illustrating differences in shoot development and root formation.

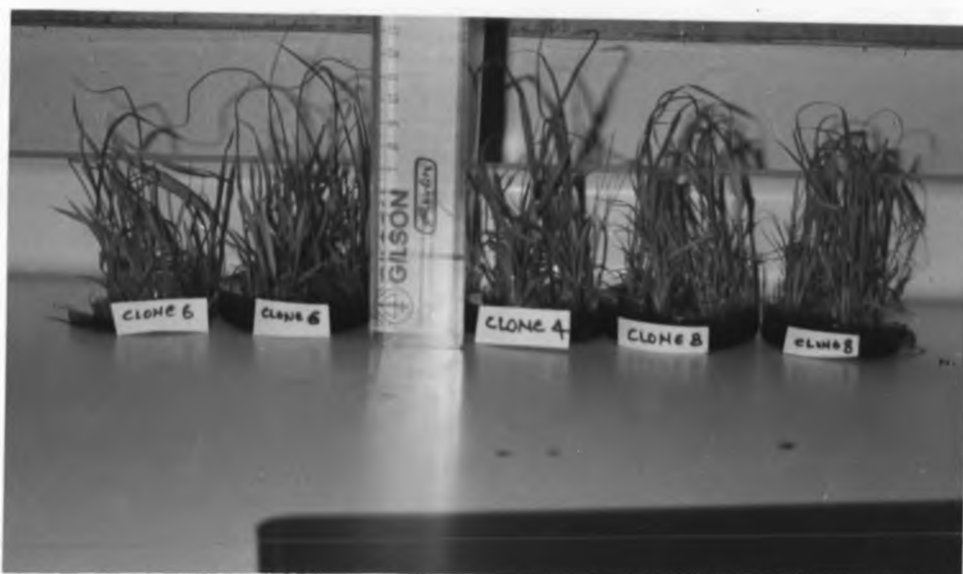

Plate 11. Clones CO 331 (6), CO 6415 (4) and EAK 71-402 (8) fully developed plantlets in a rooting medium just before transplanting to the pellets/soil medium.

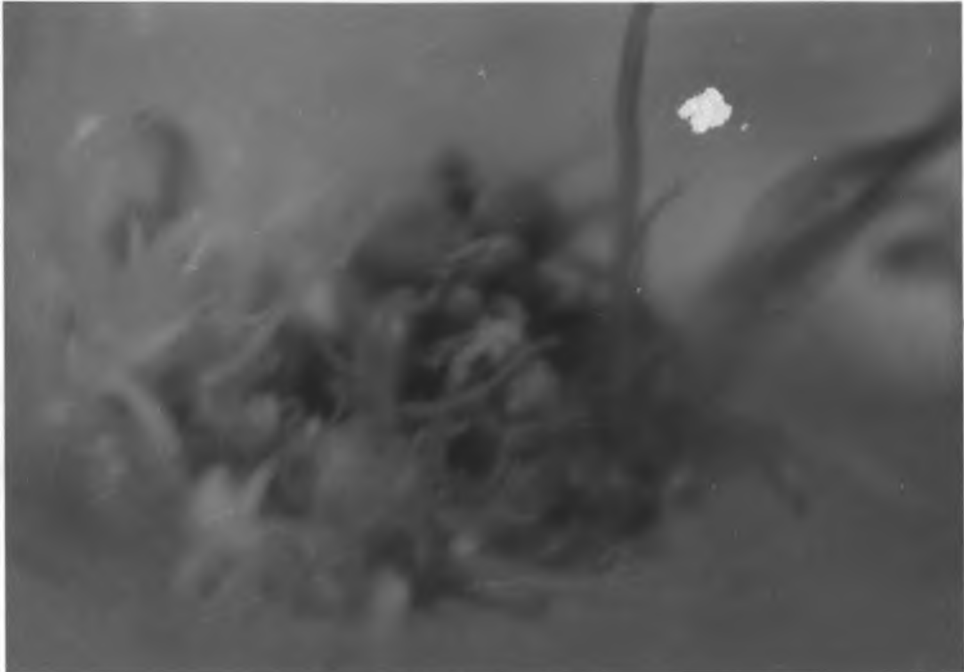

Plate 12. The highly prolific clone CO 6415 showing chlorophyll mutants (albinos) arising from the same callus with normal green shoots.

Plate 13. Clone EAK 69-47 (9) three-month old somaclones showing morphological variation in tillering abilities and in erectness.

4.3.0. ESTABLISHMENT OF THE PLANTLETS INTO THE SOIL.

Sugar cane plants were successfully established in the soil in the greenhouse within a period of five months. Sixty to seventy percent of the plantlets transfered into the soil survived. The plantlets grew to a plant height of over 50 cm in this period and developed extensive tillering.

As long as the shoots and the roots were well established, plantlets could be transferred into a medium containing white pellets and soil in equal proportions and covered with plastic containers for upto seven days. After a month, establishment was fully achieved and within three months, morphological differences could be observed in the young plants (Plate 13).

4.4.0. SUGAR CANE MICROPROPAGATION ANALYSIS.

Table 4.4 illustrates the mean number of regenerants obtained from every sugar cane callus. It also indicates the approximate number of plantlets from each somaclone in a multiplication medium after five weeks in culture. As observed earlier, the most prolific clones in callus induction are also the most prolific clones in plantlet regeneration and multiplication. The clones CO 6415 and EAK 69-47 had the highest number of plantlets per callus (32 & 31 respectively) and are also significantly different from other clones at P(0.05) but not from each other. The rest of the clones were not significantly different from each other. Clone CO 945, which was a less prolific clone, regenerated 21 plantlets per callus on average.

Clone EAK 69-47 individual somaclones regenerated a mean number of 16 plantlets in a multiplication medium (MS supplemented with 2.0mg/l BAP). This was significantly higher than all the clones. The average number of plantlets obtained per clone, after five weeks in culture, in a decreasing order were EAK 69-47 (16), CO 6415 (12), CO 331 (11), CO 617 (11), EAK 71-402 (10), EAK 71-183 (10) and CO 945 (9).

Table 4.4. The mean number of plantlets obtained per callus and the mean number of plantlets obtained five weeks after individual somaclones are cultured in a multiplication medium.

CLONE	No. of regenerants/callus	No. of plantlets/regenerant
CO 6415	31.6 a	12.2 b
CO 617	25.4 b	10.6 bc
CO 331	26.4 ab	11.0 bc
EAK 71-183	23.4 b	10.0 bc
EAK 71-402	26.6 ab	10.2 bc
EAK 69-47	30.8 a	15.6 a
CO 945	20..8 b	9.4 c

The values followed by a common letter in each column do not differ significantly at p = 0.05 based on Duncan's Multiple Range Test (DMRT) with five replicates.

4.4.1. *Estimation of Plantlets from each sugar cane shoot.*

An average six-eight week-old young sugar cane shoot can give at least six callus inducing explants. Each of the calli will give 25 young plantlets in culture. The total number of obtainable plantlets from each shoot can be calculated as follows:

Number of plantlets = No. of calli x No. plantlets/callus

= 6 x 25

= 150

Considering that 20 % of these get contaminated during subculturing, then the number of plantlets available for transplanting into the soil is at least 120. With an allowance of 70 % survival rate in the field, still 85 plants get established within three months. With an extra month for multiplication *in vitro*, each plantlet will give rise to ten more and this results in at least 850 sugar cane plants in a four-month period. This is on the lowest side as each callus can be divided and cultured to give more regenerants.

4.5.0. CHARACTERIZATION OF *IN VITRO* REGENERATED CANE CLONES.

Porosity gradient polyacrylamide gel electrophoresis (PoroPAGE) with gradient of 10 - 28 % polyacrylamide was found sufficient to give good results. The enzyme systems examined included peroxidases and esterases which were found useful in determining not only the interclonal variation but also somaclonal variations.

4.5.1. Esterases.

In all the *in vitro* regenerated plantlets, regardless of the stage of development, eight activity bands named A_7 - A_{13} and A_8 were common. A maximum of 18 bands were identified using this enzyme system (see Table 4.5). Numbers were used to designate these bands, the first ones being the least mobile and the last ones being those with higher mobilities. The bands existed in three main polymorphic zones. The middle zone had dark staining bands which constituted the common bands for all the clones assayed. The slowest migrating multiple band region had the anodic bands A_1 to A_6. Clone CO 6415 had the anodic bands A_1 to A_4 but lacked A_5 and A_6 which is a contrast of clone CO 331 that had the latter and lacked the former bands. Clones CO 617 and EAK 69-47 had none but A_4 band in this zone hence could not be differentiated in this region. Clones EAK 71-402 and CO 945 had no band in this region whereas clone EAK 71-183 had only one band A_6.

In the middle zone of polymorphic activity, all the clones had dark staining similar bands. Band A_{11} was thick and dark in all the phenotypes except clone CO 331. Only two phenotypes were identified in this region. In the last zone, all the clones had the fastest monomorphic band A_{18} in common. This region, though made of faint bands identified virtually all the clones assayed.

A comparison of esterase isozymes between plantlets in culture and established plantlets showed some considerable diversity. Generally, the established plantlets had more and dark stained bands than the plantlets in culture (Plate A). There was also some repositioning of some bands especially in the first zone of slow migrating anodic bands. Once established, the number of bands were then constant but only for the increased band intensity in older plants.

Esterase bands for plantlets arising from multiplication of individual somaclones did not show any variations at all apart from some degree of variation in stain intensity. All the bands indicated equal migrating distances (Plate B). On somaclonal variants, it was possible to detect upto 13 % somaclonal variation in the most prolific callus forming clones (see Tables 4.7 & 4.8).

Table 4.5. A comparison of esterase zymogram in regenerated sugar cane clones, two months after transplanting.

Dis.(cm)	4.2	4.5	4.8	6.0	6.2	6.4	6.6	6.8	7.0	7.1	7.3	7.5	7.7	8.1	8.3	8.6	9.0	9.2.
Number.	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9	A_{10}	A_{11}	A_{12}	A_{13}	A_{14}	A_{15}	A_{16}	A_{17}	A_{18}
CO 6415	MN	MN	MN	MN	-	-	DN	DN	DN	DN	DT	DN	DN	FN	FN	-	-	MN
CO 617	-	-	-	MN	-	-	DN	DN	DN	DN	DT	DN	DN	-	MN	MN	MN	MN
CO 331	-	-	-	-	MN	MN	DN	DN	DN	DN	DN	DN	DN	-	-	-	MN	MN
EAK 71-183	-	-	-	-	-	MN	DN	DN	DN	DN	DT	DN	DN	FN	-	-	MN	MN
EAK 71-402	-	-	-	-	-	-	DN	DN	DN	DN	DT	DN	DN	MN	-	MN	-	MN
EAK 69-47	-	-	-	MN	-	-	DN	DN	DN	DN	DT	DN	DN	-	-	-	-	MN
CO 945	-	-	-	-	-	-	DN	DN	DN	DN	DT	DN	DN	MN	-	-	FN	MN

NOTE: M - Medium staining band; F - Faint band; D - Dark staining band.
N - Normal thickness band (0.8 mm); T - Thick band (1.2 -1.5 mm)

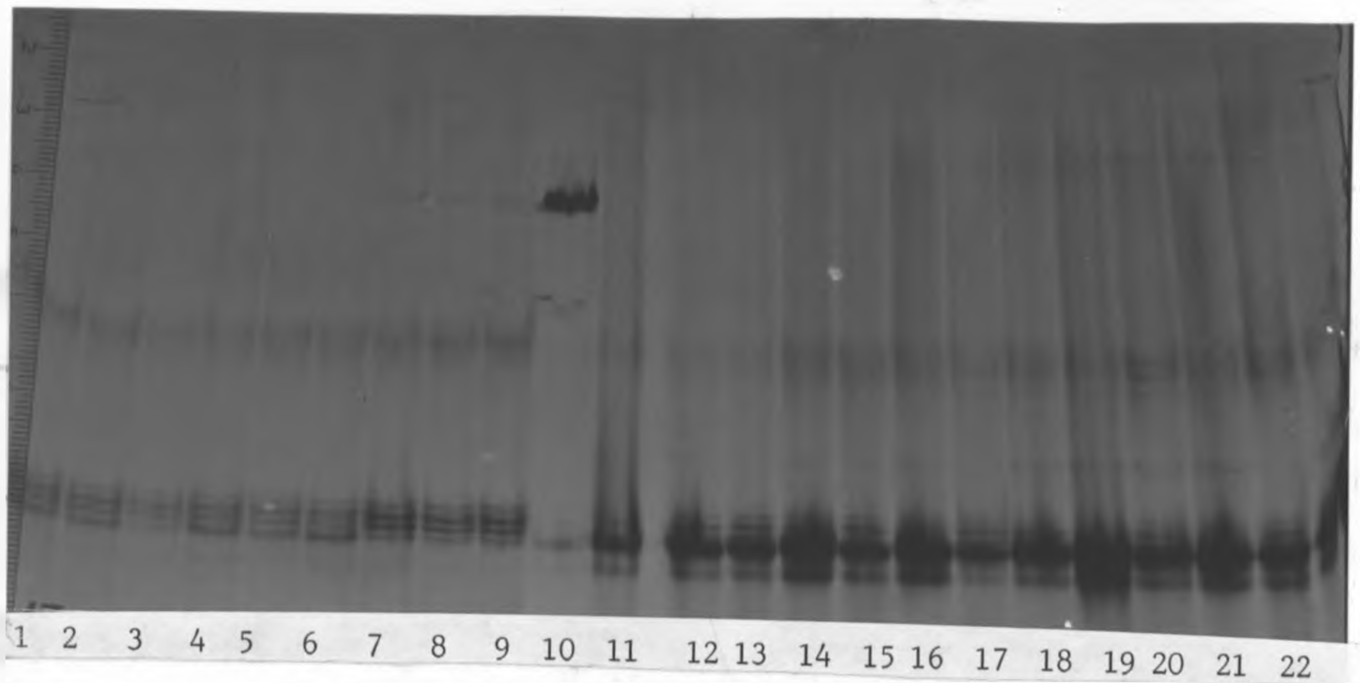

Plate A. A comparison of esterase isozymes in regenerated somaclones of CO 6415. 1-9 are of plantlets before transplanting; 10 is an albino and 11-22 are from two-month old plants after transplanting into soil.

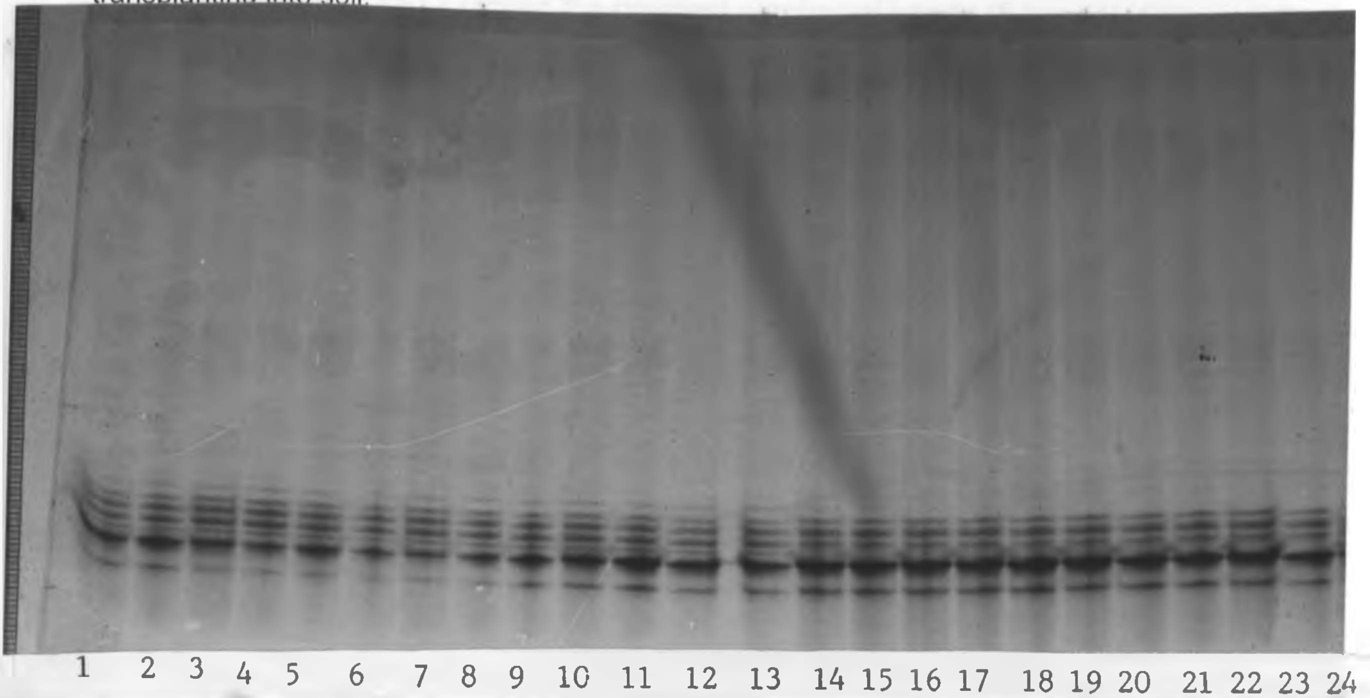

Plate B. Esterase isozymes detected in plantlets obtained from multiplication of individual somaclones. 1-13 and 24 are clone CO 331 somaclones and 14-23 are clone EAK 71-402 somaclones.

4.5.2. Peroxidases.

It was possible to detect upto 18 % somaclonal variation using peroxidases alone (See Table 4.8). In most cases, the plants which showed variation in esterases also showed differences in peroxidases.

A total of 24 activity bands could be identified and were numbered from the least mobile to the fastest as A_1 - A_{24}. Table 4.6 illustrates the variation in the peroxidase banding for the clones studied (also Plate C). The peroxidase banding could be effectively divided into two major zones. The first zone of slow migrating bands was polymorphic region which was effectively used to differentiate all the seven phenotypes studied. Like esterases, none of the clones had all the bands present. Within this zones the bands were designated A_1 to A_{12}. Clones CO 6415, EAK 71-402 and EAK 71-183 each lacked seven bands in this region, but at different loci. Clone CO 617 lacked the greatest number of bands (8 bands). Clones CO 331 and EAK 69-47 both lacked six bands whereas clone CO 945 lacked only five bands.

In the second zone of banding, basically all the clones had 12 bands in common. These region of activity portrayed double banded polymorphic zone. The bands occurred in pairs starting from a dark stained band A_{12} and ending with the fastest migrating of faint stained bands A_{23} and A_{24}.

Somaclonal variants occurred as either those having additional bands or those lacking some bands. In clone CO 331, for example, the somaclonal variants each had a band missing. The variants 7, 9, 12 and 14 had bands A_8, A_{14}, A_8 and A_{18}, respectively missing (Table 4.7 and Plate D).

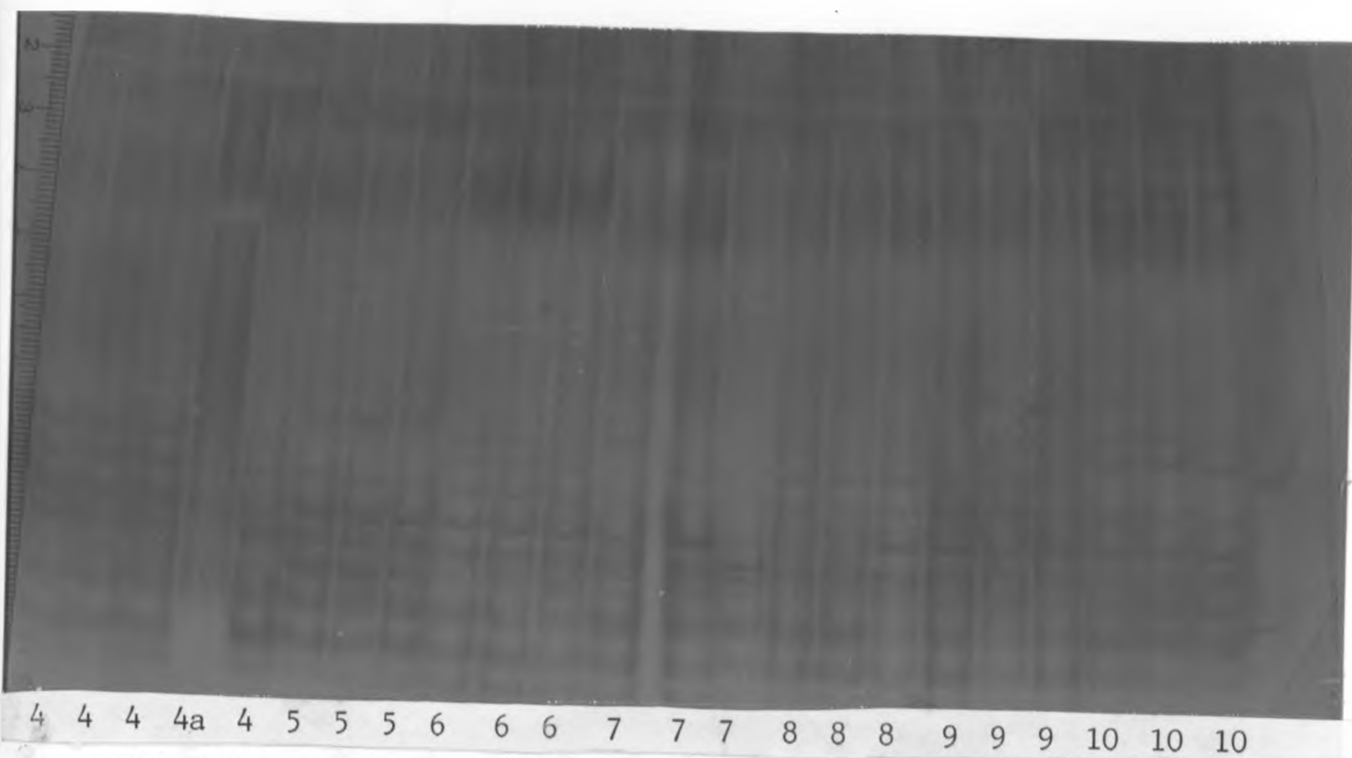

Plate C. A comparison of peroxidase isozymes as detected in regenerants before transplanting into the greenhouse. 4a is the albino plant.

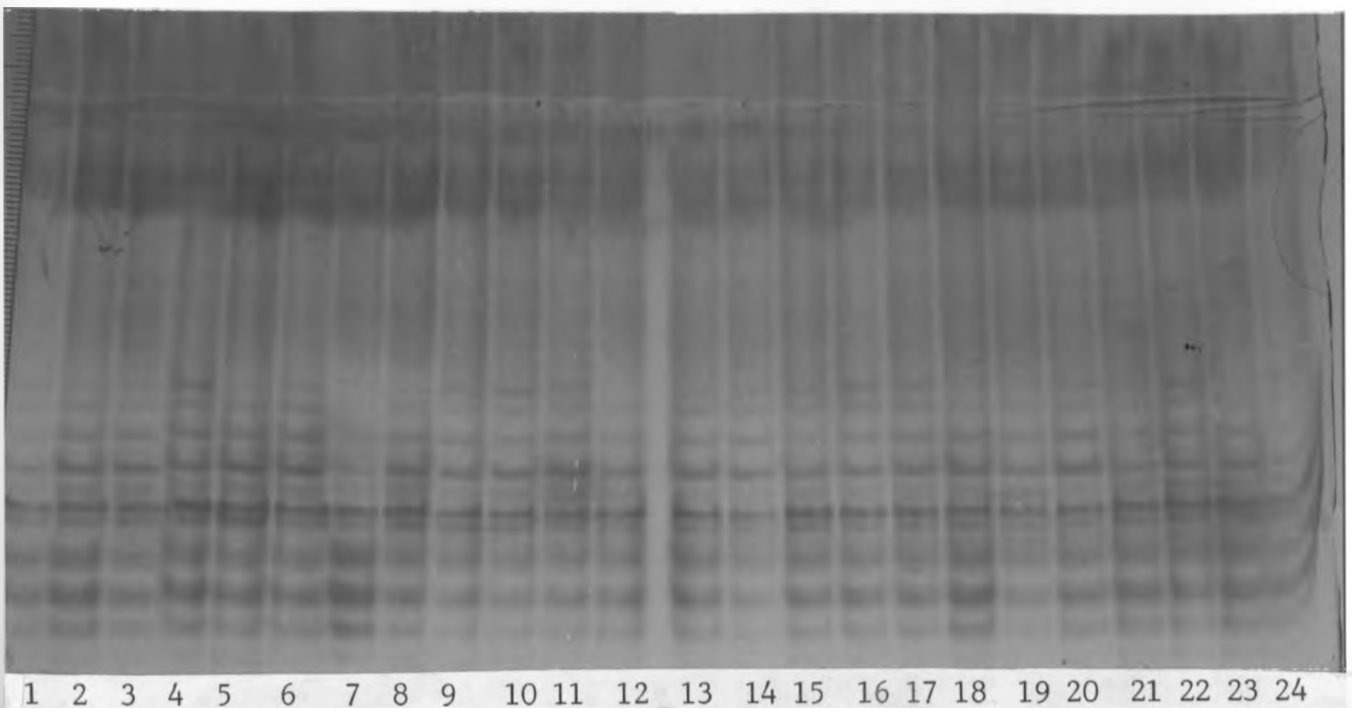

Plate D. A comparison of peroxidase isozymes as detected in 2-month old regenerants of clone 6 (CO 331).

Table 4.6. A comparison of peroxidase zymograms in regenerated sugar cane clones, two months after transplanting.

Band Dist. (cm).	1.0	1.4	1.8	2.4	2.7	4.8	5.6	6.0	6.2	6.4	6.8	6.9	7.5	7.8	8.0	8.2	8.4	8.6	8.9	9.1	9.5	9.7	10.0	10.2
Band Number.	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9	A_{10}	A_{11}	A_{12}	A_{13}	A_{14}	A_{15}	A_{16}	A_{17}	A_{18}	A_{19}	A_{20}	A_{21}	A_{22}	A_{23}	A_{24}
CLONES\																								
CO 6415	–	–	MN	MN	–	–	–	MN	–	MN	–	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
CO 617	–	–	MN	MN	–	–	–	MN	–	–	–	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
CO 331	–	–	MN	MN	–	–	–	MN	MN	MN	–	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
EAK 71-183	–	–	MN	MN	MN	–	–	MN	–	MN	–	–	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
EAK 71-402	–	–	MN	MN	–	–	–	MN	–	–	MM	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
EAK 69-47	–	MN	MN	–	–	MN	--	MN	–	MN	--	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
CO 945	MN	MN	MN	MN	–	–	MN	MN	–	–	–	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN

NOTE: D - Dark staining bands. M - Medium staining bands. F - Faint staining bands.
N - Normal thickness bands (0.8 mm). -- Missing bands.

Table 4.7. A comparison of clone 6 (CO 331) peroxidase zymogram as observed in 2-month old regenerants.

Band dist. (cm).	1.80	2.40	6.00	6.20	6.40	6.90	7.50	7.80	8.00	8.20	8.40	8.60	8.90	9.10	9.50	9.70	10.00	10.20
Band number.	A_1	A_1	A_8	A_9	A_{10}	A_{12}	A_{13}	A_{14}	A_{15}	A_{16}	A_{17}	A_{18}	A_{19}	A_{20}	A_{21}	A_{22}	A_{23}	A_{24}
1	MN	MN	MN	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
2	MN	MN	MN	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
3	MN	MN	MN	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
4	MN	MN	MN	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
5	MN	MN	MN	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
6	MN	MN	MN	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
7	MN	MN	--	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
8	MN	MN	MN	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
9	MN	MN	MN	MN	MN	DN	DN	--	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
10	MN	MN	MN	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
11	MN	MN	MN	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
12	MN	MN	--	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
13	MN	MN	MN	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
14	MN	MN	MN	MN	MN	DN	DN	MN	MN	MN	MN	--	MN	MN	MN	MN	FN	FN
15	MN	MN	MN	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
16	MN	MN	MN	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
17	MN	MN	MN	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
19	MN	MN	MN	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
20	MN	MN	MN	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
21	MN	MN	MN	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
22	MN	MN	MN	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
23	MN	MN	MN	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN
24	MN	MN	MN	MN	MN	DN	DN	MN	MN	MN	MN	MN	MN	MN	MN	MN	FN	FN

NOTE: M - Medium staining band; F - Faint staining band
N - Normal thickness band; -- Missing band.

4.6. SUMMARY RESULTS OF ISOZYME ANALYSIS.

Table 4.8 illustrates the variation obtained from the analyses of somaclones. Both isozymes in combination could detect 22 % somaclonal variants, with peroxidases being the most useful in detecting the variants. Clone EAK 69-47 had the greatest percentage (22.72 %) of plants being variants, whereas Clone CO 617 had a smaller percentage (12.50 %) of variants. The others were CO 6415 (21.62), EAK 71-402 (20.83) and CO 331 (16.67).

Relationship between biochemical variants and morphological variants in erectness and tillering was not clear. Some somaclones had similar isozyme patterns but were quite diverse in morphology, notable among them was clone EAK 69-47 (see Plate 13).

Table 4.8. Summary of isozyme analysis indicating the extend of somaclonal variation within the regenerants.

CLONE	No.of Plants	VARIANTS(Esterase)		VARIANTS(Peroxidase)		TOTAL.	
	studied	No.	%	No.	%	No.	%
CO 6415	37	5	13.51	7	18.92	8	21.62
CO 617	24	1	4.16	3	12.50	3	12.50
CO 331	24	1	4.16	4	16.67	4	16.67
EAK 71-402	24	2	8.33	4	16.67	5	20.83
EAK 69-47	22	2	9.09	4	18.18	5	22.72

Note: Plants that were found to be variants by both isozymes were counted only once.

5.0. DISCUSSION.

5.1.0. *IN VITRO* SUGAR CANE REGENERATION.

Plant tissue culture technology is an important recent technology now available to plant breeders for plant genetic improvement. In our study this technique was found useful in creation and propagation of variants. The production of agriculturally useful variants will have an impact on sugar cane improvement program in Kenya. The results are discussed below.

5.1.1. Effect of 2,4-D regulators on callus induction.

Callus induction and development was influenced by both the presence and concentration of 2,4-D. Generally, for all the clones analysed, the response to auxin concentration was similar. A low auxin concentration could still induce callus formation but of less amount. The most optimum callus initiation was obtained from an auxin concentration of 3.00 ppm. At this level, the greatest values of calli fresh weights and diameters were obtained. These two parameters showed an expected positive proportionality in their relationship. Above 3.00 ppm of 2,4-D the callus induced was not as great as at this level. The absence of significant differences among one-week cultures was because 2,4-D had not yielded significant weight increase. No callus was induced in media without 2,4-D. However, subculturing explants from an auxin-free medium to one with 3.00 mg/l led to callus induction. This illustrated that the meristematic activity was not affected by the medium without 2,4-D even after one month.

The presence of 2,4-D in MS medium was found to be essential for callus induction. Similar observation was made by Kuehnle and Sugii (1991) who induced callus in Hawaiian

Anthuriums by supplementing the medium with 0.36 mM of 2,4-D and 4.4 mM of BAP. Al-Khayri *et al.* (1991) also induced callus in spinach leaf explants from a medium supplemented with 0.5 mg/l 2,4-D. In sugar cane, an optimum level of 2,4-D obtained in this study agreed with what other researchers have used (Heinz *et al.*, 1971; Liu *et al.*, 1974 and Liu, 1984). They also found that higher levels of the auxin resulted in reduced or no callus induction at all. This could be due to the fact that herbicidal effects of 2,4-D become paramount as higher concentrations are used. Different plants have different optimum levels of 2,4-D requirements for callus induction. Reynolds and Murashige (1979), for example, reported using 100 mg/l 2,4-D in their medium for date palm. In general, however, concentration of any single hormone used in tissue culture is less than 10.0 mg/l (Conger, 1986).

Effect of tissue type and orientation on callus initiation. As observed in our results, young shoot tips gave the most prolific calli. The physiological status of the explant was of prime importance. Explants from the youngest innermost portions of the shoot tip formed the most prolific calli. Thus portions of the same explant behaved differently in culture. This can be attributed to the high meristematic activity of the young shoot tips. Powell (1976) had also noted that the basal portions of an apple stem is more dormant than the upper portions and produced different responses *in vitro*. Feucht (1975) had earlier reported that the cortex and cambium from stem explants of *Prunus* showed different growth rates as a function of hormones. Explants of adult forest trees have also been reported to be slower than rejuvenated tissues to commence growth *in vitro* (Lal and Lal, 1990). Regeneration potential of tree species diminishes with each plant maturation (Cheng, 1975).

Joarder *et al.*(1986) also noted similar results in Italian rye grass. They found that the ability of the leaf tissue to form callus in culture depended on the position of the explant and

the age of the leaf. The callusing frequency of the sectors also declined with increasing age of the leaf. The most suitable explants for tissue cultures are those in which there is a large proportion of either meristematic tissue present or cells which retain the ability to express totipotency.

Tissue orientation on culture medium showed positive effects on callus induction. A normally oriented shoot tip explant, as if on the donor plant, generally formed a bigger callus. This resulted from an initial upward expansion of the tissue followed by callus induction in the whole expanded tissue especially in the regions in contact with the medium. This may imply that the endogenous hormones in the explant induced it to expand initially. However, the exogenous auxin from the medium once absorbed by the tissue inhibited expansion and induced callusing. No work has been reported on this behaviour so far but Liu *et al.* (1972) also observed that callus mass readily formed on the portions of explant not immersed into the medium. This was also ascertained in this work.

5.1.2. Organogenesis (Shoot initiation).

Organogenesis was initiated with different hormonal composition. In sugar cane, shoot initiation (caulogenesis) occurred in MS medium supplemented with 10 % coconut water. This resulted in appearance of green dots which developed into leaf primordia and gradually became shoots. The mechanism through which this occurs is a complex process. Torrey (1966) advanced the hypothesis that organogenesis in callus starts with the formation of clusters of meristematic cells (meristemoids) capable of responding to factors within the system to produce a primordium. Depending on the nature of internal factors, the stimuli can either initiate a root, a shoot or an embryoid. The factors that regulate the origin of these meristematic zones are not well understood (Dodds and Roberts, 1985), though the fact that these zones are located at the tissue - medium interface has led to the suggestion that physiological gradients of substances diffusing from the medium into the tissue may play a role in determining the sites at which meristemoids are formed (Ross et al., 1973).

Boxus (1974) also found out that strawberry shoots were formed when callus was explanted into a medium supplemented with 1.0 mg/l BAP. Hendre *et al.* (1983) established *in vitro* shoot multiplication of sugar cane in a medium containing Kinetin and BAP. The results were quite similar with those obtained in this study though only BAP was used for shoot multiplication in the study. Plantlet regeneration and shoot multiplication was achieved in Hawaiian anthuriums when MS medium was supplemented with BAP (Kuehnle and Sugii, 1991).

5.1.3. Organogenesis (Root induction).

Rhizogenesis was initiated by subculturing into a medium with 2.00 mg/l IBA. This led to extensive root formation within two weeks and by the fourth week, the roots were developed enough to withstand hardening off effects during transplanting. Similarly, Al-Khayri *et al.* (1991) induced roots in spinach plantlets in a medium with IBA. Abbot and Whiteley (1976) had also succesfully rooted upto 80 % of their apple cultures by dipping the young shoots in 1.0 mg/l IBA for 15 minutes. Other hormones have also been found to initiate rhizogenesis. An interaction exists between clones and hormones in rooting. GA_3 for instance, was reported by Button and Bornman (1971a) to give enhanced rooting of 'Washington Navel' orange *in vitro*, but Putz (1971) noted that the same hormone inhibits rooting in raspberry. Charturvedi and Mitra (1974) reported an interaction of hormone and shoot growth vigour. They found that low vigour shoots rooted with 0.1 mg/l NAA while high vigour shoots required either 0.5 mg/l NAA or 0.25 mg/l IBA.

Control of oxidative browning. Oxidative enzymatic browning occurs extensively during the subculturing of sugar cane tissues. It is evident that sugar cane contains relatively high concentrations of phenolic compounds which cause extensive tissue necrosis during culture transfers. Polyphenoloxidases stimulated by tissue injury will oxidize these phenolic substances to growth inhibiting dark - brown compounds which lead to tissue necrosis and eventually death (Dodds and Roberts, 1985). Hu and Wang (1983) listed the various control methods for such browning as: adding antioxidants to the medium; presoaking the explants in antioxidant solutions prior to culture; subculturing to fresh medium and providing little or no light at the initial period of culture.

In sugar cane culture, it was possible to control such enzymatic browning by

incorporating 0.05 % activated charcoal into the medium. It was also observed that apart from the effective control of browning, the charcoal also led to an earlier and extensive rhizogenesis in the rooting media. Thus by suppressing the oxidation, it promoted effective hormone activation and hence root formation could be noticed even after only 2 -3 days after subculturing.

Similar results for control of browning has been reported in *Strelitzia reginae*, Kiwifruit, blackberry and somatic hybrid of sugar cane and pearl millet (Ziv and Halevy, 1983; Messina *et al.*, 1991, Fernardez and Clark, 1991 and Tabaeizadeh *et al.*, 1986). Chee (1991) reduced browning in *Cucumis melo* with 0.1 % activated charcoal in the regeneration medium. In blackberry, Fernardez and Clark (1991) found out that plants cultured in MS without the charcoal formed only shoots but no roots. The results are similar to those observed in this study. Earlier, Liu et al (1972) were able to control browning by subculturing to fresh medium with 10 times the calcium ion concentration.

5.2.0. PROPAGATION OF SELECTED VARIANTS.

Micropropagation of sugar cane results in production of a large number of plants with the economy of both space and time. Over 300 plants/month can be obtained from the protocol developed in this work. Similar results were also obtained by Liu (1971) when he raised 1,650 plants from 26 clones and established 2,700 plants in the field by the end of one year. Other researchers elsewhere were able to establish over 4,000 plants in the field from 58 clones within two years (Liu *et al.*, 1974). They also etablished that a production scale of 200-300 plants/month was possible (Liu *et al.*, 1972). These results are quite similar to those obtained in this study, though the method of regeneration varied on the type and concentration of

growth regulators used.

In vitro plant regeneration through callus culture may also give rise to a high degree of variation which can be a useful tool in selection of more suitable clones. The breeder's other most important objective after creation and selection of required traits is to be able to multiply the new clones without further genetic changes in the traits selected for. Whereas the earlier researchers did not fully address this fact, a way was devised in this study to circumvent the problem. Subculturing of individual somaclones in a multiplication medium (Refer to Appendix 4) gave rise to numerous plantlets which showed no variation in isoenzymes (Plate B) as well as in the morphological traits. This gives a breeder an avenue of multiplying the clones without much problems of unexpected somaclonal variation. It has also been reported that apical buds cultured on MS medium containing 0.2 mg/l BAP and 0.1 mg/l Kinetin developed direct shoots (Taylor and Dukic, 1993). Plants developed in such a manner will have minimal somaclonal variation (if any at all) as compared to raising plantlets through the callus stage. It is therefore not only possible to use *in vitro* techniques to create variability in sugar cane but also to use the same techniques for micropropagation.

In an effective and continuous propagation system it is possible to transplant over 10,000 plantlets into the field anually using this technique. The incoporation of this technigue into our sugar cane breeding program in Kenya will therefore solve the persistent problem of low seedling production. Apart from this *en-masse* production of seedlings, the technique is also useful in disease preclusion and future regeneration of genetically engineered plants.

5.3.0. ISOZYME ANALYSIS.

Esterases and peroxidases were found to be sufficient in detecting both the interclonal and somaclonal variations in sugar cane. Similar results were also reported by Feldman (1985) who used 18 isozymes to identify 140 clones and found out that only esterases and peroxidases were necessary to identify all the clones. Because they are direct product of genes, isozymes are particularly useful as genetic markers. Zheng (1989) reported that isozyme analysis could provide evidence for classification and form a basis for decision of the parents used before crossing in sugar cane.

5.3.1. Esterases.

Esterase zymogram showed many distinct bands that identified all the clones studied. The slowest migrating anodic bands (A_1 to A_6) could be used to distinguish all but two of the seven phenotypes assayed. The middle zone of polymorphic activity identified only two phenotypes whereas the last zone of fast migrating faint bands identified virtually all the phenotypes. The use of esterase isozymes to detect clonal variation has been found to be successful by various workers (Liu *et al.*, 1974; Zheng, 1987; Glaszmann et al., 1989; Nagai *et al.*, 1991). Unlike in maize in which three distinct loci have been identified for esterase isozymes (Swartz, 1960), no specific locus has been associated with the banding patterns in sugar cane. Glaszmann *et al.* (1990) reported that several types of zymograms could be distinguished and appeared interpretable as resulting from the dimerization of multiple protomers present in variable proportions. The seven bands A_7 to A_{13} identified in this study in the middle zone may indicate the control by specific genes contained in all the

clones studied. The loci controlling the bands could be linked and hence indicated less variations. The loci or group of loci are active and constant in the young plantlets in culture as well as in established plants in the greenhouse. The rest of the bands in the other zones were highly polymorphic, illustrating the fact that sugar cane is truly a polyploid. These were used to differentiate all the clones studied.

Somaclonal variants observed in esterase bandings mainly occurred in the other bands other than the basic bands. The observed albino plants as well as the chimeral plants indicated lack of these basic bands. The albino plants did not show A_7, A_{12}, and A_{13} bands while the chimeral plant similarly did not show A_7 and A_{12} bands among others. This could imply a mutation occurred and led to breakage of the possible existent linkage. Whereas esterase isozymes detected less somaclonal variants, it is however a useful tool for detecting the interclonal variation and hence the identification of the sugar cane clones.

5.3.2. Peroxidases.

Peroxidase isozymes showed the most diverse activity bands and extensive polymorphism. It was not only possible to identify the various clones (Plate C), but it was also possible to detect the somaclonal variants arising *in vitro* (see Table 4.7). This variation was also reported by several researchers working on various sugar cane clones (Gonzalez *et al.*, 1986; Barreto and Simon, 1982; Nagai *et al.*, 1991 and Glaszmann *et al.*, 1989). Zheng (1987) identified and correlated peroxidase bands with morphological characters. He found out that a certain zone was significantly positively correlated with stalk/clump number and negatively with the stem diameter. Zymograms of the other isozymes did not have any clear correlation with the agronomic characters studied.

It was also possible to observe variation in the number of bands between plantlets in culture and those already established in the greenhouse. This would indicate the triggering of genomic information when the young plantlets are subjected to hardening off conditions. Thus, a new set of genes are activated in the greenhouse plants and this activation does not vary at different growth periods of the established plants; atleast upto 4-month old plants studied. This observation was also reported in isozymes of chloroplasts between callus stage and in developed plants (Dviwedi *et al.*, 1985). Abbot *et al.* (1984) had also showed that some esterase isozymes present in anther before microspore formation decreased in concentration during microspore development and new isozymes appeared in the microspore around the pollen grain at mitosis I. These esterases were specified by nuclear genes and there was variation between genotypes. In this work, the albino plants lacked a good number of the basic bands (Plate D) and could not survive in the greenhouse while the chimeral plants became weakling plants during hardening off stage. This was a negative genetic change as it resulted in a genetic dead end of the albino plants.

5.4.0. SOMACLONAL VARIATION IN SUGAR CANE.

The term somaclones refers to plants derived from any form of cell or tissue culture and somaclonal variation is the variation displayed amongst such plants (Larkin and Scowcroft, 1981). In the current study, somaclonal variation was observed both in morphology and isozyme patterns. Albino plants were the most easily observed morphological variants (see Plate 12) and also the chimeral plants. The albino plants were an unfortunate genetic dead end as they could not be estalished in the greenhouse. Among the established somaclones, variation was noticed in stooling, tillering and erectness (see Plate 13). Isozyme analysis revealed upto 20 % variation in both peroxidases and esterases. This was in contrast to Zawazaki *et al.* (1989) who found out that variations only existed between clones and not somaclones using similar enzyme systems. Heinz and Mee (1971) reported variations in peroxidase isozymes of upto 62 %. Liu *et al.* (1976) also reported variation in erectness and stooling among somaclones, and also 5 % variation in isozymes. They also reported karyological variants among regenerants.

It can however be concluded that variation will vary from clone to clone and also the method used to analyse the variants. Liu *et al.* (1974) also attributed this to differences in behavic ur of the clones used or to the method by which the bands were observed. These variants form an important source of variation especially for asexually propagated plants like sugar cane. It is thus evident that creation of useful genetic variability can be obtained through a callus mass.

Little relationship has been reported between biochemical and morphological variants (Heinz and Mee, 1971; Liu *et al.*, 1975; Zheng, 1987). A great deal of progress has all the same been achieved through the use of *in vitro* techniques. A high cane yield (32 %), sugar

yield (34 %) and stalk number (6 %) than the donor parent has been reported (Liu, 1984). Krishnamurthi and Tlaskal (1974) reported resistance to Fiji disease among sugar cane somaclones and found that 8.9 % of the somaclones were highly resistant or nearly immune, yet the clones were indistinguishable from donor plants in gross morphology. Application of similar techniques would be advantageous to the current cane breeding program in Kenya especially for the control of such diseases as smuts and cane mosaic viruses.

Somaclonal variation in other crops has also been reported. Skirvin and Janick (1976b) developed an *in vitro* technique to regenerate plantlets from scented geraniums. They found out that one of the resultant calliclones "Velvet Rose" was genotypically different from the rest and was released as a new cultivar. Smith and Noris (1983) noted that the direct formation of adventitious buds from leaf buds of African Violets *in vitro* resulted in the maintenance of the plant chimera pattern.

5.5.0. CONCLUSIONS AND RECOMMENDATIONS.

From the results obtained from this study, it can be concluded that practical and potential values of sugar cane tissue culture have been achieved for both micropropagation and the development of useful variants subject to selection for required traits. The study shows the development of a practical protocol for micropropagation of the commercially produced sugar cane clones in Kenya. The use of isozyme studies was also found to be an effective means of clonal and somaclonal identification of the cane clones. It is suggested that these two approaches can complement the conventional approach of cane breeding and hence result in a more effective sugar cane improvement programme in Kenya.

The recommendations arising from this study that will be a basis for further research in

sugar cane breeding include the following:

1. A further study is required for the establishment of the relationship between biochemical variants *in vitro* and the phenotypic variations in established plants in the field for those characters of agricultural importance.

2. The use of these variants as a source of resistance to the cane pathological problems facing the sugar cane production in Kenya.

3. A comparison on the somaclonal variation of plants resulting from callus culture and those resulting from direct regeneration from shoot tip culture can also be looked into as relates sugar cane micropropagation.

4. A further study on the use of shoot tip culture to avoid callus stage is also required as it could lead to faster micropropagation with minimal variation.

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7.0. APPENDICES.

7.1. Appendix 1.

AREA HARVESTED, CANE DELIVERED AND YIELDS BY FACTORY ZONES 1983 - 1992

FACTORY	ITEM	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992
CHEMELIL	Area harvested (ha)	7,687	7,335	7,638	8,297	8,507	7,324	9,150	9,603	10,241	7,366
	Cane delivered (TC)	544,581	572,522	549,973	561,272	618,194	669,969	754,992	796,336	718,067	481,578
	Yields (TC/ha)	70.8	78.1	72.0	67.6	72.7	85.6	69.1	68.3	65.11	65.38
MUHORONI	Area harvested (ha)	4,816	5,547	5,625	5,866	6,391	5,391	6,347	5,884	7,045	3,873
	Cane delivered (TC)	392,096	490,022	461,103	427,794	428,764	411,811	531,151	494,044	447,255	211,956
	Yields (TC/ha)	81.4	88.3	82.0	72.9	67.1	76.4	64.8	66.2	63.49	54.63
MUMIAS	Area harvested (ha)	17,999	17,719	20,128	19,490	18,101	18,330	17,558	17,127	17,757	20,694
	Cane delivered (TC)	1,336,150	1,479,353	1,508,419	1,556,037	1,628,425	1,822,566	1,870,920	1,836,768	1,839,872	1,870,474
	Yields (TC/ha)	74.2	83.5	75.0	79.9	90.0	99.4	106.5	107.3	103.61	90.38
NZOIA	Area harvested (ha)	3,391	5,574	4,373	3,837	3,294	4,380	4,223	3,516	5,846	6,772
	Cane delivered (TC)	211,273	307,619	307,291	362,958	366,905	483,166	497,210	383,749	506,725	449,249
	Yields (TC/ha)	62.3	55.2	70.0	94.6	111.4	110.2	117.7	109.1	86.68	66.34
SONY	Area harvested (ha)	3,836	5,149	3,414	4,053	4,377	4,038	3,631	4,366	5,043	6,325
	Cane delivered (TC)	198,484	344,391	163,941	250,877	297,147	350,941	358,150	461,290	475,600	479,199
	Yields (TC/ha)	72.6	66.9	48.0	61.9	67.9	86.9	98.6	105.7	94.31	75.76
MIWANI	Area harvested (ha)	5,429	6,574	6,375	4,690	5,906	1,386	-	-	1,949	2,592
	Cane delivered (TC)	309,465	263,132	289,235	186,763	273,219	66,266	-	-	125,767	130,707
	Yields (TC/ha)	56.9	40.0	43.0	39.8	46.6	47.8	-	-	64.53	49.20
RAMISI	Area harvested (ha)	6,020	4,608	4,335	5,460	3,891	1,275	-	-	-	-
	Cane delivered (TC)	154,859	879,827	128,984	162,719	103,824	30,519	-	-	-	-
	Yields (TC/ha)	25.7	19.5	30.0	29.8	26.7	24.0	-	-	-	-
ALL FACTORIES	Area harvested (ha)	49,178	52,506	51,888	51,693	50,467	42,621	40,909	40,496	47,881	47,622
	Cane delivered (TC)	3,146,908	3,548,866	3,408,947	3,508,420	3,716,478	3,835,238	4,012,423	3,972,187	4,212,986	3,623,163
	Yields (TC/ha)	63.99	67.99	65.70	67.87	72.40	84.21	92.14	92.16	87.99	75.87

NB: Some factories received non-contracted cane, mainly from neighbouring factory zones, as follows:-

	1987	1988	1989	1990	1991	1992
Chemelil	62,573	193,194	122,993	135,390	39,337	-
Muhoroni	-	52,883	120,027	104,621	-	3,373
Miwani	-	-	-	-	-	3,192
Sony	-	-	-	-	-	3,556

Miwani and Ramisi were closed indefinitely in April, 1988. Miwani re-opened in July, 1991.

7.2. Appedix 2. SUGAR PRODUCTION, CONSUMPTION, IMPORTS AND EXPORTS

1983 - 1992

(TONNES)

YEAR	PRODUCTION	CONSUMPTION	IMPORTS	EXPORTS
1983	326,329	332,973	NIL	3,880
1984	372,114	348,678	4,000	4,001
1985	345,641	373,890	33,000	NIL
1986	365,796	381,394	142,500	NIL
1987	413,248	400,700	11,500	NIL
1988	411,296	462,207	42,000	NIL
1989	441,261	489,544	80,000	NIL
1990	431,836	537,999	64,050	NIL
1991	433,713	493,945	21,288	NIL
1992	371,225	552,000	124,463	NIL

NB:

Sugar consumption and imports for 1992 are provisional estimates.

7.3. Appendix 3.

(a). ANOVA of calli fresh weights of 10 clones treated with two levels of 2,4-D. Measurements were carried out after 8 weeks of cultivation

SOURCE	DF	SS	MS	F
Blocks	3	2528.37	842.80	1.95ns
Treatments	1	292270.96	292270.95	676. 50**
Error (a)	3	1296.10	432.03	
Clones	9	1633800.68	181533.41	492.21**
Clones X Treat.	9	118363.59	13151.51	35.66**
Error (b)	051	18809.57	368.81	
Total	76	2067069.27		

** means values are significant at p=0.01.

CV (a) = 3.3 % ; CV (b) = 3.05 % ; S.E = 3.29.

Appendix 3 continued.

(b). ANOVA of calli diameters of 10 clones treated with two levels of 2.4-D and measured after 8 weeks of cultivation.

SOURCE	DF	SS	MS	F.
Blocks	3	1.2219	0.4073	1.47ns
Treatments	1	142.3767	142.3767	512.36**
Error (a)	3	0.8337	0.2779	
Clones	9	706.9246	78.5472	521.97**
Clones X Treat.	9	64.0735	7.1193	47.31**
Error (b)	51	7.6747	0.1505	
Total	76	923.1050		

** Means values are significant at p = 0.001

CV (a) = 3.52 % ; CV (b) = 3.22 % ; S.E. = 0.08.

7.4. Appendix 4.

(a). ANOVA of calli fresh weights (mg) of six clones treated with four conc. levels of 2,4-D as observed oftervone week of cultivation.

SOURCE	DF	SS	MS	F
Blocks	4	167.62	41.90	0.94ns
Treatments	3	283.96	94.65	2.11ns
Error (a).	12	537.25	44.77	
Clones	5	417.07	83.41	3.15*
CLones X Treat.	15	262.89	17.53	0.66ns
Error (b)	80	2119.53	26.49	
Total	119	3788.32		

CV (a) = 16.35 % ; CV (b) = 12.58 % ; S. E. = 1.22

Appendix 4 continued.

(b). ANOVA of calli fresh weights of six clones treated with 4 levels of 2.4-D as observed after 2 weeks of cultivation.

SOURCE	DF	SS	MS	F
Blocks	4	129.45	32.36	0.50ns
Treatments	3	13988.89	4662.96	72.00**
Error (a)	12	777.15	64.76	
Clones	5	2148.34	429.67	9.63**
Clones X Treat.	15	890.76	59.38	1.33ns
Error (b)	80	3569.40	44.62	
Total	119	21503.99		

CV (a) = 13.0 % ; CV (b) = 10,79 % ; S. E. = 1.47.

Appendix 4 continued.

(c). ANOVA of calli fresh weights of six clones treated with 4 levels of 2,4-D as observed after 3 weeks of cultivation

SOURCE	DF	SS	MS	F
Blocks	4	543.28	135.82	2.06ns
Treatments	3	113781.13	37927.04	574.08**
Error (a)	12	729.78	66.06	
Clones	5	28298.50	5659.70	49.80**
Clones X Treat.	15	6598.77	439.92	3.87**
Error (b)	80	9092.73	113.66	
Total	119	159107.20		

CV (a) = 8.02 % ; CV (b) = 10.52 % ; S. E. = 1.48.

7.5. Appendix 5.

(a). ANOVA of calli Fresh weights of six clones treated with 4 levels of 2,4-D as observed after 4 weeks of cultivation.

SOUCRE	DF	SS	MS	F.
Blocks	4	1978.38	494.59	2.31ns
Treatments	3	653174.83	217724.94	1018.17**
Error (a)	12	2566.08	213.84	
Clones	5	301213.70	60242.74	214.36**
Clones X Treat.	15	67341.16	4489.41	15.97**
Error (b)	80	22483.13	281.04	
Total	119	1048757.28		

CV (a) = 8.07 % ; CV (b) = 9.25 % ; S. E. = 2.67

Appendix 5 continued.

(b). ANOVA of calli fresh weights as noted after five weeks of cultivation in callus inducing medium.

SOURCE	DF	SS	MS	F
Blocks	4	1886.93	471.73	0.85ns
Treatments	3	2326859.26	775619.75	1392.61**
Error (a)	12	6683.46	556.95	
Clones	5	561278.84	112255.77	262.78**
Clones X Treat.	15	120749.12	8049.94	18.84**
Error (b)	80	33748.04	427.19	
Total	119	3051205.65		

CV (a) = 7.88 % : CV (b) = 6.90 % ; S. E. = 4.31 .

Appendix 5 continued.

(c). ANOVA of calli fresh weights of six clones as observed after six weeks of cultivation in callus inducing medium.

SOURCE	DF	SS	MS	F
Blocks	4	2197.59	549.40	0.79ns
Treatments	3	4677664.80	1559221.60	2255.09**
Error (a)	12	8297.08	691.42	
Clones	5	786467.86	157293.57	387.79**
Clones X Treat.	15	220558.02	14703.86	36.25**
Error (b)	80	29610.29	405.62	
Total	119	5724795.64		

CV (a) = 6.62 % ; CV (b) = 5.07 % ; S. E. = 4.80.

7.6. Appendix 6.

A modified MS medium that was found most appropriate for *in vitro* regeneration of the commercial sugarcane clones in Kenya.

COMPOUND (NUTRIENT)	AMOUNTS (in mg/L) REQUIRED FOR: Callus	Caulogenesis	Multiplication	Rhyzogenesis
A: Macronutrients.				
NH_4NO_3	1650	1650	1650	1650
KNO_3	1900	1900	1900	1900
$CaCl_2.2H_2O$	440	440	440	440
$MgSO_4.7H_2O$	370	370	370	370
KH_2PO_4	170	170	170	170
B: Iron Source.				
Na_2EDTA	37.35	37.35	37.35	37.35
$FeSO_4.7H_2O$	27.85	27.85	27.85	27.85
C: Micronutrients.				
$MnSO_4.4H_2O$	22.3	22.3	22.3	22.3
$ZnSO_4.7H_2O$.	8.6	8.6	8.6	8.6
H_3BO_3	6.2	6.2	6.2	6.2
KI	0.83	0.83	0.83	0.83
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025	0.025	0.025
D: Vitamins.				
Nicotinic acid	0.5	0.5	0.5	0.5
Pyrodoxine.Hcl	0.5	0.5	0.5	0.5
Thiamine.Hcl	0.1	0.1	0.1	0.1
E: Hormones.				
2,4-D	3.0	0.0	0.0	0.0
BAP	0.0	0.0	2.0	0.0
IBA	0.0	0.0	0.0	2.0
Coconut milk	0.0 ml	150.0 ml	0.0 ml	0.0 ml
F: Others.				
Glycine	2.0	2.0	2.0	2.0
Sucrose	30.0g	30.0g	30.0g	30.0g
Agar	10.0g	10.0g	10.0g	10.0g
Myo-inositol	100.0	100.00	100.0	100.0
Active charcoal	0.0	0.0	5.0g	5.0g
pH	5.7-5.8	5.7-5.8	5.7-5.8	5.7-5.8

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