# EVALUATION OF 2, 4-DICHLOROPHENOXY ACETIC ACID AND NAPHTHALENE ACETIC ACID CONCENTRATION ON CALLOGENESIS, SOMACLONAL VARIATION AND SUGARCANE MOSAIC VIRUS ELIMINATION IN SUGARCANE (*Saccharum officinarum (L)*).

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**Evaluation Of 2, 4-Dichlorophenoxy Acetic Acid and Naphthalene Acetic Acid Concentration On Callogenesis, Somaclonal Variation and Sugarcane Mosaic Virus Elimination in Sugarcane** (*Saccharum Officinarum L*).

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**A thesis submitted in partial fulfillment for degree of Doctor of Philosophy in Biotechnology in the Jomo Kenyatta University of Agriculture and Technology**

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## DECLARATION

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

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## DEDICATION

To my wife and children for perseverance and believing in me, to my late mother Concephiter for her desire to take her children to school, to my sister Florence and her husband Philip for their enthusiastic support, to my late brother Patrick and his wife Ruth for their unrelenting encouragement and support and to all those who have contributed something consciously for the Betterment of humanity

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## TABLE OF CONTENT

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF PLATES

## APPENDICES

## ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| **AMOVA** | Analysis of molecular variance |
| **ANOVA** | Analysis of Variance |
| **2, 4-D** | 2, 4 – Dichlorophenoxy Acetic Acid |
| **BAP** | Benzyl Amino Purine |
| **Bp** | Base pair |
| **CFM** | Callus Formation Media |
| **CTAB** | Cetyl Trimethyl Ammonium Bromide |
| **CV** | Coefficient of variation |
| **DAP** | Di-ammonium Phosphate |
| **EDTA** | Ethylene Diamine Tetra Acetic Acid |
| **FAO** | Food and Agriculture Organization |
| **FAOSTAT** | Food and Agriculture Organization Statistics |
| **HCL** | Hydrochloric Acid |
| **IAA** | Indole Acetic Acid |
| **IBA** | Indole Butyric Acid |
| **IBR** | Institute for Biotechnology Research |
| **ISSR** | Inter Simple Sequence Repeat |
| **JKUAT** | Jomo Kenyatta University of Agriculture and Technology |
| **KESREF** | Kenya Sugar Research Foundation |
| **MMUST** | Masinde Muliro University of Science and Technology |
| **NAA** | Naphthalene Acetic Acid |
| **PCR** | Polymerase Chain Reaction |
| **RAPD** | Rapid Amplification Polymorphism DNA |
| **RFLP** | Restriction Fragment Length Polymorphism |
| **RNA** | Ribose Nucleic Acid |
| **RT-PCR** | Reverse Transcription PCR |
| **SCMV** | Sugarcane Mosaic Virus |
| **TC/H** | Tons Cane/Hectare |
| **TC/TS** | Tons Cane/Tons Sugar |

## ABSTRACT

Sugarcane, *Saccharum officinarum (L.),* is one of the most important crops in Kenya and has wide range of economic benefits. However, the industry has been facing several challenges including declining yields due to use of poor quality planting materials. The prolonged seed multiplication process of newly released varieties and local environmental conditions that do not favour production of flowers are some of the other major constraints to sugarcane production in Kenya. *In vitro* culture offers a practical and rapid method for mass propagation of disease-free clonal materials. Further, unintended variations observed during *in vitro* callus regeneration have proved quite promising in sugarcane improvement programs. The study therefore aimed at establishing the effect of 2, 4-D and NAA concentrations on the callogenesis, somaclonal variation and disease response of three varieties of sugarcane, namely; CO421, CO945 and N14.

The effects of 2, 4-D and NAA concentrations on *callus* induction and shoot regeneration in three sugarcane varieties; CO945, CO421 and N14 were investigated in the study. Young leaf spindle explants were cultured on MS basal medium supplemented with 2, 4-D (0.0, 2.0, 2.5, 3.0, 3.5 and 4.0 mg $L^{-1}$) and NAA (0.0 and 1.0 mg $L^{-1}$). Experiments were laid out in the laboratory in completely randomized design replicated three times. Observations were recorded on percent *callus* formation, percent shoot formation and morphological characterization of *callus*. Data was subjected to ANOVA at 5% level of significance. Significantly higher *callus* production (93%) was observed at 3 mg $L^{-1}$2, 4-D. The presence of NAA tended to depress *callus*ing and shoot production in sugarcane variety N14. The interaction between NAA and 2, 4-D had no significance on the parameters. Application of 2, 4-D at between 2.0 and 3.5 mg $L^{-1}$ gave the highest % *callus* formation and shoot formation in all sugarcane varieties. Application of 2.5 and 3.5 mg $L^{-1}$ 2, 4-D was most effective for sugarcane callogenesis and regeneration for the three sugarcane varieties. NAA treatment in *Callus* formation media was not beneficial.

The study reported the SCMV indexation of *in vitro* regenerated plants by infectivity assay method in sugarcane. The plants were developed by organogenesis through callus. For infectivity test sap of *in vitro* regenerated plants was inoculated on Sorghum one month after

hardening. All the SCMV indexed plants were grown in green house and monitored for mosaic symptoms at weekly intervals. All 36 treatments showed some levels SCMV infection. *In vitro* regeneration of sugarcane through callus induction of young leaf spindles did not eliminate SCMV and was not therefore recommended for the multiplication of viral disease free sugarcane planting materials.

Callogenesis is one of the tools in sugarcane tissue culture for generating agronomically significant variation. Tissue culture derived variations are known as somaclonal variation. The study was carried out to investigate the effect of MS media supplemented with various concentrations of NAA and 2, 4-D on somaclonal variation in sugarcane genotype CO421, CO945 and N14. Screening of somaclonal variants was done on four months old field grown *in vitro* culture generated plants. The treatments were laid out in split-split plot design. The morphological characters studied included; tillering capacity, diameter of the cane, internode length, leaf length and width. Analysis of variance was done using GenStat version 17.0 and pair wise comparison of means of phenotypic traits of all somaclones computed by calculating fisher's least significant difference (LSD) at $P \leq 0.05$. Pearson's correlation between all phenotypic traits and Turkey test of the selected clones were computed. Multivariate analysis of variance (MANOVA) done reveal the patterns of phenotypic diversity of quantitative traits studied. Means of each quantitative character were standardized before subjecting to the principal component analysis (PCA). The standardized data of 5 quantitative traits were then used as an input for the PCA biplot loading and cluster analysis. An agglomerative, hierarchical cluster classification technique with Average linkage strategy was performed. The results of the analysis of variance for the differences in morphological traits indicated that genotype, 2, 4-D and the various interactions had significant effect on the various morphological traits. Application of 2, 4-D to CFM led to somaclonal variation irrespective of the sugarcane genotype used. The observed variation however had no correlation to the hormonal concentration supplemented in the CFM. The dendrogram demonstrated variation among the somaclones based on morphological traits, could be a valuable source for sugarcane improvement program.

Application of 2.5 and 3.5 mg $L^{-1}$ 2, 4-D was most effective for sugarcane callogenesis and regeneration for the three sugarcane varieties. *In vitro* regeneration of young leaf spindles of

sugarcane through callus induction at various concentrations of 2, 4-D and NAA did not eliminate SCMV and was not therefore recommended for the multiplication of viral disease free sugarcane planting materials. Application of 2, 4-D to CFM led to somaclonal variation irrespective of the sugarcane genotype used. The observed variation however had no correlation to the hormonal concentration in callus formation media. The study therefore established that 2, 4-D concentration have an effect callogenesis, somaclonal variation and disease response of three varieties of sugarcane, namely; CO421, CO945 and N14 and could be useful in rapid multiplication of sugarcane planting materials and generation of useful variations for breeding programs.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Origin and distribution of sugarcane

The origin of *Saccharum officinarum (L. 2n=80 to 105) is* intimately associated with the activities of humans, as it is a purely cultivated or garden species which is not found in the wild (Sreenivasan *et al*., 1987). The center of origin of *S. officinarum* is thought to be in the Indonesia/New Guinea area (Daniels & Roach, 1987) where it has been grown as a garden crop since 8000 B.C. (Fauconnier, 1993). Its cultivation spread along the human migration routes to Southeast Asia, India and the Pacific, hybridizing with wild canes. It reached the Mediterranean around 500 B.C. (Fauconnier, 1993). From there it spread to Morocco, Egypt, Syria, Crete, Greece and Sicily, the main producers until the 15$^{th}$ Century, followed by introduction to West Africa and subsequently Central and South America and the West Indies (Fauconnier, 1993).

The Sugarcane plant has a very long history in Central and East Africa, having been known in the 12$^{th}$ Century on the East African coast (McMartin, 1961). Sugar cane was first introduced in Kenya in 1902 with the first sugar factory being set up at Miwani near Kisumu in 1922 and later in 1927, at Ramisi in the coast province, the area where the current Kwale International Sugar Company is located (Jamoza, 2016).

## 1.2 Taxonomy

Sugarcane belongs to the genus *Saccharum* L., traditionally placed in the grass family. Taxonomically, sugarcane belongs to the major grass family, *Poaceae*, sub-family Panicoideae, super tribe *Andropogoneae*, sub-tribe *Saccharineae* and genus *Saccharum* (Watson *et al.,* 1985). This tribe includes tropical and subtropical grasses and the cereal genera *Sorghum* and *Zea* (known as maize or corn). The tribe is further divided into groups, with sugarcane in the *Saccharinae* Benth.

The taxonomy and phylogeny of sugarcane is complicated as plants from five genera share common characteristics and form a closely related interbreeding group known as the '*Saccharum* complex'. The *Saccharum* complex comprises *Saccharum*; Erianthus section *Ripidium, Miscanthus* section *Diandra*, *Narenga* and *Sclerostachya* (Daniels & Roach 1987). These genera are characterized by high levels of polyploidy and frequently unbalanced numbers of chromosomes (aneuploidy) making it difficult to determine taxonomy and resulting in many revisions of the taxonomic relationships (Daniels & Roach 1987; Sreenivasan *et al*., 1987). More recent molecular analysis of the genera in the *Saccharum* complex has led to suggestions that the taxonomy should be rearranged as many of the divisions appear to be polyphyletic (Hodkinson *et al*., 2002).

As shown in Table 1.1, the genus *Saccharum* traditionally comprises six species: *S. spontaneum, S. officinarum, S. robustum, S. edule, S. barberi,* and *S. sinense* (D'Hont *et al*., 1998). However, Irvine (1999) suggested that the genus should be reduced to just two species, grouping together *S. robustum, S. edule, S. barberi, S. sinense* and *S. officinarum* as the species *S. officinarum (Plate 1-1(a))* and leaving *S. spontaneum* (Plate 1.1(b)) as a separate species.

*Saccharum officinarum (L.)* was named by Linnaeus in 1752 in *Species Plantarum* (Daniels & Roach, 1987). The word *Saccharum* is thought to have been derived from the Sanskrit 'sharkara' (Ritter 1841 as cited in Daniels and Roach, 1987). It is also known by the common name of noble cane. Sugarcane is thought to have resulted from complex introgression between *S. spontaneum, Erianthus arundinaceus* and *Miscanthus sinensis* (Daniels & Roach, 1987) although some data support it originated from *S. robustum* (Amalraj & Balasundaram, 2006). *Saccharum officinarum (L.)* has a chromosome number of 2n=80, with a basic chromosome number of ten, making this species octaploid (having eight pairs of each chromosome).


Plate1.1: *Saccharun officinarum L. (a) and Saccharum spontenum* L. (b) *plants Source*: (Daniels, 1987)

However, *S. officinarum* is not a simple polyploid, as it is both an autopolyploid (more than two sets of homologous chromosomes derived from a single species) & also an allopolyploid (possessing two or more unlike sets of chromosomes) (Sreenivasan *et al.*, 1987).

Table 1.1: Members of genus *Saccharum*

| **Species** | **Description** | **Sugar content** | **Chromosome number** |
|---|---|---|---|
| *S. spontaneum* L. | Wild species | Very low – low | 2n=40–128 |
| *S. robustum* Brandes | Wild species | Very low | 2n=60–200 |
| *S. officinarum* L. | Noble canes | High | 2n=80 |
| *S. barberi* Jeswiet | Ancient hybrid | Low | 2n=111–120 |
| *S. sinense* Roxb. | Ancient hybrid | Low | 2n=80–124 |
| *S. edule* Hassk. | Cultivated species | Low. Compacted inflorescence, eaten as a vegetable | 2n=60–80 with aneuploid forms |

*Source:* Purseglove 1979; Daniels & Roach 1987

*Saccharum officinarum (L.)* has chromosomes in common with both of the genera *Miscanthus* and *Erianthus* (Besse *et al*., 1997a; Daniels & Roach 1987), although molecular data has suggested that this is due to common ancestry, rather than any direct involvement of these genera in more recent introgression (Besse *et al*., 1997a; Grivet *et al*., 2004).

### 1.3 Botanical characteristics

Sugarcane is one of the most efficient photosynthesizer, a C-4 plant in plant kingdom and commercially propagated through stems cuttings (Yadav *et al*., 2012). *Saccharum* consists of six species Wild: *S. spontaneum* L. and *S.robustum*, Cultivated: *S. officinarum* L; *S. barberi; S. sinense* and *S. edule* (Daniels & Roach, 1987). The four cultivated species are complicated hybrids and all intercross readily.

Sugarcane (*Saccharum officinarum (L.)*) is a tall growing, monocotyledonous, tropical, perennial grass, belonging to the genus *Saccharum* (Purseglove, 1974). It's a major sucrose accumulator and biomass producer and is one of the most important field crops grown in the tropics and subtropics.

Sugarcane is a tall perennial tropical grass that tillers at the base to produce unbranched stems, 3-4 m or more tall and about 5 cm in diameter. The basic structure of the sugarcane is closely related to that of other members of the order *Gramineae*, of which it is a giant member (Purseglove, 1974).

The solid unbranched stem, roughly circular or oval in cross section, is clearly differentiated into joints, each comprising a node and an internode. Generally the nodes are placed at an interval of 15-25 cm, but are much closer at the top of the stem, where elongation is taking place, than at the bottom, where they form part of the rootstock and are essential to the formation of tillers (Purseglove, 1974). Sugar accumulates in the stems (canes), internodes vary in length (5-25 cm), girth (1.5-6 cm in diameter), shape (cylindrical, barrel or bobbin and circular or oval in cross-section), colour (yellow, green, red, purple, black, striped, variegated) and hardness according to the variety and growing conditions.

The leaves of sugarcane are attached to the stem at the bases, alternately in two rows on opposite side of the stem. Each leaf consists of two parts - a sheath and a blade. The leaf has a strong midrib, white and concave on the upper surface, convex and green below. Two types of root system develop shortly after a sett has been planted: those from primordial of the cutting, which are thin and branched; and those from the primordial of the tillers that are thick, fleshy and much less branched. At first the newly planted seed piece depends entirely on its own roots for the uptake of water and nutrients. Later this function is taken over by the tillers, and sett roots die. Each shoot produces its own root system.

### 1.3.2 Sugarcane varieties and their Characteristics

Commercial cultivation of sugarcane (Saccharum spp. hybrids) in Kenya begun in the early 1900s in the Kibos area by Indian settlers, who used it to manufacture jaggery (Jamoza, 2005), and by the early 1920s production had spread to the lowland coast at Ramisi. Between the mid-1960s and early 1980s, sugarcane production expanded to the Western and Nyanza provinces.

Early efforts to identify improved sugarcane varieties for the Kenyan sugar industry involved the importation and testing of varieties for adaptation to local conditions and possible production (Jamoza, 2005). This led to the commercialization of varieties such as CO 421, CO 617 and CO 331 in the 1950s and 1960s and more recently, CO 945, CO 1148, CB 38-22 and N14 were recommended for commercial production. The major commercial varieties in Kenya, Co 617, Co 421, N14 and Co 945 occupy 7.5, 15.4, 27.6 and 32.3% of the sugarcane acreage in Kenya, respectively. Crossing or hybridization is conducted at KESREF's Sugarcane Breeding Centre, situated at Mtwapa (3º56'S, 39º44'E and 15 m above sea level) near Mombasa on the Kenyan coast, where flowering occurs under natural conditions (Jamoza, 2005).

## 1.4 Ecology of sugarcane

Sugarcane, as a crop, is affected by many biotic and a biotic factors which ultimately influence productivity per unit areas. Table 1.2 shows the suitable ranges of temperatures, sunshine, humidity, and elevation required for optimum cane development, from germination to stage of ripening.

Table 1.2: Optimal ecological conditions for growth of sugarcane

| NO. | Parameter | Level/type | Remarks |
|---|---|---|---|
| **1** | Water - rains | 1200 - 1500 mm | Sugarcane grows best in warm sunny, frost free weather. It needs fertile soils at least 1500 mm of rains annually supported by supplementary irrigation |
| **2** | Sunshine | 7 to 9 Hours | Sun loving plant , greater incident radiation favours sugar yields |
| **3** | Winds | 60 Km/hour | High winds exceeding 60Km/hour cause lodging |
| 4. Optimum temperature/Cane growth | | | |
| A. Germination | | 27 - 33 $C^o$ | Optimum |
| B. Tillering | | 26 - 32 $C^o$ | Optimum |
| C. Photosynthesis | | 24 - 30 $C^o$ | Optimum |
| D. Mobilization ripening | | 16 - 26 $C^o$ | Optimum |
| **5** | Soil PH | 6 - 8 pH | Optimum |
| **6** | Soil type | Sandy loam to clay loam best. Heavy clays with proper drainage and addition of organic matter. Saline/alkaline and acidic soils are not suitable for sugarcane. | Loamy soils are ideal for growing sugarcane |
| **7** | Elevation | 0 to 1700 m | |
| **8** | Altitude | 35° N 35° S | |

Source: kenani Engineering & Technical Services, 2013

### 1.4.1 Water

As shown in Table 1.2 sugarcane requires ample supply of 1200-1500 mm per annum of rainfall supplemented with irrigation for optimum performance (Purseglove, 1974). Sugarcane grows best in warm sunny, frost free weather. The duration of the rainy season is important in sugarcane growth. If excess water is not immediately drained at the sprouting stage, it will result in rotting of setts. On the other hand, if rainfall is insufficient during the season, supplementary irrigation becomes necessary to ensure effective development of stems. In this context, Water stress occurring in the plant during stem elongation severely reduces cane production.

### 1.4.2 Temperature and elevation

Sugarcane growing areas in Kenya are warm and conducive to maximum physiological activity throughout the season (Acland, 1989). Like many other tropical cereal grasses, optimum temperature for sugarcane is essential for effective germination of setts, tiller elongation, photosynthate mobilization and ripening (Table 1.2). According to Purseglove (1974), the optimum temperature for germination of sugarcane is 27-33 ℃ while good tiller production occurs when the temperature is about 30℃. A day temperature of below 18℃ lengthens the tillering period thus resulting in uneven maturity of the canes. Stalk elongation is linearly related to temperature with an optimum at 23℃. An air temperature range of 24-30℃ and a soil temperature of around 21℃ are optimum for photosynthesis in sugarcane. Sugarcane does not grow when temperatures fall below 15℃ or rise above 38℃ (Purseglove, 1974).

The adaptability of sugarcane to high temperature conditions is mainly associated with expansion rate of the leaf area. Maximum rates of leaf expansion are observed at 22 ℃ (Bull & Glasziou 1975). Slow growth in the establishing stage of sugarcane seedlings is one of the limiting factors to utilize this crop in warm temperate zone as in Japan, where the optimum range of temperature for juvenile growth stage was studied by Ehara *et al*. (1994). Their experiments concluded that 25℃-30℃ was

favourable to increase the rate of emergence of leaves, and that 20℃-25℃ was favourable to increase the number of tillers during the juvenile phase. Under cooler conditions the juvenile growth phase is prolonged but that in the tropics results in higher relative leaf area expansion rates during periods of high radiation exposure and may exceed 150t $ha^{-1}$.

### 1.4.3 Photoperiodism and solar radiation

Insolation in a sugarcane plantation is controlled not only by day length and humidity, but also by cloudiness of the sky (Purseglove, 1979). Solar radiation is particularly important in sugarcane because absorption of mineral nutrients from the soil is enhanced by the presence of light during the day time and absorption of water from the soil is equally dependent upon solar energy. It is imperative, therefore, that the greater the exposure of sugarcane to sunlight, the greater the yield. These ecological requirements of sugarcane make Kenya and many other tropical countries best suited for its production.

In sugarcane, close spacing increases yield per area where the growing season is short. When the growing season is long enough, proper plant arrangement in rows promotes optimum exposure to solar radiation and hence, greater production (Acland, 1989). Sugarcane flowers when the photoperiod is conducive. It is optimal to harvest sugarcane immediately before or after flowering to extract high quality sugar. If harvesting is long delayed after flowering, sugar quality would be greatly reduced (Acland, 1989). This happens mainly because the sugar stored in the stem tissues hydrolyze and is transported to the inflorescence to promote development.

### 1.4.4 Soil

Sugarcane is a heavy feeder crop. The soils in which it is grown should have optimum properties. In Kenya, sugarcane is grown mainly on loamy soils with good proportions of sand, silt and clay with good water storage and drainage characteristics (Kenani Engineering & Technical Services, 2013). The soils are fairly fertile, thus supplementary fertilizers are used only when necessary. A pH range of 6-

8 is considered optimal for sugarcane production (Table 1-2). Sandy soil is less favorable for sugarcane cultivation due to its poor chemical properties, but if supplied with adequate fertilizers under usage of drip irrigation can support excellent sugarcane growth.

## 1.5 Sugarcane growth and development

The crop is usually produced from stem cuttings called setts and each node has all the qualities for growing new plants (Basnet *et al*., 2007). The shoots grow from underground nodes, and the axillary buds at these nodes give rise to tillers. The number of tillers may vary from very few to a very large number, e.g. up to 144 per stool arising from one bud (Shamel, 1974). Sett roots supply the germinating bud with water until shoot roots are formed. Root proliferation becomes abundant when growth conditions are optimum. Growth of sugarcane varies depending on the cropping cycle of one year or two year cycle. Whereas numerous stalks may arise from a single sett, over 50% may die before nine months of growth when stable stalks are established (Anon, 2011). Sugarcane selection for improved clones normally focuses on effective tillering ability and rapid growth rate to maximize exposure to solar radiation.

## 1.6 Sugarcane Production and importance

### 1.6.1 Global Sugarcane Production

At present sugarcane is grown as a commercial crop primarily in South America, North/Central America, Asia, Africa, Australia and the Pacific islands. In 2013, FAOSTAT (2015) reported world production of sugarcane at an estimated 1,911 tons from which 1,686 million tons sugar was processed. The sugarcane was grown on approximately 26.9million ha (FAOSTAT, 2015).

As shown in Table 1.3 Brazil was the largest producer at 36 million tons of sugar globally in 2015, while in Africa the largest producer was South Africa with an estimated 2.2 million tons. Other countries which produce sugar in significant

quantities from sugar cane include India, Thailand, China, Cuba, Egypt, El Salvador, Peru and Myanmar (FAO, 2013).

Table 1.3: Top five sugar producing countries in the world and in Africa 2010-2015

| **COUNTRY** | Production ('000' MT) | | | | | |
|---|---|---|---|---|---|---|
| | **2010** | **2011** | **2012** | **2013** | **2014** | **2015** |
| BRAZIL | 36,400 | 38,350 | 36,150 | 38,600 | 37,800 | 35,950 |
| INDIA | 20,637 | 26,574 | 28,620 | 27,337 | 26,605 | 30,240 |
| THAILAND | 6,930 | 9,663 | 10,235 | 10,024 | 11,333 | 10,790 |
| CHINA | 10,336 | 11,246 | 11,246 | 12,822 | 13,452 | 10,200 |
| MEXICO | 5,115 | 5,495 | 5,351 | 7,393 | 6,382 | 6,344 |
| SOUTH AFRICA | 2,265 | 1,985 | 1,897 | 2,020 | 2,435 | 2,192 |
| EGYPT | 1,027 | 1069 | 1075 | 1080 | 917 | 917 |
| SUDAN | 625 | 750 | 750 | 760 | 700 | 705 |
| SWAZILAND | 658 | 602 | 670 | 681 | 676 | 705 |
| **KENYA** | **569** | **524** | **490** | **494** | **520** | **550** |

Source:http://www.indexmundi.com/agriculture/?country=zaandcommodity=centrifugal-sugarandgraph=cane-sugar-production. Retrieved on 3rd May 2016

### 1.6.2 Sugarcane Production in Kenya

Sugarcane performance depends largely on climatic and biophysical (soil and topographic) conditions, which vary significantly throughout Kenya. Sugarcane is mainly cultivated in four major production belts– the Nyando, Western, Nyanza and Coastal Belts (Appendix 12).

As shown in Table 1.4 sugarcane was cultivated on about 85, 000 Ha of land in 2013 and the average annual production was 5, 900, 000 MT of cane making Kenya the 27th highest producer in the world after Brazil and 4th in African FAOSTAT (2013).

As illustrated in the table, production has increased considerably over the past decade.

Table 1.4: Sugarcane production in Kenya (2004 – 2013)

| **YEAR** | AREA (HA) | CANE PRODUCTION (MT) |
|---|---|---|
| 2013 | 85000 | 5900000 |
| 2012 | 84916 | 5820000 |
| 2011 | 64091 | 5338562 |
| 2010 | 68738 | 5709586 |
| 2009 | 65774 | 5610702 |
| 2008 | 54465 | 5112000 |
| 2007 | 59201 | 5204214 |
| 2006 | 54621 | 4932839 |
| 2005 | 56537 | 4800820 |
| 2004 | 54191 | 4660995 |

SOURCE: http://faostat3.fao.org/home/index.htmlDOWNLOAD Accessed on 6th May 2016.

Trends suggest that increases in production in recent years have been more correlated with increases in total land planted with cane than with increase in yield, as they were in the past (Kenya Sugar Industry, 2009).

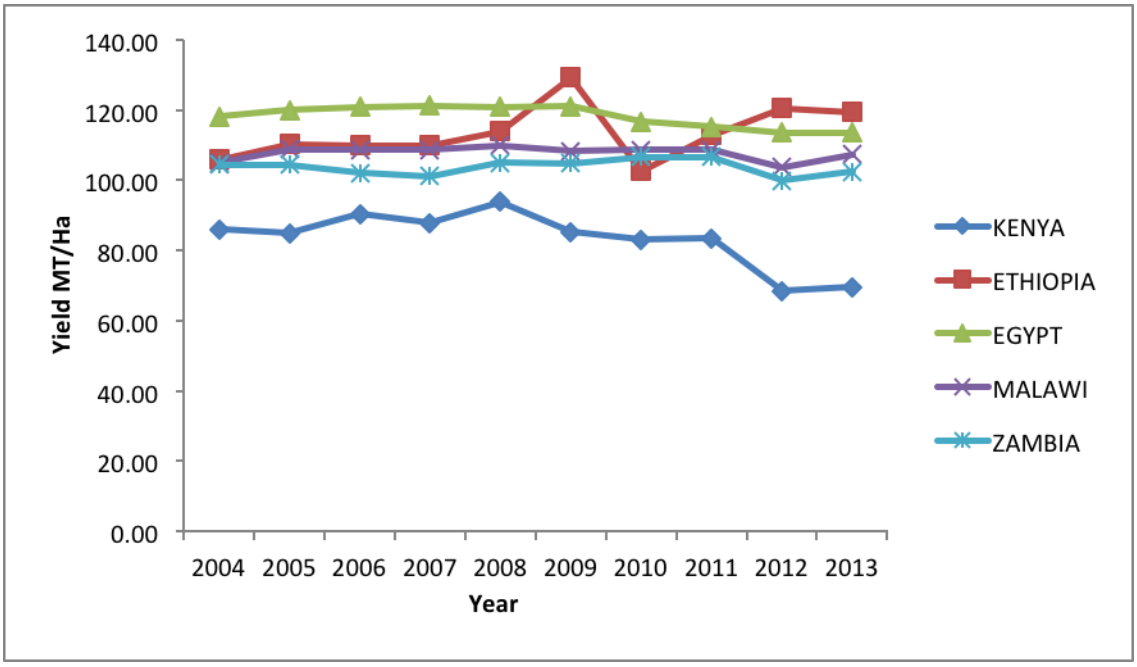


**Figure 1.1: Comparison of sugarcane productivity (MT/Ha) between Kenya and major producing countries in Africa**

SOURCE: http://faostat3.fao.org/home/index.htmlDOWNLOAD. Accessed on 6th May 2016

As shown in Figure 1.1 Ethiopia has the highest productivity in Africa and is ranked 2nd globally. Egypt, Malawi and Zambia are ranked 4th, 5th and 6th globally in productivity. Kenya is ranked 38th globally in productivity. Sugarcane productivity in Kenya has generally been on the decline in the last decade. According to Wolfgang and Owegi (2012), Kenya produces an average of 60 tons of sugarcane per hectare which is just about half of the productivity of Ethiopia (119 tons per ha), Zambia (115 tons per ha), Egypt (113 tons per ha) and Malawi (105 tons per ha).

In fact, output of sugarcane per hectare in the 2013 has seen a significant decline as compared to yields obtained in the 2004 (Figure 1.2). Potential reasons for this reduction in productivity include the widespread use of low quality sugarcane varieties, poor agricultural and land management practices and delayed harvesting of mature sugarcane (Kenya Sugar Board, 2009). However, on basis of average yield (MT/HA) Kenya is much lower in the world and even in Africa (Figure 1.2).

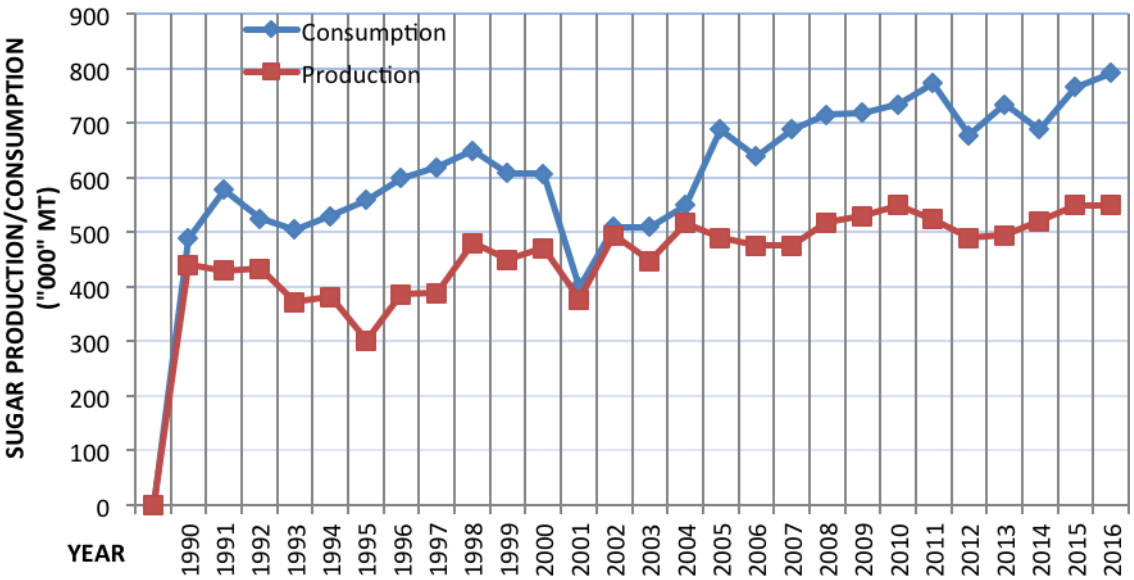


Figure 1.2: Kenya's sugar production and consumption ("000" MT) 1990-2016

**(***Source: World Bank estimates based on Kenya Sugar Board and Kenya National Bureau of Statistics data, 2016***)**

As illustrated in Figure 1.2, production has increased considerably since 1990, especially over the past two decade. Trends suggest that increases in production in recent years have been more correlated with increases in total land planted to cane

than with increases in yield, as they were in the past (Kenya Sugar Industry, 2009). In fact, output of sugarcane per hectare in the 2000s and 1990s has seen a significant decline compared to yields obtained in the 1980s. Reasons for this reduction in productivity include the widespread use of low quality sugarcane varieties, poor agricultural and land management practices, lack for incentives for the farmers and delayed harvesting of mature sugarcane (Kenya Sugar Board, 2010).

As reported by Kenani Engineering and Technical Services (2013), the productivity of sugarcane in Kenya experienced major deterioration in the past decade with the yield measured by the tonnage of cane produced for every hectare. Mumias and Nzoia experienced major hits each experiencing 58% and 36% declines in tons of Cane per Hectare respectively when compared to their ten year averages. In 2012, the Tons Cane/Tons Sugar (TC/TS) ratio was lowest in Butali sugar company (9.74TC/TS), and highest in Chemelil (18.41 TC/TS). This implies that Chemelil requires an additional 9 tons or 90% more cane in order to realize 1 ton of sugar (Kenya Sugar Board, 2012). The decline in yields and the low TC/TS ratio could be partly attributed to poor quality planting materials.

This was corroborated by Kenani Engineering and Technical Services (2013) in a feasibility study in which they identified a number of factors as the main reasons behind the low sugarcane yields in Kenya. These factors include weakness in the spreading of new high yielding sugarcane varieties and reliance by the farming system on old low yielding varieties and low quality seed cane material for plant crop establishment affecting the crop stand, vigor and the final yield among others;

### 1.6.3 Importance of Sugarcane

Sugarcane (*Saccharum officinarum (L.)*), the major source of sugar and alcohol, is a crop of prime importance due to its high agro-economic values (Nawaz *et al*., 2013). It is considered the world's most valuable crop and accounts for approximately 70% of the world's sugar (Tecson-Mendoza, 2000).

The sugar sub-sector plays a major role in the Kenyan economy and is a source of livelihood for millions of citizens (VAS Consultants., 2012). It plays a significant role in Kenya's economy, contributing about 15 percent to the country's agricultural Gross Domestic Product (Kenya Sugar Industry, 2009). It is one of the most important crops alongside tea, coffee, horticulture and maize (Kenya Sugar Industry, 2009). Currently, the industry directly supports approximately 250,000 small-scale farmers who supply over 92 per cent of the cane milled by the sugar companies. An estimated six million Kenyans derive their livelihoods from the industry. In 2008, the industry employed about 500,000 people directly or indirectly in the sugarcane business chain from production to consumption.

In addition, the industry saves Kenya in excess of KSh.19.3 billion in foreign exchange annually and contributes tax revenues to the exchequer (VAT, Corporate Tax, personal income taxes, cess). In the sugar belt zones, the sugar industry contributes to infrastructure development through road construction and maintenance; construction of bridges; and to social amenities such as education, health, sports and recreation facilities.

According to Hussain (1996) fresh sugarcane contains about 90% juice, 80% of this juice is water. 12-17% consists of sucrose. The major products and by-products of sugarcane (sucrose, bagasse and molasses) have varied and numerous applied uses. Bagasse is composed of cellulose, hemicelluloses and lignin (Jalaja, 2008). The bye products of the sugar industry are bagasse, molasses, filter cake, and waxes. The sugarcane industry provides raw materials for other industries such as bagasse for power co-generation and molasses for a wide range of industrial products such as ethanol. Molasses is also a key ingredient in the manufacturing of various industrial products such as beverages, confectionery and pharmaceuticals.

Fiber products primarily paper can be obtained from the pentosanes and plastics are potentially derived from the lignin. Molasses solids consist of 60% combined sucrose and inert sugars and about 13% inorganic salts. The principle product of sugarcane is sucrose, primarily a food but research has shown that this also can be used as a raw material for production of higher value products. Some natural pharmaceutical

compounds are derived from sugarcane (Menéndez *et al.,* 1994); additionally, agricultural and industrial by-products of the sugar production process are extensively employed for animal nutrition, food processing, paper manufacturing and fuel (Patrau, 1989). The sugarcane juice is used as antidote, antiseptic, antivenous, bactericide, cardiotonic, demulcent, diuretic, intoxicant, laxative, pectoral, pesticide, refrigerant and stomachic (Yadav *et al.,* 2012).

### 1.7 Statement of problem

Most of the sugarcane producers in Kenya and indeed in most producing countries in Africa are small scale and are confronted with problems of low yields due to poor quality seed cane and prevalence of pests and diseases among others (Jalaja *et al*, 2008). According to Kenya Sugar Research Foundation (2005), Sugarcane yields in Kenya have been on the decline from an average of 100 t $ha^{-1}$ in the early 1980's to 69.4 t $ha^{-1}$ in 2013.

Malik (1990); Aamir *et al*. (2008); Kenya Sugar Research Foundation (2009); and Kenani Engineering and Technical Services (2013) attributed yield decline to; susceptibility to diseases and insect pests; low quality seed cane for plant crop establishment and monoculture among others.

The normal way of propagation sugarcane is by vegetative means using nodal sections of sugarcane with 2 or 3 nodes pieces (referred to as setts). Vegetative methods results in less variability. However a particular variety grown year after year accumulates various disease pathogens (Jamoza, 2005). Cultivation of such cultivars lead to transmission of diseases generation after generation which results in less vigorous growth and per hectare yield declines after 15-20 years (Basnet *et al.,* 2007). Thus loses its yield potential and other economically important traits (Lal & Sigh 1994).

The shrinking agricultural lands and increasing demand for sugar have compelled the agricultural scientists to increase the sugarcane and sugar productivity per unit area through the development of varieties with high yielding potential. Vegetative

multiplication of this crop is common in many regions of the world. Its heterozygous and perennial nature, along with a lengthy juvenile period, is an inevitable hurdle in the fast genetic improvement of the crop through traditional breeding programs (Khan *et al*., 2008)

New varieties of sugarcane are developed through breeding methods involving a multi-stage selection procedure which takes about 8–10 years (Lal *et al*., 2014). Due to slower multiplication ratio, it takes a further period of 10–12 years to reach all the zones for general planting, if multiplied through conventional means (Jalaja *et al*., 2008). By that time the varieties start deteriorating in yield and quality parameters.

Lack of rapid multiplication procedures has long been a serious problem in sugarcane breeding programs as it takes 10-15 years of work to complete a selection cycle. Sugarcane being a vegetatively propagated crop has low multiplication rate of 1:6 to 1:8 (Jalaja *et al*., 2008). Hence non-availability of quality seed material is one of the major problems faced by farmers. This problem is further compounded by lack of disease free elite stock for seeding. The bulky cuttings used for planting as seed harbour insect pests and diseases thereby decreasing cane yield and quality drastically. Accumulation of diseases over vegetative cycles leads to further yield and quality decline over years. Indeed, poor quality seed is a major constraint in sugarcane production.

This study was therefore undertaken to establish the effect of 2, 4-D and NAA concentrations on the callogenesis, somaclonal variation and SCMV disease response of three varieties of sugarcane, namely; CO421, CO945 and N14.

### 1.8 Justification

The global biotechnology business is estimated to be around 150 billion US dollars, of which 50-60% is agri-business. Annual demand for tissue culture raised products constitutes about 10% of the total with annual growth rate of about 15 percent ( Bhojwani & Dantu, 2013).

In recent years, Kenya's sugar industry has faced several key challenges, including high costs of production compared to other sugar producing countries in the region, declining sugarcane yields, and inadequate research and extension services among others (Kenya Sugar Industry, 2009).

In a report by Kenani Engineering and Technical Services (2013) a number of factors that lead to the high cost of sugar in Kenya were identified. These included: low utilization of new high yielding sugarcane varieties and reliance by the farming system on old low yielding varieties; and poor quality seed cane material for plant crop establishment affecting the crop stand, vigor and the final yield among others.

Sugarcane is highly heterogeneous and generally multiplied vegetatively by stem cuttings in many countries including Kenya. Flowering and seed set under natural conditions of Kenya is a very serious problem in sugarcane that hampers varietal improvement. Further, the basic facilities for hybrid seed production and variety development are lacking. Though the coastal belt is endowed with specific climatic conditions where sugarcane plants flower at local spots, non-synchronization in genotypes for cane flowering reduces the possibility of hybridization (Tiawari *et al*, 2009). Therefore, sugarcane variety development in Kenya is mainly based on imported germplasm from the cane breeding stations abroad and also through exotic or locally collected fuzz. In most of the cane breeding programs large numbers of seedlings are grown from fuzz, selections are made in subsequent generations to obtain superior clones/genotypes for release as new varieties.

Seed multiplication rate using vegetative methods is too low (1:6 to1:8) which makes the spread of newly released varieties slow, taking over 10 years to scale up a newly released variety to the commercial level (Sengar, 2010; Cheema & Hussain, 2004), and also it facilitates the spread of pathogens and may result in epidemics (Schenck & Lehrer, 2000). Moreover, the method requires large nursery space: one hectare nursery for 10 to 15 hectares of field planting (Sundara, 2000). This leads to slow release of new sugarcane varieties and hence spread of diseases. It is worth noting that Kenya still relies on the Coimbatore varieties of sugarcane that were introduced

in the country over 50 years ago despite the availability of better and improved varieties from research institutions (Jamoza, 2005).

Diseases of sugarcane range from bacterial, fungal, viral and phytoplasmal origin (Jamoza, 2016). Under field conditions occurrence of new fungal pathogenic strains has been reported from time to time. The red rot pathogen *Colletotrichum falcatum L* is a facultative parasite, which keeps on mutating in nature and as a result new races of the pathogen frequently emerge. Existence of several pathogenic races of smut pathogen has been reported throughout the sugarcane growing zones in Kenya (Jamoza, 2016). There are several known sugarcane viruses in Kenya. The Sugarcane Mosaic disease occurs throughout the world except in a few countries.

The author has observed that in Kenya sugarcane planting materials are subjected to hot water treatment by sugar millers as a way of controlling diseases. These materials are then multiplied through several cycles before they are released to farmers. However, hot water treatment alone does not guarantee eradication of all the diseases, especially viral, in the materials (Bhojwani & Dantu, 2013). Furthermore, the long multiplication period exposes the materials to re-infection by diseases.

It is therefore imperative that technological interventions that circumvent the problems associated with the conventional propagation methods are found and implemented to address the problem of low sugarcane productivity in Kenya. *In vitro* culture technology is a tool for obtaining rapid mass multiplication of disease free and true to type planting materials (Singh, 2003).

Plant tissue culture technique is now emerging as a powerful tool for rapid multiplication of sugarcane varieties. From just one established propagule, several thousands of plants can be produced within a year (Jalaja, 2008). It is expected that micropropagation technique may prove to be an efficient alternative tool to the conventional multiplication system for producing sufficient quantity of sugarcane seed material of newly released varieties within a short period of 1–2 years only. Since the plants are propagated from small explants (shoot tips/meristems), they are

also free from diseases and insect-pests. Thus, yield losses incurred due to diseases and insect-pests can also be minimized.

Adoption of tissue culture in sugarcane therefore offers many unique advantages over conventional propagation methods such as rapid multiplication of valuable genotypes, expeditious release of improved varieties, production of disease-free planting materials, non-seasonal year round production, germplasm conservation and facilitating their easy international exchange. Further the technique has major commercial significance.

It is hoped that the findings of this study will benefit Kenyan researchers and other stakeholders by providing relevant information and adding more to the already existing knowledge on sugarcane in vitro culture. It may also assist policy makers in the Ministry of Agriculture in evaluation of its policy on biotechnology in order to make it more effective in developing suitable programs to address the problems in agriculture. The gaps that may emerge may serve as an eye opener for further research in this area.

## 1.9 General objectives

The study aimed at establishing the effect of 2, 4-D and NAA concentrations on the callogenesis, somaclonal variation and disease response of three varieties of sugarcane, namely; CO421, CO945 and N14, in the western Kenya sugarcane zone.

### 1.9.1 Specific objectives

The following specific objectives were used in this study:-

(i) To establish the concentration of 2, 4-D and NAA that will give the highest callus induction in three sugarcane varieties CO421, CO945 and N14.

(ii) To determine whether *callus* culture at selected concentration of 2, 4-D and NAA will eliminate sugarcane mosaic virus from three sugarcane varieties CO421, CO945 and N14.

(iii) To determine the effect of sugarcane varieties, NAA and 2, 4-D concentration on somaclonal variations.

## 1.10 Hypotheses

The following specific hypotheses were tested in this study:-

a) Varying concentration of 2, 4-D and NAA will not increase callus induction in the three sugarcane varieties.

b) *Callus* culture at selected concentrations of 2, 4-D and NAA does not eliminate sugarcane mosaic virus from the three sugarcane varieties.

c) Sugarcane genotype, 2, 4-D and NAA concentration have no effect on somaclonal variations.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Concepts of Tissue Culture

### 2.1.1 Introduction

Plant tissue culture, also known as plant cell, sterile, axenic or *in vitro* culture, is a technique of growing plant cells, tissues or organ in an artificial gel or liquid media supplemented with nutrients, vitamins and plant growth regulators under controlled and sterile conditions (Bhojwani & Dantu 2013; Thorpe, 2013; García-gonzáles *et al*. 2010; Singh & Kumar, 2009). Tissue culture is a conventional technique, which is mainly concerned with the optimization of *in vitro* conditions for growth of plants, exploring the nutritional and hormonal requirements (Kane et at., 2015). It offers many unique advantages over conventional propagation methods such as rapid multiplication of valuable genotypes, expeditious release of improved varieties, production of disease-free plants, non-seasonal production (round the year), germplasm conservation and facilitating their easy international exchange (Govil & Gupta, 1997: Jalaja *et al*., 2008; Kane et at., 2015).

According to Caponetti *et al*. (2005) Henri-Louis Duhumel du Monceau probably pioneered plant tissue culture with studies on wound-healing in plants in 1756 in which he observed callus formation. The first reports regarding tissue culture date back to the beginning of the 20$^{th}$ century when Gottlieb Haberlandt developed experiments to maintain mesophyll cells in culture based on postulates which established the "totipotentiality of plant cells" (García-gonzáles *et al*. 2010; Bhojwani & Dantu 2013). Thorpe (2013) further states that extensive microscopic studies led to the independent and almost simultaneous development of the "Cell Theory in 1930s by Scheilden and Schwawn. Later on, White (1939), Gautheret (1955), Stoutmeyer & Britt (1969) achieved notable success in tissue culture by experimentation on different plants like tobacco and carrot (Bhojwani & Dantu 2013).

### 2.1.2 Cell Totipotency and Plasticity

The development of tissue culture techniques rest upon two properties of plant cells: cell totipotency and cell plasticity (Thorpe, 2007). The concept of totipotency is central to understanding *in vitro* regeneration. The term is used in the context of differentiation not being an irreversible process as a cell undergoes maturation, i.e., a living plant cell with overt functional and structural specialization still carries all the information necessary to divide and undergo a morphogenetic process in the form of either organogenesis [which can be either rhizogenesis (root formation), caulogenesis (shoot formation) or, occasionally, flower formation] or embryogenesis, or to develop directly into a specialized cell type (e.g., as seen in xylogenesis)(Maroon-Lango, 2004).

Cellular plasticity is cell characteristic which marks the difference between plant and animal cells in their capacity for multiplication, division, differentiation and formation of a new individual (García-gonzáles *et al*., 2010). As opposed to animals, plants are sessile organisms often with long life cycles that have been forced to develop defense and survival mechanisms in order to face different negative biotic as well as abiotic factors (García-gonzáles *et al*., 2010). This capacity of modifying response allows plant cells to respond to external stimuli directed towards the achievement of a specific response.

According to Gorst (2004) the first step in the expression of totipotency, where it occurs, is for mature cells to re-enter the cell cycle and resume cell division (a process known as dedifferentiation). The next step is redifferentiation, either through direct formation of organized structures (direct regeneration) or by the formation of an intervening callus stage from which organized structures may later be induced (indirect regeneration). An early appreciation of the mechanisms underlying regeneration of whole plants, or parts of plants from cells came with the classic observations of Skoog and Miller (1957) that the direction of differentiation could be influenced by the ratio of the exogenously supplied growth regulators auxin and cytokinin. They observed in tobacco stem pith cultures that a high ratio of auxin to cytokinin led to initiation of roots, whereas a low ratio led to development of shoots.

Although there are many species for which this simple manipulation will not work, in principle, this is the basis for regeneration in plant tissue culture systems (Gorst 2014). The two groups of growth regulators play a pivotal role in unlocking and realizing totipotent expression by influencing both dedifferentiation and redifferentiation (Bhojwani & Dantu, 2013).

### 2.1.3 Competence and Determination

The process whereby differentiated cells respond to inductive phenomena leading to organogenesis involves two major phases; competence and determination (Gorst 2004). These phases reflect the two-stage practice of exposing cultures first to an ‘‘induction’’ medium and then to a ‘‘regeneration’’ medium during the regeneration process (Christianson & Warnick, 1983), although there are cultures for which both phases will occur on the same medium, particularly in the case of direct regeneration.

Competence is a transient state in which cells can be induced to follow an organogenic pathway (Christianson & Warnick 1983; Sugiyama 1999) and mechanical wounding is the most effective biological trigger for shifting cells into the competent state. Competence can be thought of as having two distinct components, one for cell division and the other for organogenesis. Determination is a process in which cells follow a specific developmental pathway (Gorst 2004).

The distinction between determination and competence can be illustrated by the work of Christianson and Warnick (1983). They found that callus produced on *Convolvulus* explants was initially developmentally interchangeable, i.e., it was competent to follow two developmental pathways; root formation and shoot formation. Once induction of shoots began, the cells involved in shoot formation became determined, and transfer to a root-inducing medium did not affect the formation of shoots. In other words, as determination proceeds, cells become more and more committed, and the developmental potential becomes restricted unless there is a catastrophic event, such as wounding, that cuts across the deter- mined state. The realizing of commitment is considered to be a third phase in the process of organogenesis (Christianson & Warnick, 1983).

### 2.1.4 Organogenesis and Somatic Embryogenesis

There are two methods of whole plant regeneration: organogenesis and somatic embryogenesis, irrespective of their source (root, leaf, stem, floral parts, pollen, and endosperm) and ploidy level (haploid, diploid, triploid) (García-gonzáles *et al*. 2010; Bhojwani & Dantu 2013).

Organogenesis is the formation of plant organs from a determined tissue in order to form complete plants, characterized by being polar, which means that only one aerial organ or root is emitted and from this a new complete plant is regenerated (García-gonzáles *et al*., 2010). According to Skoog and Miller (1957) Organogenesis can occur directly from the explants depending on the hormonal combination of the medium and the physiological state of the explants. At the same time, organogenesis may be direct, if the organogenic shoot is directly obtained from the explants, or indirect, if the organogenic process occurs from previously formed callus in the initial explants (Vijaya & Giri, 2003).

According to García-gonzáles *et al*. (2010) somatic embryogenesis is the production of embryos from somatic plant cells (any non-sexual cell) to obtain a complete plant. Unlike organogenesis, this is a polar process where the aerial structures and roots of the plants are obtained from the somatic embryo. It can also be direct or indirect, if the process originates from the initial explants or from previously induced callus. Somatic embryogenesis consists of four fundamental stages: A) Callus induction; B) Embryo formation and proliferation; C) Embryo maturation; and D) Embryo germination (García-gonzáles *et al*. 2010). At the same time, the embryos may pass through four stages in their development, the globular form, the heart form, the torpedo and the cotiledonary forms (Ammirato, 1983). Each one of the stages of somatic embryogenesis, just as the different phases of normal embryo development, depends on the species and on the genotypes which are being cultured.

Callus represents an unorganized or undifferentiated mass of cells generally composed of parenchymatous cells and usually undergo division (Beyl & Trigiano, 2015). When an explant is cultured in a medium supplemented with sufficient

amount of auxins, it starts producing mass of cells from the surface of the explant. The concentration of auxins required for each type of explant is different and is mainly dependent on the physiological state of the explant tissue (Gorst 2014). Callus cultures can be maintained for a very long time by intermittent sub-culturing to a fresh medium (Bhojwani &Dantu, 2013).

Plant tissue culture is the best technique to exploit the cellular totipotency of plant cells for numerous practical applications, and offers technologies for crop improvement (haploid and triploid production, *in vitro* fertilization, hybrid embryo rescue, variant selection), clonal propagation (Micropropagation), virus elimination (shoot tip culture), germplasm conservation, production of industrial phytochemicals, and regeneration of plants from genetically manipulated cells by recombinant DNA technology (genetic engineering) or cell fusion (somatic hybridization) (García-gonzáles *et al*. 2010; Bhojwani & Dantu 2013).

**2.2 Callogenesis in Sugarcane**

Tissue culture is a conventional technique which is mainly concerned with the establishment of the conditions for growth of plants explants, exploring the nutritional and hormonal requirements. *In vitro* studies have been successfully carried out in species of numerous families of dicotyledonous plants (*Flick et al.,* 1983) but monocotyledonous plants have been more recalcitrant, particularly the cereals (Vasil, 1987). Cereals comprise crop species which are most vital source of nutrition. Due to high agricultural interest in these crops a number of investigators from all over the world have seriously engaged in potential improvement of cereals through *in vitro* manipulation.

In cereals sugarcane is considered one of the most valuable cash crops, which serves as a prominent source of carbohydrates. In view of its agricultural importance the production of new varieties is underway using both the conventional and tissue culture techniques.

Significantly the earlier awareness of the potential of sugarcane improvement appeared when tissue culturists were closely associated with plant breeders and pathologists. This was the case in the Hawaiian Sugar Planters Association Experimental Station (Heinz & Mee 1969; Nickell & Heinz 1973). They induced callus culture from shoot apices, leaves and inflorescence of *Saccharum spp* hybrids. The callus were initiated on MS medium supplemented with 2, 4-D and 10% coconut water. Removal of 2, 4-D from the media caused differentiation of plantlets. Cytokinin (1mg/l) has been added in some regeneration media (Sreenivasan *et al*., 1987)(Heinz *et al*, 1979; Liu, 1983). However, Liu *et al* (1972) reported that addition of cytokinin may be unnecessary. Ho & Vasil, (1983) reported evidence of embryogenic callus development in monocots. Somatic embryogenesis has been reported from a large number of commercial sugarcane clones and can be obtained directly or indirectly from the leaf tissues (Wekesa *et al*. 2014; Raza *et al*. 2012; Manickavasagam M, Ganapathi A 1998; Guiderdoni *et al*. 1995). Embryogenic callus can be maintained for several months without losing its regeneration potential to a significant level (Fitch & Moore, 1993).

Regeneration of sugarcane plants *in vitro* can occur through two main routes, organogenesis and somatic embryogenesis, both of which have well established protocols (Hendre *et al*. 1983; Burner & Grisham 1995; Lakshmanan et al. 2006; Lorenzo *et al*. 2001; Hoy *et al*. 2003; Behera & Sahoo, 2009; Meyer *et al*. 2009; Nkwanyana *et al*. 2010; Ramgareeb *et al*. 2010).

Direct organogenesis involves the regeneration of shoots directly from either apical meristems (Saini *et al*. 2004; Pathak *et al*. 2009; Sandhu *et al*. 2009; Ramgareeb *et al*. 2010) or immature leaf discs (Burner & Grisham, 1995; Lakshmanan *et al*. 2006; Gill *et al*. 2006; Ali *et al*. 2007) after exposure to at least one cytokinin (e.g. 6-benzyladenine and kinetin) and an auxin (e.g. naphthalene acetic acid (NAA), at a high cytokinin : auxin ratio. This is followed by the induction of rooting in response to an auxin (e.g. 3-indole butyric acid) (Cheema & Hussain 2004; Ali *et al*. 2008) or with the removal of growth regulators from the medium (Lakshmanan *et al*., 2006) and supplementation with high sucrose (Singh *et al.*, 2006).

However, there are reports of direct organogenesis in sugarcane under constant light & NAA alone (Garcia *et al*., 2007). Plants can also be regenerated indirectly through the *de novo* organization of shoot meristems from an intervening callus stage, also in response to auxins (shoot formation) and cytokinins (Wekesa *et al*. 2014; Garcia *et al*. 2007; Behera & Sahoo 2009).

Somatic embyrogenesis, the production of embryos from somatic cells, is induced in sugarcane explants in response to auxins, particularly 2, 4-dichlorophenoxyacetic acid (Heinz & Mee 1969; Ho & Vasil 1983; Behera & Sahoo 2009). Embryos develop from single, small, thin-walled, tightly packed, richly cytoplasmic cells that contain many vacuoles and starch grains (Ho & Vasil 1983; Guiderdoni & Demarly 1988). This process, up to embryo germination, is analogous to that of zygotic embryo formation (Evans *et al.*, 1984). As in organogenesis, plant regeneration is possible without a callus phase, if explants are exposed to low levels of 2,4-D (Snyman *et al*. 2001; Franklin *et al*. 2006; Ali *et al*. 2007), thidiazuron (Gallo-Meagher *et al.*, 2001) or chlorophenoxy acetic acid and NAA (Lakshmanan 2006). In these cases, highly embryogenic young explants, such as immature leaf roll discs and pre-emergent inflorescences, are the explants of choice (Snyman *et al*. 2006).

Indirect somatic embryogenesis requires auxin-induced development of embryos from callus, with concentrations of 2–4 $mgl^{-1}$ 2, 4-D being the most effective (Guiderdoni & Demarly 1988; Ali *et al*. 2007a). Once auxin is removed, the embryos terminate without addition of other plant growth regulators (Heinz & Mee 1969; Ho & Vasil 1983; Ali *et al*. 2007). However, in some sugarcane varieties, NAA and kinetin stimulate root formation (Aftab & Iqbal 1999; Gill *et al*. 2002).

More detailed structural investigation of sugarcane callus has revealed, like many other cereals, that it's comprised of different morphological pattern of growth. It consisted of nodular compact regions which expressed better regeneration capacity while loosely packed friable part of callus had shown poor response to differentiation (Ho & Vasil, 1983; Guiderdoni & Demarly, 1988).

Three morphologically distinct types of callus were also reported by Chan *et al* (1988). The first white compact callus exhibited better morphogenetic potential, second was non-morphogenic callus and the third one was mucilaginous nodular callus. Mucilaginous callus on changing in 2, 4-D concentration in culture medium reverted into the two other types. He also observed that leaf explants formed from excised shoot apices. Variation in response to callus sugarcane species varieties and common cultivars were observed. To obtain morphogenetic callus visual selection of suitable callus at each sub-culture and use of alternating high/low 2, 4-D concentration in culture medium were essential. This type of strategy maintained the regeneration capacity over 30 months of callus proliferation. Liu (1983) reported gradual decline in regeneration frequency.

In sugarcane culture Murashige and Skoog (1962) medium is most widely used as basal medium, significantly effective auxin used for callus initiation was 2, 4-D alone or 2, 4-D with kinetin. Wekesa *et al*. (2014) observed highest percent of callus and shoot formation in sugarcane varieties at between 2.0 and 3.5 mg $L^{-1}$ 2, 4-D supplementation to MS basal media. However, NAA treatment in callus formation media was not beneficial. Rooting was stimulated by replacing 2, 4-D with BAP in the medium. Genotypic response may vary in culture, requiring refinement of plant growth regulator concentration and ratios for improved propagation efficiency (Khan at el. 2009; Khan *et al*. 2007).

Increased numbers of reports are available on differentiation of sugarcane callus through shoot formation (Heinz & Mee 1969; Rashid *et al*. 2009; Tarique *et al*. 2010; Sani & Mustapha 2010; Tahir &Victor 2011; Nawaz *et al*. 2013; Dibax *et al* 2013).

In study to establish appropriate conditions for obtaining plant regeneration and acclimatization of the 'RB92579' and 'RB93509' sugarcane cultivars and to elucidate the shoots origin through histological analysis, Dibax *et al*. (2013) showed that for both cultivars, obtaining shoots showed better results with the culture of explants on a callus induction medium containing 2.0mg $L^{-1}$ 2,4-dichlorophenoxyacetic acid, followed by cultivation on a shoot induction medium containing 0.1mg $L^{-1}$ kinetin and 0.2mg $L^{-1}$ benzil amino purine. Histological

analysis revealed that the origin of the shoots in both cultivars occurred through indirect organogenesis.

There are no reports on the effect of 2, 4-D and NAA concentration on callus formation on the sugarcane varieties CO421, CO945 and N14. This study was therefore conducted to establish the effect of 2, 4-D and NAA concentrations on the selected sugarcane varieties.

### 2.3 Diseases in Sugarcane

Pests and pathogens cause considerable damage and economic losses to agricultural and horticultural crops (Bhojwani & Dantu, 2013). A significant proportion of the total world crop production is lost each year because of various pathogens like viruses, bacteria, fungi and nematodes (Ahmad *et al*., 2007). A good amount of this damage is caused by virus infections. The crop plants, such as potato, sweet potato, banana, cassava, sugarcane, potato, horticultural crops (e.g. citrus, pome and stone fruits) and ornamentals, which are generally propagated by vegetative means, are particularly prone to losses caused by viruses that are transmitted from generation to generation (Bhojwani &Dantu, 2013). A rough estimate of annual global losses of agricultural produce by virus infection is to the tune of US$ 6.9 billion (Thompson & Tepfer, 2010). The losses could be in terms of yield and quality of fruits and flowers, vigour and longevity of the productive life of the perennial crops, increased susceptibility of the host plant to other phytopathogens and the severity of the damage caused by them (Wateworth & Hadidi, 1998). Virus infection is also known to reduce the rate of clonal propagation.

Diseases caused by fungi and bacteria have been successfully controlled chemotherapeutically. Unlike fungi and bacteria there is no chemical or physical treatment to eradicate effectively viruses from infected plants. This is mostly due to the fact that viruses do not have independent metabolism (Rao *et al.,* 2001). They mobilize the metabolic machinery of the infected plant so that they multiply at the expense of the host metabolism. These events in the virus infected plant lead to depletion of or accumulation of or appearance of new compounds and induce biotic

stress to the host. Chemotherapeutic interference of viral replication and synthesis could not be done without adverse effect on the host nucleic acid and protein synthesis mechanism (Rao *et al*., 2001).

Sugarcane, a monoculture crop grown in tropical and subtropical climatic regions, is prone to a variety of pests and diseases, the spread of which is exacerbated by vegetative propagation on a commercial scale (Snyman *et al.*, 2011). Different kinds of plant pathogens viz. fungi, bacteria, virus and phytoplasma infect sugarcane (Anon, 2011). At least 150 diseases have been recorded in sugarcane in different countries (Viswanathan, 2002). Among them red rot, smut, wilt, sett rot, grassy shoot, ratoon stunting, leaf scald and mosaic are the major diseases seriously affecting sugarcane production (Viswanathan, 2002).

In sugarcane, there are five viral infections viz; mosaic, sereh, streak, ratoon stunting and Fiji (Khani *et al*., 2012). Viruses that are of notable concern in the global sugarcane growing are sugarcane mosaic virus (SCMV) and sugarcane yellow leaf virus (SyLMV) (Ramgareeb *et al.*, 2010). Mosaic is one of the most important diseases of sugarcane (Saccharum interspecific hybrids). It is widely distributed and can cause significant yield losses (Grisham, 2000). Virus infected plants either deteriorate quality or reduce the yield to a significant level (Rassaby *et al*. 2003; Naz *et al*. 2009).

It has been reported that replacement of virus infected stock with the healthy stock (virus free) led to 300% yield increase (Murashige 1980; Schenck & Lehrer, 2000). It is an established fact that vegetatively propagated plants once systematically infected with a virus, the pathogen passes from one vegetative generation to the next. The entire population of a given clonal variety plant may over a year be infected with the same pathogen (Schenck & Lehrer, 2000).

Historically, the causal agent of sugarcane mosaic was attributed to a single potyvirus called sugarcane mosaic virus or SCMV with numerous strains, or possibly to a complex of potyviruses (Koike & Gillaspie Jr, 1989; Shukla *et al*., 1994). Differentiation of the strains was based on symptom expression on differential hosts

and serological properties. These sugarcane infecting potyviruses were recently included in a SCMV subgroup consisting of four related but distinct species of potyviruses (McKern *et al*., 1991; Shukla *et al*, 1992; Shukla *et al*., 1989; Shukla & Ward, 1994; Shukla *et al*., 1994): Sugarcane mosaic virus (SCMV), Sorghum mosaic virus (SrMV), Maize dwarf mosaic virus (MDMV) and Johnsongrass mosaic virus (JGMV). Zea mosaic virus (ZeMV), a novel potyvirus isolated from maize in Israel, may also be included in this subgroup (Seifers *et al*., 2000). Among these viruses, only SCMV and SrMV are known to infect sugarcane under natural conditions and are considered as the causal agents of sugarcane mosaic (Grisham, 2000; Xie *et al*., 2009). At least eight stains have been reported (Mali & Thakur, 2000), including five from SCMV and three from SrMV. Mixed infection of different virus strains also occurs (Marcos *et al*., 2012; Mali & Thakur, 2000; Xie *et al*., 2009), and dominant virus strains are variable (Grishamand & Pan, 2007).

According Teakle (1989) these viruses have flexuous rod-shaped particles of about 75O x 12 nm, with ssRNA enclosed by spirally wound protein subunits of a single type. The viruses are transmitted in a non-persistent manner when certain species of aphid probe diseased and then healthy plants (Teakle, 1989). The author further states that the viruses are also transmissible in vegetative planting material (sugarcane setts) and by sap inoculation, especially when pricking or severe abrasions cause sufficient wounding of the younger leaves of sugarcane. Symptoms of mosaics are a patchwork of green, pale green and yellow areas on the leaf blade and are most marked on the younger, basal portion of the fast-growing upper leaves and tend to disappear as the leaf ages (Teakle, 1989). In some sugarcane clones, necrotic red or brown spots and streaks can occur. It primarily damages chloroplasts, blocks photosynthesis, and decreases photosynthetic products, thus resulting in a decline in yield and sugar content (Chen *et al*, 2011).

Sugarcane mosaic has been reported in more than 70 countries (Grisham, 2000) and because the reported strains were only from the U.S.A. and Australia, the number of existing SCMV strains is expected to be much greater. In Surveys for sugarcane mosaic virus (SCMV) made in 34 of 41 districts in Kenya Louie (1980) reported

prevalence of SCMV in 20 districts mainly in the western plateaus, Central Highlands, and Rift Valley. Provinces with high incidence of SCMV included Nyanza (15.2%), Rift Valley (15.8%), and Western (19.6%). SCMV was not found in Coast or Nairobi provinces. No recent reports exist on the prevalence of the disease in Kenya; however, since most regions have embraced the growing of maize (Co-host), prevalence SCMV is expected to have increased.

Moreover, numerous isolates or strains have not yet been investigated such as SCMV-C, F, G, K and L from the U.S.A. (Shukla *et al*., 1994) and CMV-N from India (Kondaiah & Nayudu, 1985). Three mosaic strains were reported to occur in sugarcane growing areas in Africa, including strain D in Cameroon, Egypt and South Africa (Koike & Gillaspie Jr, 1989). However, these descriptions were made more than 20 years ago with only a few isolates and recent information regarding mosaic strains occurring in Africa is not available. The characterization of symptoms produced on differential hosts is time consuming, and reliable studies require the use of standard differential hosts and of previously described strains. These conditions are rarely met. Yang and Mirkov (1997) recently sequenced several strains of SCMV and SrMV and developed a RT-PCR-RFLP method for strain discrimination.

The simplicity of pathogenic virus genome quickens the change of dominant strains. Coupled with the complexity of the genetic background of sugarcane, the difficulty in the crossbreeding of virus-resistant varieties is obvious, especially for breeding sugarcane varieties resistant to multiple virus strains. Vasil and Vasil (1987) reported that through tissue culture three methods (namely; apical meristem, organogenesis and somatic embryogenesis) are in practice to obtain virus free plants and their clonal propagation.

Given the widespread occurrence of SCMV in Kenya and the impact it has on the yield and quality of sugarcane, it is important that suitable in vitro cultural techniques that eliminate the virus be established. More specifically that the 2, 4-D and NAA concentrations in MS media effective in eliminating the SCMV be determined.

## 2.4 Virus Elimination in Sugarcane

### 2.4.1 Introduction

Sugarcane breeding programs have focused on generation of varieties with increased yields, higher sucrose content, pests and disease resistance, tolerance to biotic and abiotic stress and improved ratooning ability (Brumbley *et al*., 2008). A significant amount of cane production is lost due to biological pests like viruses. There are no chemical agents to eliminate virus from infected plants. Unlike fungal and bacterial pathogens, viruses are difficult to eradicate by hot water surface sterilization treatments used in quarantine protocols (Saboohi *et al*., 2014). Control of these viruses by use of resistant varieties has been limited. The spread of the viruses can be controlled if seed cane nurseries ensure that newly propagated materials are virus-free. Hence, rapid in vitro multiplication of virus-free plants sugarcane plants is indispensable.

### 2.4.2 Sugarcane Mosaic Virus

Sugarcane mosaic virus (SCMV) is the most widely distributed and it is found in almost all the cultivars of sugarcane ( Xie *et al*., 2009; Gemechu *et al*. 2006; Oertel *et al*. 1997; Koike & Gillaspie, 1989). The crop yield is significantly reduced (10–22%) when incidence of infection level reaches 50% ( Xie *et al*. 2009; Viswanathan 2002). Due to this a large number of traditionally high yielding sugarcane varieties have gone out of cultivation in Kenya (Osoro, 1997). SCMV has been reported to be prevalent in more than 70 countries (Jeffery *et al*., 1998). The initial symptoms of the mosaics disease are the chlorosis and yellowing of the green tissue of the leaf followed by reddening and finally necrosis. The majority of plant viruses are transmitted by vector organisms that feed on the plant. The most common vector organisms of plant viruses are insects, particularly aphids (Wang & Wang, 2012).

Seeds and pollen, once thought to be comparatively free of viruses, are now known to transmit a large number of viruses (Mink 1993). It is difficult to get rid of these viruses and incipient diseases in field conditions.

Whereas fungal and bacterial diseases can be controlled by the application of fungicides and bactericides respectively, control of viral diseases is a serious problem as commercial chemical control methods are either not available or are not economical (Bhojwani & Dantu, 2013). Eradication of viruses and other pathogens is highly desirable to optimize the yield, to facilitate the movement of plant materials across international boundaries and for long-term germplasm storage, ex vitro or *in vitro* (Button 1977; Sediva *et al*. 2006).

There are a number of established and routinely used treatments for the elimination of pathogens from vegetatively propagated sugarcane. For example, soaking stem sections in cold running water for 48 hours, followed by a hot water treatment of 50°C for 3 hours removes *Xanthomonas albilineans* L. (causal agent for leaf scald) or a hot water treatment of 50°C for 2–3 hours eliminates *Leifsonia xyli* subsp. *xyli* L.) (Dookun *et al*. 1996; Guevara & Ovalle 2005; Wekesa *et al*. 2015). However, these stem-based treatments do not remove viruses, and the logistics of treating large amounts of seed cane for commercial planting is complicated (Snyman *et al*., 2011). Surface decontamination of sugarcane setts using detergent and/or ethanol, followed by germination of buds *in vitro*, has been attempted for the removal of bacterial contaminants and to eliminate Sugarcane Fiji disease virus (Wagih *et al*., 1995). However, only 28% of the germinated plants were virus free, suggesting that, in addition to hot water treatments of setts and thermotherapy during germination of shoots, alternative methods and virus-free cells (e.g. apical meristems) should be considered for propagation of virus-free material.

Thermotherapy has been used effectively for a long time to obtain virus-free plants from infected plants of diverse species. Traditionally, thermotherapy of the infected plants has been used to obtain virus-free plants in sugarcane planting materials by sugar milling companies in Kenya. Many viruses are killed or inactivated at higher temperatures without causing serious injury to the host plant (Bhojwani & Dantu, 2013). Bhojwani and Dantu stated further that high temperatures reduce replication of viruses significantly and may be inhibiting the synthesis of the virus coat protein and virus-encoded movement proteins, which help in cell-to-cell movement of

viruses. Thermotherapy is usually effective against isometric and thread-like viruses and mycoplasmas, but is ineffective against many other viruses (Saboohi *et al*. 2014; Bhojwani & Dantu 2013). For instance Krizan *et al*. (2009) reported that grapevines were rid of the Grape Fanleaf Virus by subjecting soft cuttings from the infected plants to $37^0$C in a thermal box under relative humidity of 80% and light intensity of 22 **1**mol $m^{-2}s^{-1}$ for 45 days. The new shoots arising from these treated plants were rooted and cultivated for 12 months in a green- house. These plants were found to be free of virus. However, thermotherapy is not only a cumbersome process but also not all viruses are eliminated by this method, and many host plants are thermosensitive (Saboohi *et al*. 2014; Bhojwani & Dantu 2013).

The knowledge of the gradient of virus distribution in the shoot tips enabled Holmes (1948) to raise virus-free plants from infected individuals of Dahlia by shoot-tip cuttings (Bhojwani & Dantu, 2013). Morel and Martin (1952) further refined the technique and developed the meristem-tip culture technique to eliminate viruses. This *in vitro* technique soon became the most popular technique to eradicate virus from infected plants and is being widely used for the purpose in sugarcane. In vitro methods used to eliminate viruses from infected cane include either apical meristem culture only (Chatenet *et al*. 2001; Fitch *et al*. 2001), or combination of thermotherapy and meristem culture (Flynn *et al*., 2005). Some studies reported that virus-free plants could be obtained through callus culture derived from young buds or leaf tissue (Irvine & Benda 1985; Anderlini TA & Arnaldos 1986; Peros *et al*. 1990).

Since late 1960s, meristem-tip culture has become the most popular method of virus elimination. Many of the viruses that could not be eliminated by thermotherapy alone have been eradicated by meristem-tip culture alone or in combination with thermotherapy and/or chemotherapy. The additional advantages of meristem- tip culture are as follows: (i) the potential of removing fungal and bacterial infections from the donor plant; (ii) *In vitro* clonal propagation with high genetic fidelity; (iii) the practical propagule for cryopreservation and other techniques of germplasm storage; (iv) easily acceptable by quarantine regulations for inter-national exchange; and (v) the technique is suitable for precise *in vitro* multiplication of chimeras.

The distribution of viruses in plants is uneven. The apical meristems of infected plants are either free or carry a very low concentration of viruses (Wang & Hu, 1980). The virus titre in the plant increases as the distance from the meristem-tip increases (Holms 1948; Kassannis 1957). Several reasons have been proposed for the lack of viruses in the meristem-tip (Reddy & Sreenvasulu 2011): (i) Virus multiplication is dependent on the metabolism of the host plant. High metabolic activity in the actively dividing meristematic cells does not allow virus replication, (ii) The rapid spread of viruses in the plant is through the vascular system which is absent in the meristem. Those viruses invading non-vascular regions move from cell to cell via the plasmodesmatal connections, which is rather slow to keep pace with rapidly growing tip region, (iii) A high endogenous auxin level in the shoot tips may be inhibitory to the viruses, and (iv) The meristem is probably protected by certain 'virus inactivating systems'.

The first report in sugarcane of a successful *in vitro* intervention for pathogen-free-plant (Dookun, 1998) production was the recovery of 90% of plants free of Sugarcane mosaic virus (SCMV) from diseased material, through plant regeneration directly from excised apices (Hendre *et al.,* 1975). Subsequent, less successful (12–94% pathogen-free regenerated plants) investigations utilized either immature leaf roll disc explants followed by direct (Irvine *et al.,* 1991) and indirect shoot multiplication (Leu 1978; Dean 1983)or leaf discs taken through direct embryogenesis (Snyman *et al*., 2007). It is likely that the larger immature leaf roll explant 5×40 mm vs. 1–2 mm for excised meristems) had virus particles in some cells that then regenerated into contaminated plants.

Another possible reason for the success of the earlier study is that the shoot and root tip meristems have been shown to be free from viruses (Grout, 1990). Nevertheless, in at least two studies where leaf roll discs were excised from diseased material, 100% pathogen-free plants were produced via direct somatic embryogenenesis of cultivars infected with *Lxx* or *ScYLV* (Snyman *et al*. 2007) and by indirect embryogenesis of 19 cultivars containing both *ScYLV* and sugarcane yellows phytoplasma (Parmessur *et al*., 2002). The most widely used technique for cleaning

up diseased germplasm involves meristem excision (Grout 1990). The use of meristems as explants for indirect (Leu 1978; Fitch *et al*. 2001) and direct shoot organogenesis (Chatenet *et al*. 2001; Ramgareeb *et al*. 2010) results in 80–100% pathogen-free plantlet production. Success rates for recovery of virus-free cells are influenced by the size of the meristem, the susceptibility of the cultivar, and the consequent viral load (S.J. Snyman et al., 2007).

For propagation of virus-free cells in sugarcane, a suitable meristem size likely to be free of virus is in the range of 0.2–1.5 mm in length (Chatenet *et al*. 2001; Fitch *et al*. 2001; Ramgareeb *et al*. 2010). Although it is difficult to regenerate plants from explants less than 0.5 mm long (i.e. meristem dome only), excision and subsequent *in vitro* establishment and recovery of plants have been accomplished from meristems sized 0.2 and 0.3 mm (Chatenet *et al*. 2001; Fitch *et al*. 2001). Ramgareeb *et al*. (2010) demonstrated that virus-free sugarcane plants from material infected with ScYLV and SCMV can be obtained from 0.2 to 0.5 mm long meristems.

However, most *in vitro* techniques, regardless of the explant source or morphogenic route, do not result in 100% of the regenerated plants being virus free, emphasizing the need to verify the status of derived material using diagnostic molecular techniques (Chatenet *et al*. 2001; Fitch *et al*. 2001; Parmessur *et al*. 2002; Ramgareeb *et al*. 2010). Consequently, the following two-stage approach for the production of disease-free seedcane would be judicious: (1) a cohort of indexed, healthy plants is obtained by *in vitro* regeneration from excised meristem-domes; (2) pathogen elimination is verified and phenotypic integrity established. These plants become the foundation for large-scale micropropagation.

There are published few reports that compare the yields of pathogen-free plants produced from propagules recovered from infected plants, through i*n vitro* strategies, with those of treated seed cane (i.e. hot water treatment of stalks). Exceptions are the study undertaken by Hoy *et al*. (2003a), where no differences were observed between plants produced by meristem culture and conventional germplasm and improved yields (in respect of biomass and sucrose) from i*n vitro*-derived plants (Flynn *et al*., 2005).

The technique of meristem-tip culture involves isolation of meristematic tip under aseptic conditions and its culture on a suitable medium under optimal conditions, clonal multiplication of the surviving tips, virus indexing the regenerated shoots/plants using suitable methods and selecting virus-free plants and transferring the plants to insect-proof net house or glasshouse for their periodic check for freedom from virus. Hence, rapid multiplication of virus free in vitro sugarcane plants is indispensible. Therefore, the present study was envisaged to contribute to development of reproducible protocols for mass multiplication of elite cultivars of sugarcane and validation of their virus free nature using RT-PCR technique.

## 2.5 Virus Indexing

Not all plants produced through meristem-tip culture alone or in combination with thermotherapy and/or chemotherapy or by any other method of virus eradication program are virus-free. Therefore, it becomes imperative to check each and every plant produced through any of the virus eradication programs for the presence of viruses before a plant is labeled as free of specific virus and released for commercial use. In cultures, many viruses have a delayed resurgence. Hence, the plants should be indexed several times during the first 18 months, and only those plants which give consistently negative results should be treated as free of specific virus (Walker, 2009). Since virus-free plants can get re-infected after transfer to the field, it is necessary to repeat virus indexing at regular intervals.

The objective of certification schemes is to identify healthy, disease-free plant sources for large-scale multiplication through the use of well-established indexing procedures (Rowhani *et al*., 2005).

According to Bhojwani and Dantu (2013) some of the methods used for virus indexing are: (i) biological indexing, (ii) electron microscopy, and (ii) molecular indexing. The authors recommended that more than one method should be used to index any system reliably. The methods are briefly described below.

### 2.4.5.1 Biological Indexing

Observation for visible symptoms characteristic for a virus in the plants is the simplest test for knowing the presence or absence of viruses (Walker, 2009). However, symptoms take a long time to develop on the host plant. Therefore, more sensitive herbaceous plants, such as *Chenopodium, Cucumis, Lycopersicon, Nicotiana* and *Scopollia* species (indicator plants), are used for indexing sap-transmitted viruses in insect-proof glass-house or nethouse. Bioassay or biological indexing was performed as described by Reddy and Sreenvasulu (2011).

The succulent tissues, such as young leaves and shoot tips, from test plant are triturated in 10 mM phosphate buffer pH 7.5 containing 2 % nicotine (1:10, weight of tissue: ml buffer) and rub inoculated into the indicator plant, using 600-grade carborundum. Nicotine neutralizes the inhibitory effects of polyphenolic compounds and inhibitors from host cells on virus infectivity and also facilitates virus transmission (Rowhani *et al*. 2005). The inoculated plants are maintained in an insect-proof greenhouse separated from each other and other plants. Successful virus transmission is indicated by the development of primary symptoms such as local lesions, ring spots within a few days of incubation. The systemic infections of mosaic, vein clearing, leaf deformation, tissue necrosis, etc. take a longer time to appear (Walker, 2009).

The sap transmission method is widely used to detect viruses and viroids. Though slow, it is a sure method. However, it is not suitable to detect latent viruses that do not show visible symptoms on the host plant and where different strains of a virus produce markedly different symptoms on the same host (Lawson 1986). Molecular assays have been developed, which are much more sensitive and rapid and can handle thousands of samples in a short time. These molecular assays are performed in addition to the biological indexing.

### 2.4.5.2 Molecular Assays

Polymerase chain reaction (PCR): PCR identifies the pathogen through their DNA. PCR assays are fast, reliable, highly sensitive and very versatile. To detect plant

viruses which have RNA, the reverse transcriptase-PCR (RT-PCR) has been developed (Hanson & French 1993; Candresse *et al*. 1998). Preparation of the plant material and isolation of DNA/RNA are very critical because of the high levels of polysaccharides and phenolic compounds that mask the activities of the enzymes used (Borja & Ponz 1992). This problem can be partly overcome by using special resin columns that bind to RNA or by the use of inhibitors and absorbents of contaminants. Alternatively, the viruses can be trapped specifically and separated from the rest of the extract. The development of automated RNA extraction instruments and protocols has eased the situation (Wells & Harren 1998).

To detect very low amounts of viruses, several variations of RT-PCR have been developed, such as the nested-, one-step-, duplex-, multiplex- and real-time RT-PCR (Foissac *et al*. 2001; Dovas & Katis 2003; Viswanathan *et al*. 2008). Another efficient and sensitive diagnostic method, based on detection of the coat protein gene, is the loop-mediated isothermal amplification (LAMP) (Fukuta *et al*. 2003). It explicitly and specifically detects viral RNA.

## 2.5 Callogenesis and Somaclonal Variation

### 2.5.1 Callogenesis

*In vitro* cultures, if the level of growth hormone is altered, particularly auxin, the meristematic or parenchyma tissue will proliferate continuously but in a disorderly manner. Instead of producing new shoots it will simply form amorphous mass of cells called callus. Due to capacity of the cellular totipotency, organization of callus can be induced through manipulation of nutrients and growth regulators. Hence full plant can be regenerated either through adventitious shoots or embryos formed on callus. So far in the past few years significant progress has been made in the callus induction and subsequent plant differentiation from different explants sources in some cereal crops. Some important recent works are outlined below:-

In sugarcane culture, 2, 4-D has proved to be indispensable for callus induction, proliferation and even embryogenesis (Brisible *et al*., 1994, Chengalrayen & Gallo-

meagbar, 2001, Kenia, *et al*., 2006). In various studies it was observed that Callus Induction was more profuse at 2.5 mg/l of 2, 4-D with full potential of callus regeneration from the explant of the various cultivated varieties (Khan *et al.*, (1998), Khatri *et al.*, (2002) Anita *et al.*, (2000) and Aamir *et al.*, (2008).

Other investigators for example Begum *et al.* (1995) found that 3.5 mg/l of 2, 4-D produced highest percentage of callus induction from leaf base explant in Bangladesh Nagabari variety of sugarcane. Islam *et al.* (1982) also reported that 5.0 mg/l of 2, 4-D showed callus induction from leaf tissue on MS medium. In these cases callus induction was highest at when a higher concentration of 2, 4-d was used.

In a related investigation, Sani and Mustapha (2010) evaluated a range of 2, 4-D concentrations (2.5-4.0mg/L) for callus induction and embryogenic callus production. In the investigation all the genotypes highest percentage of explants forming callus was recorded with 3.5mg/L of 2,4-D and callus formation slightly decreased when 2,4-D was increase to 4.0mg/l (83.7%) and progressively decreases with decrease in the 2,4-D concentration. The promotive effect of 2.5 to 4.0mg/L of 2, 4-D for callus induction and proliferation in sugarcane has been frequently reported (McCallum *et al*., 1998; Somashekhar *et al*., 2000; Javed *et al*., 2001, Ali *et al*., 2008). In the investigation although callus induction was higher when MS was supplemented with 3.5 and 4.0 mg/L, embryogenic callus production was significantly higher when MS was supplemented with 3.0mg/L (83.75%) and 2.5mg/L (81.25%) (Sani & Mustapha, 2010). The same concentrations were reported optimum for embryogenic callus production in sugarcane by many authors (Khan *et al*., 1998 & 2004). The high specificity for 2, 4-D for callogenesis in sugarcane could be attributed to the presence of putative 2, 4-D receptors (auxin-binding protein) present on the surface of cell membrane of the explant. It is believed that 2, 4-D plays an important pivotal role in the dedifferentiation of somatic cells into embryogenic callus cells. Michalczuk *et al*., (1992) reported that culture of explant in 2, 4-D containing medium, increases the endogenous auxin levels in the explants. Polar transport of the endogenous auxins (IAA) is essential for the establishment of bilateral symmetry during embryogenesis in monocot (Fisher & Neuhaus, 1996). The

efficacy of 2.5 and 3.0mg/L 2, 4-D in the induction of embryogenic callus in sugarcane, proved that, these concentrations are optimum for stimulating considerable increase in the IAA levels in the cultured explant.

In the study by Sani and Mustapha (2010) the response of the sugarcane genotypes to callus induction and embryogenic callus production was also evaluated. Optimum response to callus induction was recorded in the sugarcane hybrids; M1176/77 (65.00%), B47419 (61.00%) and M2119/88 (60.75%) and were significantly higher than SP726180 (45.75%). Genotypic response to embryogenic callus production demonstrated that, M2119/88, B47419 and M1176/77 exhibited optimum embryogenic callus production of 50.50%, 49.00% and 51.50% respectively, while SP726180 exhibited a significantly lower embryogenic callus production of 32.75%.

This variation in the response of sugarcane genotypes to in vitro callogenesis could be attributed to the physiological differences, particularly the endogenous hormones levels. Endogenous hormones levels were postulated to be the main difference between genotypes with various grades of embryogenic competence in sugarcane (Bhaskaran & Smith, 1990). In conclusion, 2.5 and 3.0mg/L 2, 4-D were found to be optimum for embryogenic callus induction and this effect could be attributed to their activity on the endogenous hormone levels. It is suggested that, this activity might be the secret behind achieving the induction of high frequency embryogenic callus and subsequent plant regeneration in many sugarcane genotypes.

The foregoing investigations indicate that the amount of 2, 4-D required for callus induction will depend on the genotype of sugarcane. There is therefore a need to determine the optimum concentration 2, 4-D for best callus induction for sugarcane varieties CO 945, CO 421 and N14.

### 2.5.2 Somaclonal variation

The term somaclonal variation has been proposed to describe the variability produced by i*n vitro* multiplication (Cote *et al.* 2001). Somaclonal variation can be identified as either somatically or meiotically stable events (Peredo *et al.*, 2006). The meiotically stable variation can be termed 'mutation'. However, not all of the somaclonal variation is meiotically heritable, especially the reversible epigenetic somaclonal variation (Oono, 1985), and throughout this area the term 'variation' is used instead (Cote *et al*. 2001; Kaeppler *et al*. 2000). Meiotically heritable somaclonal variation should also be somatically stable and some of the altered phenotypes are readily observed in the newly produced plants themselves. Others appear in their progeny owing to recessive mutation or additional causes (Phillips *et al.*, 1994). Somatically stable somaclonal variation includes phenotypes such as habituation of cultures (Meins and Thomas 2003; Pischke *et al*. 2006) and physiologically induced somaclonal variation observed among regenerants but not in the regenerant-derived progeny (Kaeppler *et al*. 2000).

Plant tissue culture can be seen as an effective tool for the large scale, controlled production of plant material and represent an option for mass clonal propagation (Chandrika & Rai, 2009). Even though plant tissue culture provides many advantages, one major drawback associated with it is mutations that can cause phenotypic and genetic changes i.e. somaclonal variation (Smiullah *et al*., 2012). Mutations can cause phenotypic alteration, genetic variation and genome instability among the regenerated plants from a single donor clone. Possible mechanisms that may cause a mutation to occur during the tissue culture process are under the controls of genetic and epigenetic systems where it can affect the genetic and genome stability of the plant (Temel *et al*., 2008).

Somaclonal variation may arise due to point mutations, the activation of mobile genetic elements, chromosomal rearrangements, or ploidy level changes (Jaligot *et al*., 2000). Any genetic changes induced by tissue culture condition will probably produce a plant with unique heritable characteristics (Soniya *et al*., 2001). Even though the resulting mutant phenotypes of the plants (e.g. alteration in leaf shape,

dwarfing, and other changes in growth habit) are unique compared to the mother plant, they are not normally deemed useful in micropropagation or for crop improvement (Evans, 1989).

Somaclonal variations can be detected easily by morphological characteristics, such as cane height, leaf morphology, bud shape, number of milable cane, sugar concentration etc. (Doule, 2006). Chromosomal abbreviation and ploidy changes are highlighted by cytogenetic analysis, including chromosome counting under microscope / flow cytometry (Rastogi *et al.*, 2015). Proteins and isozymes also have been used as markers for recognizing somaclonal variants in many fruit species but they are limited in their sensitivity. Cytological evaluation is not often used and can be complicated to detect in numerous crops (Rastogi *et al*., 2015).

Various molecular marker techniques are available for use to detect somaclonal variation. Most of the molecular marker techniques involve amplifications of genomic DNA with short random or specific primers. For example, tissue culture induced mutations of different plant have been screened using Random Amplified Polymorphic DNAs (RAPD) (Qin *et al*., 2007; Sianipar *et al*., 2008; Elmeer *et al*., 2009), Amplified Fragment Length Polymorphism (AFLP) (Puente *et al*., 2008; Chuang *et al*., 2009), microsatellite markers or simple sequence repeats (SSR) (Lopes *et al*., 2006; Burg *et al*., 2007), Sequence-Specific Amplified Polymorphism (SSAP) (Venturi *et al*., 2006; Du *et al*., 2009; Wegscheider *et al*., 2009) and Methyl-Sensitive Amplified Polymorphism (MSAP) (Jaligot *et al*. 2004; Lu *et al*. 2008). Recently, another modified advanced method called Methyl-Sensitive Transposon Display or MSTD, was shown to be useful for detecting genetic variation, changes in transposable element banding pattern and DNA methylation all in one technique (Parisod *et al*., 2014).

Choosing a molecular marker technique depends on its reproducibility and simplicity (Almeida *et al*., 2014). The molecular marker technique inter-simple sequence repeat (ISSR) was developed in 1994 (Zietkiewicz *et al*., 1994). ISSRs are semi-random markers that are amplified by polymerase chain reaction (PCR) in the presence of an oligonucleotide complementary to a particular microsatellite. ISSR is a PCR-based

marker that has some advantages over other markers. Amplification requires no succession information regarding the genome or from highly polymorphic standards (Zietkiewicz *et al*., 1994). Each band corresponds to a DNA sequence delimited by 2 inverted microsatellites. Additionally, ISSR target sequences are abundant throughout the genome of eukaryotes and evolve rapidly (Almeida *et al*., 2014). Thus, ISSRs are useful within populations of genetic studies, particularly in clonal detection, diversity, and identification of closely related individuals (Almeida *et al*., 2014). Further, use of ISSR circumvents the challenge of characterizing individual loci that other molecular approaches require (Srivastava *et al*., 2007). It was therefore for this reason that ISSR was used for the detection of somaclonal variation in the experiment.

### 2.5.3 Somaclonal Variation in tissue culture regenerated Sugarcane

Sugarcane is a genetically complex crop with 10-15 years conventional breeding, selection cycle and vegetative propagation of resulting cultivars (James, 2004). Use of somaclonal variation that results from either *in vitro* culture or mutagenic treatments is one of the ways of diversifying the genetic pool and potentially introducing desirable traits (Snyman et al., 2011). The term "somaclonal variation" is used to describe any kind of genetic or epigenetic variation detected in plants derived from cell cultures, irrespective of the morphogenic route or explant used (Lal *et al*. 2014 ;Larkin & Scowcroft 1981). However, other names such as protoclonal, gametoclonal and mericlonal variation are often used to describe variants from protoplasts, anthers and meristem culture, respectively (Bairu *et al*., 2010). Larkin and Scowcroft (1981) have discussed in detail, various factors responsible for somaclonal variation which include karyotype changes, cryptic changes associated with chromosome rearrangement, transposable elements, somatic gene rearrangements, gene amplification and depletion, somatic crossing over and sister-chromatoid exchanges.

The choice of morphogenic route influences the frequency of somaclonal variation, with indirect somatic embryogenesis resulting in sugarcane plants that are highly variable in chromosome number and agronomic characteristics (Larkin and

Scowcroft, 1981; Irvine *et al*., 1991). Somaclonal variation is a random event, so the identification of desirable somaclones is critical. Selection should be performed either *in vitro*, by the addition of a selective agent (e.g. incorporation of a fungal culture filtrate), through field-based screening of plantlets, or both (Snyman *et al*., 2011).

There are various reports of somaclonal variation induced by culture media (exposure to growth regulators and length of time in culture) that resulted in desirable traits in sugarcane (Sengar 2010; Rajeswari *et al*. 2009; Patade & Suprasanna 2008; Singh *et al*. 2008; Khan *et al*. 2007a; Gandonou *et al*. 2006; Doule 2006; Abo-Elwafa 2004; Zambrano *et al*. 2003; Zambrano *et al*. 1999; Peros *et al*. 1994; Krishnamurthi & Tlaskal 1974; Heinz 1973; Heinz & Mee 1969). Variations in morphology, chromosome number and enzymatic pattern in sugarcane plants derived from callus have been reported.

In sugarcane, somaclonal variation has been exploited for the improvement of many economically important traits like salt tolerant, eye spot and red rot resistant. The first in vitro screened somaclone of commercial sugarcane for resistant to Fiji disease was reported by Heinz (1973). Patade *et al*. (2006) studied the effects of salt and drought stresses on irradiated cells of sugarcane and obtained plants tolerant to higher salt stress. Gandonou *et al*. (2006) deliberated the effects of salt stress by exposing the callus to a single level of 68mM NaCl, and observed that physiological and biochemical indicators could play a crucial role in salt tolerance. Salt (NaCl) tolerent sugarcane cultivar CP65-357 developed from callus culture (Gandonou *et al*., 2006). Wagih *et al*. (2004) developed eight drought tolerant variants from embryogenic callus of sugarcane (Saccharum hybrids) and grew in a greenhouse for further testing under water stress. They found improved tolerance to drought in amongst the somaclonal variants for different areas of tropics and sub-tropics. Four salt tolerant somaclonal variants were developed from embryogenic calli of sugarcane variety CP48-103 (Shomeli *et al.*, 2011). Clonal variation in combination with in vitro mutagenesis and selection has been applied for the isolation. However,

there are no indications in open-access literature sources as to the use of such lines for commercial purposes.

To further capitalize on *in vitro* somaclonal variation and to increase the frequency at which it occurs, physical and chemical mutagens maybe applied to callus cultures ( (Snyman et al., 2011). Such induced mutagenesis has the potential to elicit beneficial modifications in cultivars (Patade & Suprasanna, 2008). Both physical (Saif-Ur-Rasheed 2001; Zambrano *et al*. 2003; Khan *et al*. 2007a; Patade & Suprasanna, 2008) and chemical (Kenganal *et al*. 2008; Koch *et al*. 2010) mutagens have been used successfully in sugarcane to increase somaclonal variation.

Sood *et al*. (2006) demonstrated that tissue culture derived sugarcane var. CoJ 64 plants attained better height, millable cane height, a greater number of live buds, increased cane yield and sugar recovery % as compared to conventionally propagated sugarcane plants under parallel agronomic practices in the field. They also reported that high tillering is resulted in thinner canes because thickness of the canes is directly proportional to the number of tillers per clump and is also related to the cytokinin effect. Siddiqui *et al*. (1994) compared the brix % of canes of somaclones with those of their parents and found the somaclones were better than their parents in this character. On the other hand, Khan *et al*., (2004) reported that brix % of canes of somaclones was less than those of their parents. They also reported that the somaclones were found better in the characters of tillers/plant, stalk height, number of nodes/stem and root band width but they found no difference in the length of internodes of somaclones and source plants.

The culture environment particularly the choice and the concentration of growth regulators in the medium influence the somaclonal variation (Karp, 1992). It is possible that growth regulators act as mutagens. The synthetic auxin (2,4-D) has been shown to increase the frequency of blue to pink mutation in the *Tradescantia* stamen hair system (Dolezal & Novak, 1984) and to induce significant increases in the frequency of sister chromatid exchanges in root tip cells of *Allium sativum* (Dolezal *et al*. 1987). However, there is a paucity of examples of this kind and most evidence points to growth regulators influencing somaclonal variations during the

culture phase through their effects on cell division (Gould, 1984), the degree of disorganized growth (Karp, 1992), and selective proliferation of specific cell types (Ghosh & Gadgil, 1979).

It is therefore evident that tissue culture causes somaclonal variation and most of the variation may be attributed to the use of the synthetic auxin particularly 2, 4-D. However, there are no reports on the effect of 2, 4-D and NAA concentration on somaclonal variation in selected sugarcane genotypes. This study was therefore set to determine the effect of 2, 4-D and NAA concentration on somaclonal variation in selected sugarcane varieties.

### 2.5.4 Characterization of somaclonal variation

When discovered by Larkin and Scowcroft (1981), somaclonal variation was considered as a new source of variation, as novel changes were recorded in the variations, which were not possible by conventional breeding, could be produced by somaclonal variation. After employment of different approaches of plant tissue culture viz, micropropagation; cryopreservation and storage, and regeneration via cell and callus culture, it was necessary to test the cultures for their genetic stability or possible variation. These parameters manifesting stability or variation can be morphological, physiological and genetical:-

a) Phenotypic parameters-
   - Qualitative e.g. leaf size, plant height etc.
   - Quantitative e.g. branching patterns, flower colour etc.

b) Physiological parameters:-
   - Protein pattern by electrophoresis for enzyme, or total content
   - Secondary products formation e.g. alkaloid, steroids etc.

c) Genetic parameters:-
   - Chromosome number and structure
   - Giemsale – banding pattern of chromosome inversion, deletion
   - RFLP, RAPD analysis for alteration in DNA segment

Some of the works where somaclonal variations in cereals and sugarcane are characterized based on morphological markers are discussed below:-

### 2.5.4.1 Morphological markers in sugarcane

The most valuable and reliable information needed for cultivar identification can be obtained through morphological data (Bonnel *et al* 1986) based on phenotypic observation. Moreover, identification and description of cultivars, varieties, wild species and land races are important particularly when breeders need to describe their newly produced varieties.

Mandal *et al*. (2000) characterized somaclonal variation in rice. In the work, primary regenerants of tall salt tolerant rice were produced through in vitro culture from mature seed derived from calluses of Sc2 regenerants, 26 promising lines with superior agronomic traits were selected for evaluation. Somaclones varied significantly from parents with respect to yield attributes. Grain quality and biochemical parameters were different from those of the parents.

In a study on induction and Evaluation of Somaclonal Variation in Sugarcane (*Saccharum officinarum (L.)*) variety Isd-16 by Roy *et al*. (2010) a large number of somatic embryos derived plants (SEDPs) were found morphologically different with some distinct characters such as stool habit, tillering habit, tillering density, auricle, legule, stalk colour and bud shape as compared to setts derived plants (SETDPs). Significant differences were also noted between SEDPs and SETDPs in respect of stalk height, tillers/plant, five internodes length/stem, single stalk weight, individual clump weight, millable cane/clump, and stalk density. Biochemical properties of juice for SEDPs and SETDPs were almost similar in SETDPs and SEDPs. Significant difference between SEDPs and SETDPs was noted only in HR brix% of juice.

### 2.5.4.2 Molecular markers in sugarcane

Oropeza *et al*. (1995) identified somaclonal variations in sugarcane resistant to SCMV via RAPD markers. Somaclonal variants resistant to the virus were obtained from susceptible CV PR62258. The somaclones AT 626 and BT 627 were selected by their resistance to SCMV and were characterized by using RAPD assays.

The RADP (Random amplified polymorphic DNA) technique was also used to detect tissue-culture-induced variations in sugarcane (Zucchi *et al*., 2002). Plants of the Brazilian variety RB83-5486 propagated via rhizomes and via meristem cultures were studied. The polymorphism rate for 98 RAPD loci was 6.93% when the plants derived from meristems. Besides, in order to evaluate the influence of the number of subcultures on the generation of somaclonal variation, field-grown RB83-5486 plants derived from 10 meristems were studied after five sub-cultivations. Although different rates of polymorphism were observed, there was no direct association with the stage of sub-cultivation. The analysis of plants of two sugarcane varieties cultivated in vitro from meristems showed that variety RB83-5486 was more unstable than variety SP80-185.

In a study on *in vitro* regeneration, detection of somaclonal variation and screening for mosaic virus in sugarcane (*Saccharum* spp.) somaclones Smiullah *et al*. (2012) used simple sequence repeat (SSR) markers to evaluate the genetic variation at DNA level between the parent's plants and regenerated somaclones of the accession HSF-240. For the detection of somaclonal variation, 38 primers pair were used and 15 simple sequence repeats (SSR) primer pairs were found to be polymorphic with 51.61% polymorphism. The study demonstrates that SSR genetic markers are the best tool for the investigation of genetic variation in sugarcane.

In a study on ten elite sugarcane clones for genetic diversity through RAPD, Khan *et al*. (2013)revealed that genetically most similar genotypes were Thatta-10 and AEC82-223 (80.4%) and most dissimilar genotypes were AEC712011 and NIA-2004 (49.8%). On the basis of dendrogram, the varieties could be divided into four clusters (A to D). Variety AEC82-223 produced a specific allele of 311bp with primer B-02. Primer sucrose synthase amplified three alleles which were polymorphic and allelic size were 561, 327 and 222bp. Of 10, seven varieties tagged the specific gene responsible for drought tolerance in the genome. L116 containing a different allele of 912bp amplified with DREB-2 showed the specificity of the variety. Maximum sugar recovery % (14.82) and cane yield (t/ha) (156t/ha) were recorded in AEC81-0819.

# CHAPTER THREE

# EFFECT OF 2, 4-DICHLOROPHENOXY ACETIC ACID AND NAPHTHELENE ACETIC ACID CONCENTRATIONS ON CALLOGENESIS IN SUGARCANE


## ABSTRACT

The study investigated the effect of 2, 4-D and NAA concentrations on *callus* induction and shoot regeneration in three sugarcane varieties; CO945, CO421 and N14. Young leaf spindle explants were cultured on MS basal medium supplemented with 2, 4-D (0.0, 2.0, 2.5, 3.0, 3.5 and 4.0 mg $L^{-1}$) and NAA (0.0 and 1.0 mg $L^{-1}$). Experiments were laid out in completely randomized design replicated three times. Observations were recorded on percent *callus* formation, percent shoot formation and morphological characterization of *callus*. Data was subjected to ANOVA at 5%. Significantly higher *callus* production (93%) was observed at 3 mg $L^{-1}$2, 4-D. The presence of NAA tended to depress *callus*ing and shoot production in sugarcane variety N14. The interaction between NAA and 2, 4-D had no significant on the parameters. Application of 2, 4-D at between 2.0 and 3.5 mg $L^{-1}$ gave the highest % *callus* formation and shoot formation in all sugarcane varieties. Application of 2.5 and 3.5 mg $L^{-1}$ 2, 4-D was most effective for sugarcane callogenesis and regeneration for the three sugarcane varieties.


## 3.1 Introduction

Sugarcane (*Saccharum officinarum (L.)*. L), belongs to genus *Saccharum*, family *Poaceae* and is characterized by high levels of polyploidy (2n=80~270) and frequently aneuploidy (Heinz & Mee., 1969). It is an economically important cash crop in the tropical and sub-tropical regions of the world (Chengalrayan & Gallomeagher, 2001). It accounts for approximately 80% of world sugar production (FAO, 2013). The sugar industry plays a significant role in Kenya's economy, contributing about 15% to the country's agricultural Gross Domestic Product (Kenya

Sugar Industry, 2009). Sucrose is the most important type of sugar produced from sugarcane and supplies about 13% of all energy derived from foods (Escalona *et al.*, 1995). It is used as a sweetening agent for foods and in the manufacture of cake, candies, preservatives, soft drinks etc. Sugarcane is also used for making paper, livestock feed, chipboard, cane wax, fertilizer, bioethanol, syrup and mulch ( Garcia *et al*., 2007; Chaudhry & Naseer, 2008).

Sugarcane varieties are highly heterogeneous and generally clonally propagated by stem cuttings. It is especially vulnerable to diseases and propagation from cuttings facilitates the build-up of pathogens and may results in epidemics (Schenck & Lehrer, 2000). Sugarcane stalks can be infected by various pathogens without exhibiting any symptoms, and therefore there is a high risk of disease transfer during exchange and transport of sugarcane cuttings (Parmessur *et al.*, 2002). Being highly cross pollinated in nature, this crop requires specific, hot and humid climate for flowering (Gill *et al*., 2006). The area under cultivation and the yield of this crop has stagnated over the years. It is therefore desirable to sustain the yield without expansion of the area. Introduction of high yielding disease free varieties in short period of time is therefore the best strategy.

Due to its global importance as an agricultural commodity, much research has been focused on sugarcane crop improvement through breeding and biotechnological approaches like micropropagation (Hoy *et al.,* 2003). Micropropagation allows for an extended growing season because shoots can be regenerated i*n vitro,* graduated into the field and harvested early. The system also reduces planting cost by 6%, yield plantlets that perform similar to the conventionally grown sugar cane (Lorenzo & Gonzalez, 1998) and ensure multiplication of disease free plants (Khan *et al.*, 2009).

The most commonly used methods of sugarcane micropropagation are shoot tip culture (Burner & Grisham, 1993) and *callus* culture (Liu, 1993). However, somaclonal variations has been reported by many researchers in *callus* culture raised sugarcane plants ( Hoy *et al*., 2003; Burner & Grisham, 1995). Pandey *et al*. (2012) pointed out that the occurrence of phenotypic instability is a major problem, when the objective is to produce the true copies of original plant. Somaclonal variations

maybe beneficial as an alternative method to sort out many barriers of traditional breeding programs in crops like sugarcane, however, the resulting variations tend to be unstable and may show undesirable features like reduced fertility, growth and even performance (Rastogi *et al*., 2015)

The success of *in vitro* culture depends mainly on the growth conditions of the source material (Caswell *et al.*, 2000; Delporte *et al.*, 2001), medium composition and culture conditions (Saharan *et al.*, 2004) and genotypic variation of donor plants. Among those factors, the genotype and medium composition appear to be important factors influencing the efficiency of i*n vitro* culture.

Bhajwani & Razdan (1996) stated that 2, 4-D and NAA are both auxins and are involved in elongation of stems and internodes, tropism, apical dominancy, abscission and rooting. Further, 2, 4-D is used for induction and growth of *callus* and induction of somatic embryogenesis, NAA is mainly used for rooting and in smaller concentrations for *callus* induction. Several Investigations have indicated that the sugarcane genotype and 2, 4-D concentration affect *callus* induction (Raza *et al.*, 2010; Sani & Mustapha, 2010; Gandonou *et al.*, 2005). However, information on the use of NAA in *callus* induction of the selected sugarcane varieties is limited.

Standardization of protocols for i*n vitro* multiplication of sugarcane through *callus* culture, axillary bud and shoot tip culture have been reported by many authors (Behera & Sahoo, 2009; Anita et. al, 2000). However, reports are scarce on young spindle leaf *callus* culture of sugarcane varieties CO421, CO945 and N14. There is therefore a need to determine the optimum concentration of 2, 4-D and NAA for optimum *callus* induction for the three sugarcane varieties.

The objective of the study was therefore to determine sugarcane varietal differences in response to different levels of 2, 4-D and NAA concentration for callus formation and regeneration.

## 3.2 Materials and Methods

### 3.2.1 Collection of germplasm

Plant materials were collected from Mumias Sugar Company Nucleus Estate located in Kakamega County June 2013. The explants were collected from 6-8 months old seed cane of the varieties Co 945, Co 421 and N14. Leaves and a piece of the cane collected from each plant were sampled and tested for the presence of the SCMV and only plant materials testing positive for sugarcane mosaic virus were used in the study. The collected materials were kept in cool boxes to prevent dehydration.

### 3.2.2 Excision procedure and surface sterilization

Apical portions were stripped to the terminal bud and the young meristems cut into thin smaller pieces of 1.0 to1.5 cm length. The outer immature leaf rolls were removed under aseptic condition using sterile forceps and surgical knife.

The outer leaf sheaths of the terminal part of the cane were stripped off. To obtain the apical stem portion leaf sheaths were unrolled carefully. Other tender sheaths were trimmed until only the innermost leaf sheaths remain attached to the shoot apex. The shoot tip, spindle leaf near the meristem (0.5-1.0 cm in length), pith parenchyma and first internode adjacent to the apical dome were excised for callus culture.

The explants were further treated with commercial bleach containing 5% Sodium hypochlorite supplemented with about 1 ml of Tween 20 for 20 minutes. They were finally rinsed 3 times with sterile double distilled water. Under aseptic conditions, additional outer leaves were removed to isolate immature leaf rolls (1 cm diameter) formed by the innermost 5–6 tightly furled spindle leaves. Each portion was then cut sequentially into 1–2 mm thick transverse sections, beginning from the basal end of the roll. Ten to 30 transverse section explants were prepared from each leaf roll.

### 3.2.3 Sterilization of glassware

The glassware were cleaned and sterilized by the following steps:-

1. All the glassware was dipped in sodium hypochloride for 30 minutes.
2. The hypochloride was removed by washing the glassware first with running tap water, then with household detergent under running tap water followed by many a rinsing in distilled water.
3. Finally, sterilization was completed by placing the glassware in an oven at $180^{o}$ C for about 6 hours.

### 3.2.4 Media preparation

The appropriate composition of the medium largely determines the success of cultures. Plant material does vary in their nutritional requirements and therefore it is often necessary to modify the medium to suit a particular tissue.

The basal medium employed for the culture of sugarcane is MS medium (Murashige & Skoog 1962). A variety of growth regulators such as 6- Benzyl amino purine (BAP), alpha-Naphthalene acetic acid (NAA), 3-Indole Butyric acid(IBA) and 2,4-dichlorophenoxy acetic acid (2,4-D) were added to the medium singly or in combinations at various concentrations and used for initiating different experiments. The concentrated stock solutions of the major salts, minor salts and vitamins were prepared to be used in the preparation of the media and stored under refrigeration. Auxins were dissolved in 1N KOH and cytokinins in 1N HCL before making up the final volume with distilled water. Iron EDTA stock solution was stored in amber coloured bottle. The medium was prepared by adding appropriate quantities of the stock solutions and correct volumes were made up with the distilled water. The pH was adjusted in all cases to 5.8 by using 1 M KOH and 1 M HCL. Sucrose was added at the rate of 2.5% and the media jelled with gelrite. Before autoclaving, the media were poured into washed and dried jam jars (upto 50ml) which were then capped and labeled properly.

### 3.2.5 Inoculation

Immediately after excision explants were transferred into the pre-sterilized jam jars containing 50 ml agar jelled medium prepared for callus induction, shoot proliferation and rooting. 100 ml jars containing 50 ml medium were used. The number of explants to be cultured was 5 per vessel used. All the explants were semi-submerged in the agar medium.

For callus proliferation, shoot differentiation and root induction the culture was transferred to wide mouth jars containing 50 ml fresh medium and subsequently the cultures were sub-cultured in the respective jar after 4 weeks.

### 3.2.6 Regeneration of explants

The young meristem cutting explants were inoculated on sterilized semisolid basal MS medium (Murashige & Skoog's, 1962) supplemented with different concentrations and combinations of plant growth regulators.

#### 3.2.6.1 Callus induction and maturation

*Callus* induction media was prepared using Murashige and Skoog (1962) medium supplemented with different concentrations of 2, 4-D (0.0, 2.0, 2.5, 3.0, 3.5, 4.0 mg $L^{-1}$), NAA (0.0 and 1.0 mg $L^{-1}$) and 30 g $L^{-1}$ sucrose . The pH of the media was adjusted to between 5.7 and 5.8. Activated charcoal was added at 2.5 g $L^{-1}$ and the media solidified using 3 g $L^{-1}$ gelrite. The prepared media was then autoclaved at 121°C and 15 psi for 15 min. Three explants were inoculated in each jam jar containing 35 ml of the prepared *callus* induction media with the distal end facing the media. The explants were then incubated at 27°C and kept in darkness for 4 weeks. The resulting *callus* materials were sub-cultured on the same type of media but with a reduced concentration of 2, 4-D (0.5 mg $L^{-1}$) to induce somatic embryogenesis for another 4 weeks.

### 3.2.6.2 Shoot regeneration

White friable *calli* were cultured on MS medium supplemented with 2.0 mg $L^{-1}$ BAP plus 0.5 mg $L^{-1}$ IBA for multiple shoot regeneration using the protocol as described by Rastogi *et al*. (2015) and (Behera & Sahoo, 2009).

### 3.2.6.3 Root regeneration

Elongated micro shoots measuring about 8-10 mm in length were excised from culture tube and transferred to half-strength (1/2 MS) MS medium supplemented with 1.0 mg/l IBA for multiple root regeneration (Sabaz *et al*., 2008).

### 3.2.7 Physical environment

**Temperature**

For callus induction the cultures were incubated at $28\pm1^{o}$ C. The calli were sub-cultured on the same medium and same temperature condition after 4 weeks of incubation.

**Light**

All the cultures were incubated in a growth room with a 16h photoperiod (cool, white fluorescent light 2000-3000 lux). Unless specifically mentioned, all cultures were grown in an air conditioned room illuminated by 40 W white fluorescent tubes fitted at a spacing of 40-50 cm.

### 3.2.8 Acclimatization and Transfer of Plantlets to Soil

Plantlets with well-developed roots were removed from the culture medium. The roots were washed gently under running tap water and transferred to plastic trays for hardening which contain autoclaved garden soil, farmyard manure and sand (2:1:1). The hardened plantlets in the plastic trays were covered with porous polyethylene sheets for maintaining high humidity and kept under shade in a net house for further

growth and development. All the treatments were irrigated with 1/8 MS basal salt solution devoid of sucrose and inositol every 4 days for two weeks.

After sufficient extension growth the transplants were transferred to pots containing soil with manure. The potted plants were nurtured in polythene chambers placed in the open sunlight for further acclimatization. After 30 days the plantlets were transplanted in to the field conditions for further growth.

### 3.2.9 Plan of experiment, data collection and analysis

Laboratory experiments were set up in a completely randomized design (CRD) replicated three times. 5 explants were used per each of the 36 treatments shown in Appendix 2. Observations were recorded on the days to callus induction, percentage of explants formed callus, days to shoot initiation, number of shoots per callus, shoot length, number of shoots with roots, number of roots per shoot, rooting percentage and root length. Data was analyzed using the product moment correlation to determine whether there was correlation between *callus* induction percentage and embryogenic *callus* production percentage, *callus* induction percentage and shoot formation percentage and embryogenic *callus* formation percentage and shoot formation percentage.

## 3.3 Results

### 3.3.1 *Callus* induction

Initial observations on leaf spindle transverse section culture indicated that phenolic exudation was a significant problem. Swelling of the explant was observed one week after inoculation. However, *callus* initiation was observed in the second week after inoculation of the explants, from the three varieties of sugarcane, in MS media containing different concentrations on NAA and 2, 4-D.

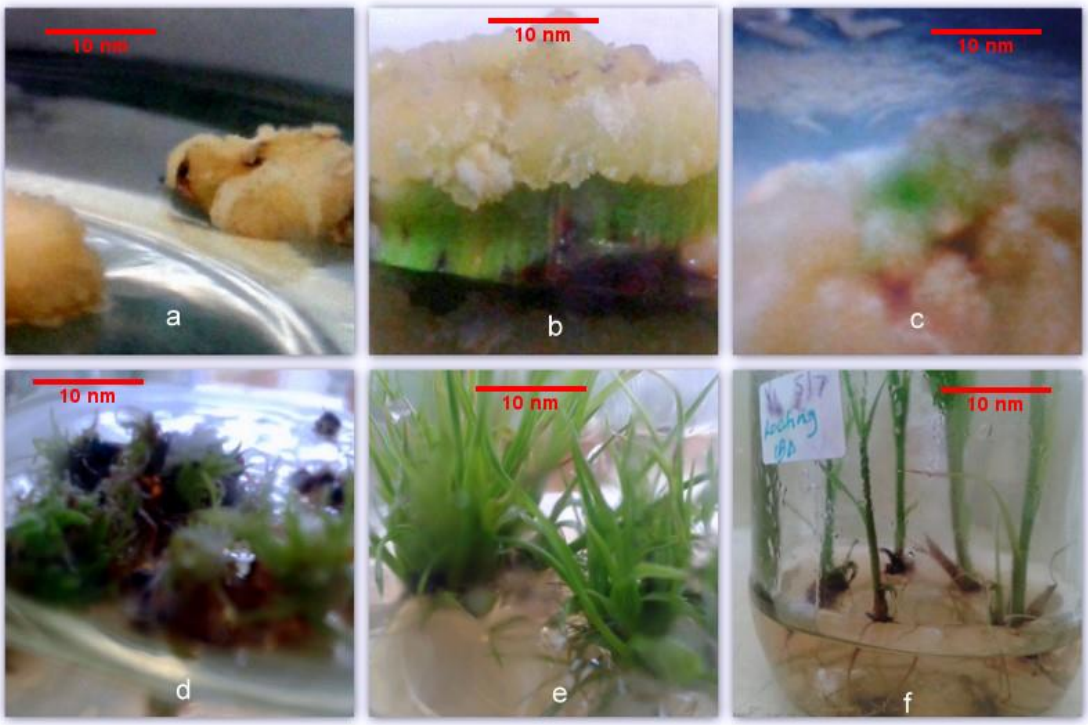


Plate 3.1: Stages of callus formation in sugarcane variety N14: a) Swelling of explant 4 days after initiation b) Callus induction at 14 days c) Callus tissue at 28 days d) Embryogenic callus formation e) Shoot formation f) Multiplication stage

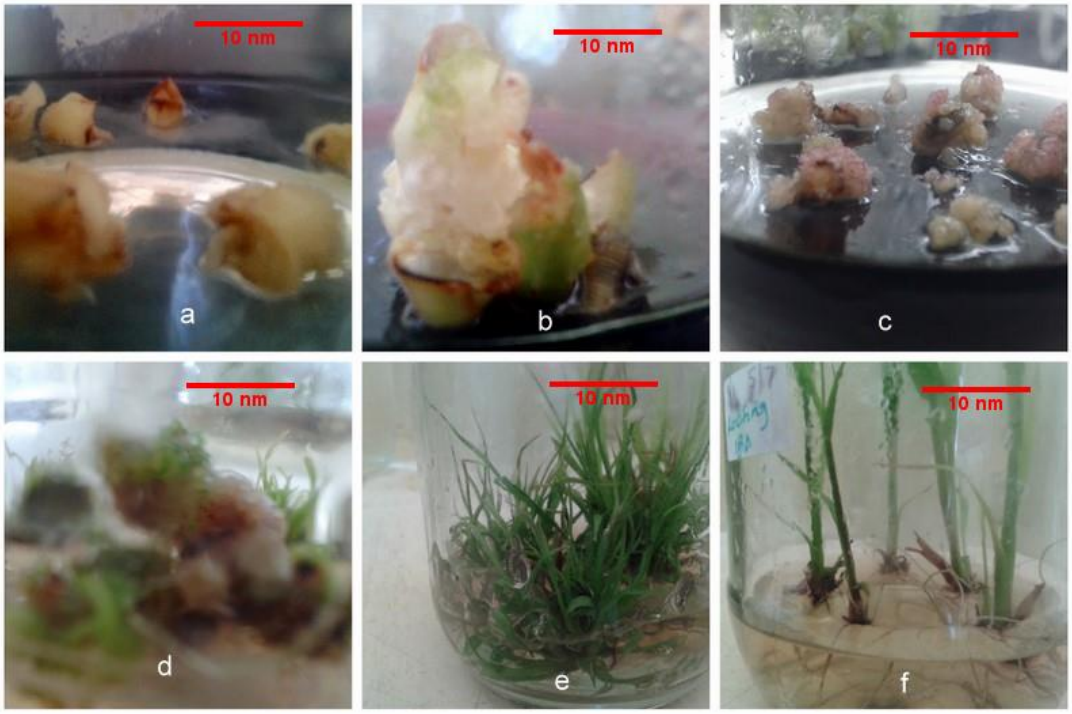


Plate 3.2: Stages of callus formation in sugarcane variety CO421: a) Swelling of explant 4 days after initiation b) Callus induction at 14 days c) Callus tissue at 28 days d) Embryogenic callus formation e) Multiplication stage f) Rooting

stage


Plate 3.3: Stages of callus formation in sugarcane variety CO945: a) Swelling of explant 4 days after initiation b) Callus induction at 14 days c) Callus tissue at 28 days d) Embryogenic callus formation e) Multiplication stage F) Rooting stage

Four weeks later, *callus* initiated at the cut edge of the explants had developed into a full grown *callus*. The *callus* for the three sugarcane varieties was generally white to creamy-white, compact, dry and nodular (Plate. 3.1 to 3.3).

### 3.3.2 Effect of sugarcane variety, NAA and 2, 4-D on callogenesis and regeneration

Callogenesis and regeneration response was studied under the effects of varieties, 2, 4-D and NAA levels and their interactions. The analysis of variance for percentage *callus* induction (Appendix 1) showed highly significant differences ($P \leq 0.01$) due to 2, 4-D concentration. However, the other treatment effects were not significant. Highly significant differences ($P \leq 0.01$) were observed in embryogenic *callus* formation due to 2, 4-D treatment (Appendix 2). The interaction between 2, 4-D × NAA was significant ($P \leq 0.05$) on embryogenic *callus* formation too. However, the other effects were not significant on embryogenic *callus* formation. The analysis of

variance for shoot regeneration showed highly significant (P≤0.01) difference due to sugarcane genotype, 2, 4-D concentration and the interactions between NAA × genotype (Appendix 3). The other treatment effects were non-significant shoot formation.

### 3.3.3 Effect of 2, 4-D concentration on callogenesis and organogenesis in the three sugarcane varieties

As shown in Table 3.1, although in all concentrations of 2, 4-D *callus* induction was triggered, significantly more profuse *callus* induction was observed at 2, 4-D concentration between 2.0 and 3.0 mg $L^{-1}$ with 2.5 mg $L^{-1}$ giving the highest induction (93.07%). While for embryogenic *callus* formation 2, 4-D application of between 2.0 and 3.5 mg $L^{-1}$ gave significantly higher percent than the other concentrations (Table 3.1). The same was observed in percent shoot formation with application between 2.0 and 4.0 mg $L^{-1}$ 2, 4-D giving significantly her percent and the highest percentage explants (55.72%) forming shoots being observed at 3.0 mg $L^{-1}$ (Table 3-1). In general significantly higher percentage of the *callus* initiated in between 2.0 and 3.0 mg $L^{-1}$ 2, 4-D successfully formed embryogenic *callus* and shoots than at other levels (Table 3.1). For successful regeneration of sugarcane therefore application of 2.0 mg $L^{-1}$ 2, 4-D is recommended.

Table 3.1: Percentage mean callus formation, embryonic callus formation and shoot formation on the three varieties of sugarcane on MS media supplemented with 2, 4-D.

| **2.4-D** (mg $L^{-1}$) | ***Callus* formation (%)** | **Embryogenic *callus* (%)** | **Shoot formation (%)** |
|---|---|---|---|
| 0.0 | $48.71^{a}$ | $42.16^{a}$ | $35.09^{a}$ |
| 2.0 | $91.81^{d}$ | $74.82^{c}$ | $59.19^{c}$ |
| 2.5 | $93.07^{d}$ | $70.61^{c}$ | $52.81^{c}$ |
| 3.0 | $85.30^{cd}$ | $72.29^{c}$ | $61.35^{c}$ |
| 3.5 | $78.34^{c}$ | $67.54^{c}$ | $55.72^{c}$ |
| 4.0 | $58.27^{b}$ | $53.98^{b}$ | $43.81^{ab}$ |

*Means sharing similar letter in a column are statistically non-significant at P≤0.05*

### 3.3.4 Effect of NAA concentration on callogenesis and organogenesis in the three sugarcane varieties

As shown in Table 3.2, addition of 1.0 $mgl^{-1}$ NAA to the media led to no significant difference in percent primary callus formation, embryogenic callus formation and shoot formation. Addition of 1 $mgL^{-1}$ NAA to *callus* induction media is therefore not necessary for sugarcane callogenesis and regeneration.

Table 3.2: Percentage mean callus formation, embryonic callus formation and shoot formation on the three varieties of sugarcane on MS media supplemented with NAA.

| **NAA concentration** ($mg\ L^{-1}$) | ***Callus* formation (%)** | **Embryogenic (%)** | ***callus*Shoot formation (%)** |
|---|---|---|---|
| 0.0 | 76.4[a] | 63.4[a] | 50.7[a] |
| 2.0 | 75.9[a] | 63.7[a] | 51.9[a] |

*Means sharing similar letter in a column are statistically non-significant at P≤0.05*

However, as shown in Plate 3.4, application of alone seemed to induce formation of aerial roots in the explant instead of callus tissue.

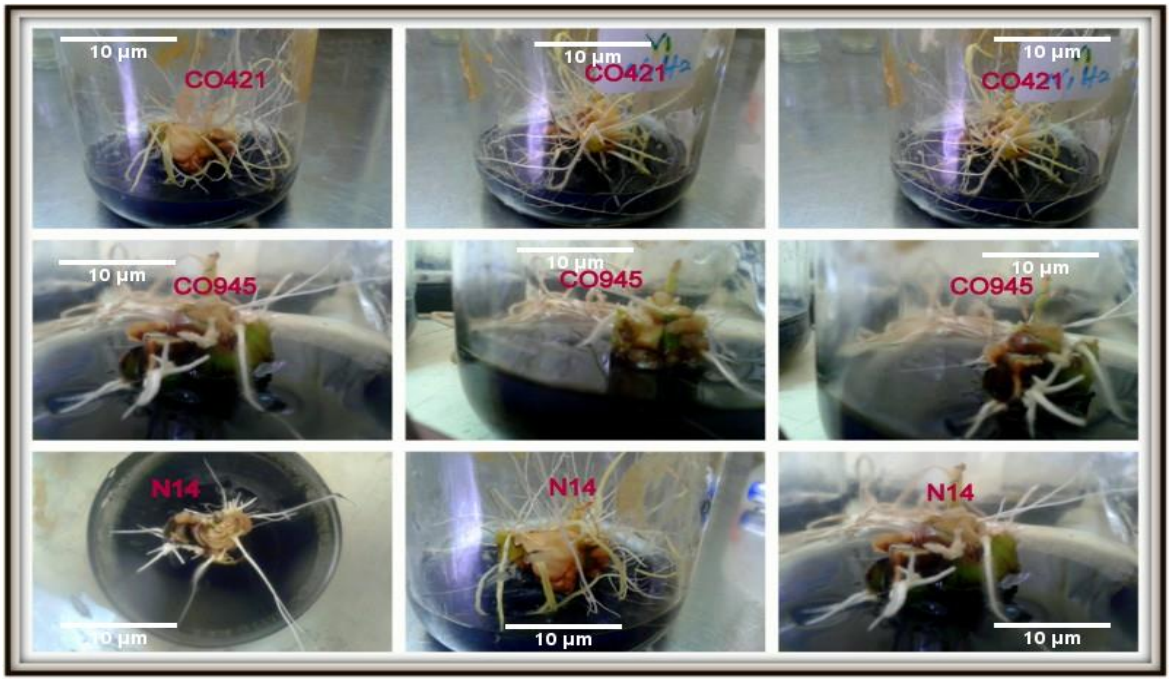

Plate 3.4: Sugarcane explants of various genotypes (V1=CO421, V2=CO945, V3=N14) producing roots instead of callus/shoots in MS media supplemented with 1 $mg^{-1}$ NAA and low levels of 2, 4-D after 45 days of incubation.

The phytohormone NAA is an auxin that induces rooting in plant materials. It therefore induces undesirable reactions when used to induce callus in sugarcane explants.

### 3.3.5 Effect of sugarcane genotype on callogenesis and organogenesis

As shown in Table 3.4 sugarcane variety CO421 gave significantly higher percentage (57.26%) of explants forming shoots compared to CO945 (50.24%) and N14 (46.48%) varieties. However, no significant differences were noted in percent primary callus and embryogenic callus formation among the sugarcane varieties. The significantly in Callus formation could indicate that the three varieties are not related genetically.

Table 3.3**:** Percentage callus formation, embryonic callus formation and shoot formation on MS media as affected by sugarcane genotype.

| **Sugarcane** | ***Callus* formation (%)** | **Embryogenic *callus* (%)** | **Shoot formation (%)** |
|---|---|---|---|
| CO421 | 75.7[a] | 65.2[a] | 57.26[b] |
| CO945 | 75.9[a] | 63.8[a] | 50.24[a] |
| N14 | 76.2[a] | 61.6[a] | 46.48[a] |

*Means sharing similar letter in a column are statistically non-significant at P≤0.05*

### 3.3.6 Effect of interaction between genotype, NAA and 2, 4-D concentration on callogenesis and organogenesis in the three sugarcane varieties

As indicated in appendices, except for the effect of the interaction between 2, 4-D × NAA on primary *callus* formation (Appendix 1) and the interactions between NAA × genotype (Appendix 3) on shoot formation, all the other interaction effects were not significant.

As shown in Table 3.5 application of 1 $mgL^{-1}$ NAA in *callus* formation media led to a significantly decrease in primary *callus* (73%) *than* in the controls (79.4%) for the sugarcane genotype N14. However NAA application had no significant effect on

*callus* formation in the other varieties. Similar results were observed in shoot formation with significantly fewer shoots (42.2%) formed with NAA application than in controls (50.8%) in variety N14 and the other varieties showing no significant differences (Table 3.3). However, NAA applications seem to have led to an increase in shoot formation in varieties CO421 and CO945 though the increase was not significant.

Table 3.4: Effect of the interaction between sugarcane genotype and NAA concentration on % callus formation, embryogenic callus formation and shoot formation in sugarcane

| NAA concentration/ Genotype | *Callus* formation % | Embryogenic *callus* formation % | Shoot formation % |
|---|---|---|---|
| **0 mg/l NAA** | | | |
| CO421 | 75.6[b] | 64.7[a] | 54.8[b] |
| CO945 | 74.1[b] | 62.1[a] | 46.6[b] |
| N14 | 79.4[b] | 63.4[a] | 50.8[b] |
| **1 mg/l NAA** | | | |
| CO421 | 75.8[b] | 65.8[a] | 59.8[b] |
| CO945 | 77.6 [b] | 65.6[a] | 53.8[b] |
| N14 | 73.0 [a] | 59.9[a] | 42.2[a] |

*Means sharing similar letter in a column are statistically non-significant at P≤0.05*

No significant differences in embryogenic *callus* formation were observed among the sugarcane varieties due to NAA application.

It is worth noting that application of NAA at 1 $mgl^{-1}$seem to depress primary *callus* formation and shoot formation in variety N14 and stimulate callus and shoot formation effect in CO421 and CO945. The sugarcane varieties CO421 and CO945 were both developed at Coimbatore Sugar research Institute in India and could be genetically related, while N14 is a South African variety that might be genetically different from the other two. Hence the probable reason for the differences in response. Application of 1.0 $mg/L^{-1}$ NAA might not be necessary for callogenesis and regeneration of the three sugarcane varieties.

As shown in Table 3.5, significant differences were observed in percent primary *callus* formation, short formation and embryogenic *callus* formation due to the interaction between at NAA and 2, 4-D.

Table 3.5: Effect of the interaction between sugarcane 2, 4-D and NAA concentrations on % callus formation, embryogenic callus formation and shoot formation in sugarcane

| 2, 4-D/NAA concentration () $mgL^{-1}$ | *Callus* formation % | Embryogenic *callus* formation % | Shoot formation % |
|---|---|---|---|
| **0 mg/l NAA** | | | |
| 0 | 49.7[a] | 47.1[a] | 30.8[a] |
| 2.0 | 94.0[b] | 77.3[b] | 58.7[a] |
| 2.5 | 93.2[b] | 72.3[b] | 52.8[a] |
| 3.0 | 86.9[b] | 64.9[b] | 64.8[b] |
| 3.5 | 80.0[b] | 40.3[a] | 57.7[a] |
| 4.0 | 54.4[a] | 48.6[a] | 39.6[a] |
| **1 mg/l NAA** | | | |
| 0 | 47.7[a] | 43.5[a] | 39.4[a] |
| 2.0 | 89.6[b] | 72.3[b] | 59.7[b] |
| 2.5 | 92.9[b] | 68.9[b] | 52.8[a] |
| 3.0 | 83.7[b] | 70.2[b] | 57.9[b] |
| 3.5 | 76.7[b] | 44.0[a] | 53.7[a] |
| 4.0 | 62.2[a] | 59.4[a] | 48.0[a] |

*Means sharing similar letter in a column are statistically non-significant at P≤0.05*

Application of 1.0 $mgL^{-1}$ NAA to the media had no significant effect on all the parameters measured as compared to the control. However, application of 2, 4-D at between 2.0 and 3.5 $mgL^{-1}$ led to significantly higher percent primary *callus* formation and embryogenic *Callus* formation in both the NAA controls and treatments. A significantly higher percent shoot formation was observed in 2, 4-D treatments in both cases of NAA treatment with 2.0 and 3.0 $mg/L^{-1}$ 2, 4-D giving higher percent shoot formation than in the other levels. NAA treatment in *callus* formation media is therefore not necessary for sugarcane callogenesis and regeneration. However, application of 2, 4-D at between 2.0 and 3.5 $mgL^{-1}$ is essential.

### 3.3.7 Correlation between *callus* induction, embryogenic *callus* formation and shoot regeneration ability

Data was analyzed using the product moment correlation and it was observed that there was significant positive correlation between *callus* induction percentage and embryogenic *callus* production percentage ($r=0.7780$, value significant at $p<0.05$ at 144 degrees of freedom), *callus* induction percentage and shoot formation percentage ($r=0.5293$, value significant at $p<0.05$ at 144 degrees of freedom) and embryogenic *callus* formation percentage and shoot formation percentage ($r=0.7278$, value significant at $p<0.05$ at 144 degrees of freedom). The high correlation observed indicate that *callus* induction and embryogenic *callus* production constitute a good index for the ability of sugarcane leaf disk explants to regenerate plants after several weeks of i*n vitro* culture.

## 3.4 Discussion

### 3.4.1 Effect of 2, 4-D concentration on callogenesis and organogenesis in the three sugarcane varieties

It is evident from the study that 2, 4-D concentration of between 2.0 and 3.5 mg L-1 gave the highest percent young leaf spindle disks explants forming primary *callus,* embryogenic *callus* and shoots (Table 3.1). The *callus* produced by the three sugarcane varieties was generally white to creamy-white, compact, dry and nodular (Plate. 3.1, 3.2 and 3.3). Days to callus initiation varied among genotypes and were inversely related to the intensity of browning of media because it hampers the uptake of nutrients by the explant, thus resulting in the decrease in callusing. When plants are injured during the preparation of explants, the phenolic compounds that are largely located in the vacuoles are mixed with the contents of the plastids and the other organelles and consequently the dark pigmentation appears (Kaur & Kapoor, 2015). These are highly reactive compounds that polymerize rapidly and form bonds with proteins and also inhibit enzyme activity and thus may result in lethal browning of explant and medium (Preece & Compton 1991).

These results are consistent with the report of Shafique *et al*. (2015), Haq & Memon (2012) and Mamun *et al.* (2004) who studied in vitro, micropropagation of in various sugarcane varieties and found that 3 mg/I of 2, 4-D was the best concentration for callus induction in sugarcane. Many other scientists have used 2, 4-D for callus formation and found it effective. Like Ather *et al.* (2009) obtained 100% callus induction in 3.0 mg/L of 2,4-D. Badawy *et al*. (2008), Pandey *et al.* (2011) and Gandonou *et al*. (2005) also obtained embryogenic callus from leaf bases at 3 mg/L 2,4-D. Jahangir *et al*. (2010) and Shahid *et al*. (2011) worked on callus inductions of sugarcane using different hormonal levels and found satisfactory results at 3.0 mg/L of 2,4-D.

Smiullah *et al.,* (2012), in a study on *in vitro* regeneration, detection of somaclonal variation and screening for mosaic virus in sugarcane (*Saccharum* spp.) somaclones, observed that the best callogenesis was obtained when Murashige and Skoog (MS) was fortified with 3 mg/L 2, 4-D with an average of 3.2 callus score. In contrast, Eldessoky *et al.* (2011) used sugarcane GT54-9 (C9) cultivar and obtained best results producing embryonic calli at 4 mg/L 2, 4-D. The three sugarcane varieties under investigation produced poor *callus* with poor regeneration ability at the controls and lower concentration of 2, 4-D in combination with MS basal media. Similar results with higher concentration of 2, 4-D were recorded by other investigators. For instance Kaur and Kapoor (2015), Gadakh *et al*. (2014) and Nawaz *et al*. (2013) obtained maximum callus induction when they used over 4 $mgL^{-1}$. The studies showed that the three sugarcane varieties require a moderate range of 2, 4-D concentration for maximum *callus* induction and successful shoot formation.

### 3.4.2 Effect of sugarcane genotype on callogenesis and regeneration

As shown in Table 3.2 significant differences were noted in percent primary callus and embryogenic callus formation among the sugarcane genotypes, These results revealed the fact that callogenesis response is genotypic dependent. The three genotypes might not be related genetically. These findings are in agreement with those of Sani and Mustapha (2010); Raza *et al.* (2010) and Gandonou *et*

*al.* (2005) who observed in various studies that callogenesis response is genotype dependent.

Sani and Mustapha for instance in a study on the response of the sugarcane varieties to *callus* induction and embryogenic *callus* production noted that optimum response to *callus* induction was recorded in the sugarcane hybrids; M1176/77 (65.00%), B47419 (61.00%) and M2119/88 (60.75%) and were significantly higher than SP726180 (45.75%). Genotypic response to embryogenic *callus* production demonstrated that, M2119/88, B47419 and M1176/77 exhibited optimum embryogenic *callus* production of 50.50%, 49.00% and 51.50% respectively, while SP726180 exhibited a significantly lower embryogenic *callus* production of 32.75%.

From these results it can be noted that sugarcane varieties that might not be closely related respond differently to 2, 4-D supplementation in MS media. However, Khan *et al.* (2009) observed non-significant difference in shoot induction from three different sugarcane cultivars. In this study genetic difference among the three sugarcane genotypes was not investigated.

### 3.4.3 Effect of the interaction between sugarcane genotype, NAA and 2, 4-D levels on callogenesis and organogenesis

As observed in Table 3.3, application of 1 $mgL^{-1}$ NAA to *callus* formation media tended to significantly depress *callus* formation and shooting in the sugarcane genotype N14. However, the three varieties showed no significant differences in primary *callus* formation, embryogenic *callus* formation and shoot formation in either the controls or the 1.0 $mgL^{-1}$ NAA application. This indicates that NAA treatment in *callus* formation media mighty not be necessary for callogenesis and regeneration of the three sugarcane varieties through callogenesis. These findings are consistent with those of Khattak *et al,* (2014) and Behera & Sahoo (2009). In studies on the effect of different media on callogenesis in sugarcane it was observed that the addition of NAA at the concentration of 2.0 and 3.0 $mgL^{-1}$ led to production of small amount of *callus* that was grayish globular and hardy in nature. This *callus* turned non-regenerable in subsequent sub-culture. However, in the present study on second

sub-culture the *callus* NAA tended to induce early rooting (Plate 3.4) instead of shooting in the explants which is undesirable.

Application of 1.0 $mgL^{-1}$ NAA to the media had no significant effect on all the parameters measured as compared to the control (Table 3.1). However, application of 2, 4-D at between 2.0 and 3.5 $mgL^{-1}$ led to significantly higher percent primary *callus* formation and embryogenic *Callus* formation in both the NAA controls and treatments. A significantly higher percent shoot formation was observed in 2, 4-D treatments in both cases of NAA treatment with 3.0 $mg/L^{-1}$ 2, 4-D giving higher percent shoot formation than in the other levels. NAA treatment in *callus* formation media is therefore not necessary for sugarcane callogenesis and regeneration. However, application of 2, 4-D at between 2.0 and 3.5 $mgL^{-1}$ is essential.

Similar results were reported by Khattak *et al*, (2014); Sani and Mustapha (2010). For instance in an evaluation of a range of 2, 4-D concentrations (2.5-4.0mg L-1) for *callus* induction and embryogenic *callus* production Sani and Mustapha (2010) noted that for all sugarcane varieties, highest percentage of explants forming *callus* was recorded with 3.5mgL-1 of 2, 4-D and *callus* formation slightly decreased when 2, 4-D was increase to 4.0mgL-1 (83.7%) and progressively decreases with decrease in the 2, 4-D concentration.

The high specificity for 2, 4-D for callogenesis in sugarcane could be attributed to the presence of putative 2, 4-D receptors (auxin-binding protein) present on the surface of cell membrane of the explant. It is believed that 2, 4-D plays an important pivotal role in the dedifferentiation of somatic cells into embryogenic *callus* cells. Michalczuk *et al*, (1992) reported that culture of explant in 2, 4-D containing medium, increases the endogenous auxin levels in the explants. Polar transport of the endogenous auxins (IAA) is essential for the establishment of bilateral symmetry during embryogenesis in monocot (Fischer & Neuhaus, 1996). The efficacy of 2.0 to 3.5 $mgL^{-1}$ 2, 4-D in the induction of *callus* in sugarcane in the study proved that these concentrations are optimum for stimulating considerable increase in the IAA levels in the cultured explant.

This response of sugarcane varieties to i*n vitro* callogenesis could be attributed to the physiological factors, particularly the endogenous hormones levels. Endogenous hormones levels were perhaps causes of the difference between varieties with various grades of embryogenic competence in sugarcane.

### 3.4.4 Correlation between *callus* induction, embryogenic *callus* formation and shoot regeneration ability

The study showed that there was significant positive correlation between *callus* induction percentage and embryogenic *callus* production percentage, *callus* induction percentage and shoot formation percentage and embryogenic *callus* formation percentage and shoot formation percentage. The high correlation observed indicate that *callus* induction and embryogenic *callus* production constitute a good index for the ability of sugarcane leaf disk explants to regenerate plants after several weeks of *in vitro* culture.

These findings are in agreement with those of Gandonou *et al*. (2005) in a similar study in which high correlation was observed between the ability of sugarcane cultivars to produce embryogenic *callus* and their capacity for plant regeneration. They concluded that embryogenic *callus* percentage constitute a good index for *callus* ability to regenerate later on plantlets. In contrast, they observed no correlation between *callus* induction percentage and embryogenic *callus* percentage and between *callus* induction percentage and plant regeneration percentage indicating that *callus* induction and regeneration capacity may have been controlled by different mechanisms in the study. In the current study this attributes were therefore controlled by the same mechanism as evidenced by the positive correlations.

## 3.5 Conclusion and Recommendation

*Callus* can be induced from totipotent tissues of plants including sugarcane young leaf spindle disks. The callogenic response from dissected tissues of the young leaf roll confirmed that leaf sections beyond apical meristematic region could be used for *callus* induction and subsequent regeneration into plants. It is evident that *callus*

induction was triggered in all concentration of the hormones. However, the best *callus* induction and regeneration was observed on MS medium supplemented with between 2.0 and 3.5 mg $L^{-1}$ of 2, 4-D in the three sugarcane varieties.

# CHAPTER FOUR

# ELIMINATION OF SUGARCANE MOSAIC VIRUS


## ABSTRACT

The SCMV is the most widespread sugarcane disease and yield losses have been reported at 39-46%. Sugarcane is a vegetatively propagated plant hence propagules of infected plant transmits SCMV generation after generation. The main objective of the investigation was to determine whether *in vitro* culture technique through callus culture at different concentration of 2, 4-D and NAA supplemented in MS media could eliminate sugarcane mosaic virus in i*n vitro* regenerated sugarcane genotypes. Sugarcane leaf samples from callus culture generated plants were separately macerated at: 1:10 dilution in chilled 10mM Potassium phosphate buffer, pH 7.0 containing 0.2% 2 mercaptoethanol and used for mechanical inoculation of sorghum bicolor seedlings at 3-4 leaf stage (appropriately 14 days old). Each treatment inoculum was separately inoculated onto not less than 20 seedlings and appropriate healthy and positive controls included. Leaves of two week old indicator plants of sorghum propagated in the greenhouse were slightly dusted with 600 grade carborundum. Wearing gloves the inoculum was applied with a finger to the leaves in one movement starting from the base of the leaf to the top, supporting the leaf in the other hand. The sap was rubbed hard enough to infect the surface of the leaf. All the SCMV indexed plants were grown in green house and monitored for mosaic symptoms at weekly intervals. Data was collected on the number of test plants showing symptoms of SCMV and analyzed. All the 36 treatments showed on average 36.3% SCMV infection. *In vitro* regeneration of sugarcane through callus induction of young leaf spindles did not eliminate SCMV and was not therefore recommended for the multiplication of viral disease free sugarcane planting materials.

## 4.1 Introduction

Sugarcane is one of the important cash crops in Kenya. It is a major source of edible sugars and many other by-products (Raja & Abbas, 2006). Wide use of sugar and its relevant products have created a challenging situation for sugarcane researchers and growers. In spite of extensive research the average yield of sugarcane in Kenya is very low as compared to other cane producing countries of the world (Kenya Sugar Board 2009). There are many factors responsible for low yield but the most striking one is extreme susceptibility of the plant to pathogens, especially viruses (Naz *et al*. 2009).

Sugarcane is infected by five major viral diseases, namely; mosaic, streak, sereh, Fiji and ratoon stunting (Khan *et al*., 2012). Viruses that are of notable concern in the global sugarcane growing zones are sugarcane mosaic virus (SCMV) and sugarcane yellow leaf virus (SyLMV) (Ramgareeb *et al*. 2010). Amongst the viral diseases of sugarcane, SCMV is the most important. The SCMV is the most widespread and almost all the cultivars grown in Kenya are infected with the virus (Jamoza, 2005). It is believed that SCMV is found in all the sugarcane growing countries of the world to an extent that it is almost difficult to get single healthy sugarcane in the field (Naz *et al*., 2009). Yield losses due to SCMV have been reported from almost 39-46% (Hema *et al*., 1997). Sugarcane is a vegetatively propagated plant hence propagules of infected plant transmits SCMV generation after generation. Lack of flowering potential, virus resistance and efficient multiplication procedures have long been serious problem in sugarcane breeding (Jalaja *et al*., 2008). Unlike fungal and bacterial pathogens, viruses are difficult to eradicate by hot water surface sterilization treatments used in quarantine protocols (Cheong, *et al.*, 2012).

The conventional methods to overcome the viral problem have been exhausted. However, for the past two decades *in vitro* techniques have played significantly more effective role in solving the problems of plant viral infection (Cheong *et al*., 2012 ;Ahmad *et al*., 2007). Several authors (Dean, 1983; Kartha 1986; Chatenet *et al*., 2001; Naz *et al*., 2009; Ramgareeb *et al*. 2010) have reported virus elimination

through apical meristem from food crops, including sugarcane, *Brassica oleracea, Pisum sativum, Glycine max* and *Solanum tuberosum*.

Significantly the earliest awareness of the potential for sugarcane improvement dawned when tissue culture was closely associated with plant breeders and pathologist. Initiation of sugarcane tissue culture was first reported by Heinz and Mee (1969). An intensive work for sugarcane improvement by using this technique has been initiated by Liu (1983) by getting callus induction and subsequent regeneration by using immature inflorescence, apical meristem, young leaves and pith parenchyma. With the passage of time, more emphasis was focused on the elimination of viruses by using apical meristem (Ali, *et al*., 2007).

Since the invention of *in vitro* techniques, a lot of interest has been generated in the recent years for the rapid multiplication of virus free sugarcane through apical meristem, (Ali *et al*. 2007), somatic embryogenesis (Ali & Siddique, 2008) and callus cultures (Ali et al., 2008). Parmessur *et al*. (2002) reported the use of tissue culture as a means to eliminate both SCYLV and SCYP from exotic varieties undergoing quarantine in Mauritius. Yellow leaf syndrome (YLS) is a recently reported disease of sugarcane, characterized by yellowing of the leaves. Two pathogens: a virus, Sugarcane yellow leaf virus (SCYLV); and a phytoplasma, sugarcane yellows phytoplasma (SCYP) are associated with the disease. However, elimination of SCMV in sugarcane through callus culture of young spindle leaves has been reported (Dean, 1983 Naz *et al*., 2009 & Ramgareeb *et al*. 2010). Ramgareeb *et al*. 2010 and Naz *et al*. (2009) observed that the size of the meristem played a pivotal role in the elimination of virus in micropropagated plants. They noted that Plants regenerated from meristems of size 0.2-3.0 mm were all free of SCMV symptoms, while one plant derived from 4.0 mm and 5.0 mm size meristems showed SCMV symptoms. Since not all cells in a shoot apical meristem are infected with the virus, it is possible to dissect out a non-infected region and manipulate this explant in vitro to produce virus-free plants (Kane 2005 & Ramgareeb *et al.*, 2010). As only the meristematic dome and the immediate covering (1st leaf primordia) are usually virus-free, the size of the meristem excised is critical. This indicates that the

size of the meristem was also a determining factor in the elimination of the virus. The larger apices were more prone to retaining virus compared to smaller one.

Successful elimination of sugarcane mosaic virus by tissue culture methods has been reported by many workers (Peros *et al* 1994; Dean 1982). Much of the interest was focused on explant source, effect of media composition on virus eradication and frequency of regeneration and serological technique for detection of SCMV. The evidences also indicated that SCMV positive tissue in cultures of certain incubation periods produce negative symptoms (Wang & Hu, 1980). The intensive bioassays regenerated plants revealed the substantial number of symptom-less stock successfully rose through *in vitro* techniques.

The phenomenon of virus elimination through apical meristem is based on the fact that apical meristems of infected plants are generally either free or carrying very low titer of the virus (Kartha 1986b; Naz *et al*., 2009). In a related study it was observed that only 40% of the single cells mechanically separated from tobacco mosaic virus infected callus contained the virus (Hansen & Hilderbrandt, 1966). Virus free plants have also been regenerated from shoot-tip calli of several other plant species (Pillai & Hilderbrandt 1968; Simonsen & Hilderbrandt 1971). The possible reasons proposed for absence of virus were lack of vascular system, high metabolic activity of meristematic cells, resistance by some cells to infection through mutagenesis and higher endogenous and exogenous level of hormones in cultured meristem which cause inhibition to viral multiplication among other reasons (Wang & Hu 1980; Kartha, 1986).

Therefore, in view of the above findings, the main objective of the investigation was to determine whether *in vitro* culture technique through callus culture at different concentration of 2, 4-D and NAA could eliminate sugarcane mosaic virus in i*n vitro* regenerated sugarcane genotypes.

## 4.2 Materials and methodology

The off shoots of *Saccharum officinarum (L.)* CV CO421, CO945 and N14 were obtained from Mumias Sugar Company, Nucleus Estate. Young leaf spindle were used as explants for callus induction in MS media supplemented with various concentration of 2, 4-D and NAA. Sugarcane explants raised by these methods were used for virus assay. Six weeks old plants from green house were randomly selected from each treatment and used for further study. To determine whether *in vitro* culture eliminates sugarcane mosaic virus (SCMV) in sugarcane tissue, bioassay/infectivity method was used.

### 4.2.1 Bioassay/Infectivity method

For Sugarcane Mosaic Virus Indexation, most of the authors use infectivity tests and commonly used *Sorghum bicolor* and other *gramineae* members as indicator plants (Dean, 1983; Lockhart *et al*., 1992; Naz *et al*., 2009; Reddy & Sreenvasulu, 2011). Infectivity test or sap transmission of virus is one hundred time more sensitive than serological tests and electron microscopy (Kartha 1986; Rao *et al*., 2001).

Bioassay was carried out using the procedure as described by Reddy and Sreenvasulu (2011). To determine for the presence of SCMV, the i*n vitro* regenerated sugarcane plants raised from callus and growing in Green House were used. After sixth week of establishment of plants, a part of the youngest visible leaf from each source were triturated in a few drops of distilled water and ground into sap. Sugarcane leaf samples from callus culture generated plants were separately macerated at 1:10 dilution in chilled 10mM Potassium phosphate buffer, pH 7.0 containing 0.2% 2 mercaptoethanol and used for mechanical inoculation of sorghum bicolor seedlings at 3-4 leaf stage (appropriately 14 days old).

Leaves of two-week-old indicator plants of Seredo cultivar of Sorghum propagated in green houses were slightly dusted with 600-grade carborundum. Sorghum plants were chosen as assay host because of their extreme susceptibility to SCMV and intense symptoms (Dean, 1982). The sap (inoculum) was mechanically rubbed hard

enough to infect the surface cell of the leaf. Wearing gloves the inoculum was applied with a finger to the leaves in one movement starting from the base of the leaf to the top, supporting the leaf in the other hand. The sap was rubbed hard enough to infect the surface of the leaf. After five minutes the inoculated leaves were gently washed with water to remove the residual inoculum. Each plant inoculum was separately inoculated onto not less than 20 seedlings and appropriate healthy and positive controls included. Inoculated plants were maintained under the glass house for several weeks for symptom development. Symptoms on the sorghum plants were noted at weekly intervals for four weeks.

Symptoms on the sorghum indicator plants were noted at weekly intervals for 4 weeks. Sugarcane plants indexing negative were further assayed for virus presence using restriction fragment length polymorphism.

## 4.3 Results

To develop the SCMV-symptoms, sap of severely infected sugarcane plants (control) was used to inoculate mechanically the host plants i.e., *Sorghum bicolor* at 3 leaf stages. All the inoculated plants developed severe mosaic symptoms. The first post inoculation symptom on sorghum consisted of small chlorotic spots appearing at the terminal whorl of youngest leaf. The number of spots increased as the disease progressed; spots became linearly elongated as the leaves increased in size resulting in chlorotic stripes (Plate 4-1e). All these symptoms closely correlate with SCMV symptoms in sugarcane (Plate 4-1f).

### 4.3.1 Infectivity/bioassay test:

The first post inoculation symptom on sorghum consisted of small chlorotic spots appearing at the terminal whorl of youngest leaf. The number of spots increased as the disease progressed; spots became linearly elongated as the leaves increased in size resulting in chlorotic stripes (Plate 4.1).

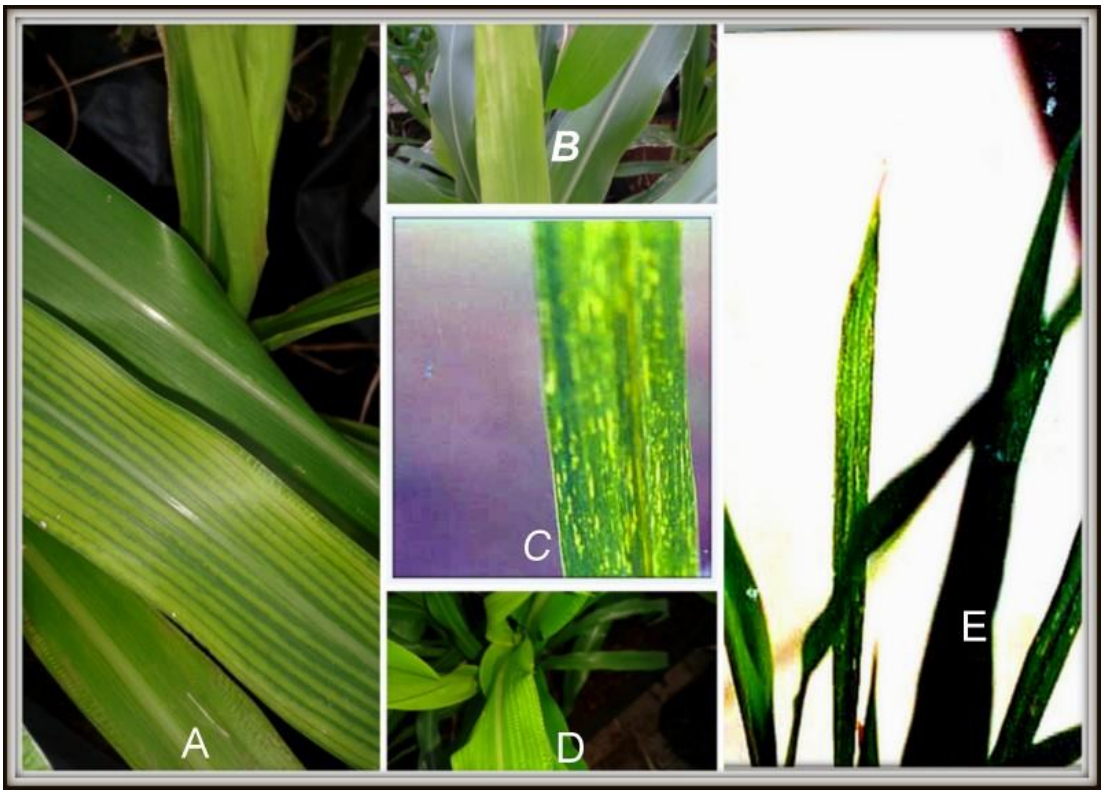


Plate 4.1: Infectivity test, stages of development of mosaic symptoms in *Sorghum bicolor* (A-B) inoculation, (C) Symptoms of SCMV on sorghum leaf. D and E) Sorghum test plant infected with SCMV.

As shown in Table 4.1, 63.7% of inoculated plants produced symptoms of the infection in sorghum, while the rest of the plants did not produce viral symptoms. All the control plants exhibited clear SCMV symptoms. In terms of percentage, 35.9% virus-infected plants were obtained from sugarcane genotype CO421, 34.4% from CO945 and 38.5% from N14 (Table 4.1). The difference in infected among the three genotypes was therefore insignificant. However, N14 showed more infectivity than the other two genotypes.

From Table 4.1 it can be noted that there was in general SCMV infection of the sorghum test plant as a result of the inoculation with sap from *in vitro* generated sugarcane plants. However, the infection was generally insignificant among the three sugarcane varieties. The explants used for initiation of *callus* were from the young leaf discs that were used to regenerate the materials. It is worth noting that length of

the spindles varied and possibly materials that had the virus were incorporated hence the spread of the virus. The treatment effect was generally insignificant.

Table 4.1: SCMV indexation of sugarcane plants regenerated through callogenesis at variation concentration (mg/L) of 2, 4-D and NAA

| NAA/2, 4-D mg/L | | **Sugarcane genotype/ % infected** | | | **Mean** |
|---|---|---|---|---|---|
| | | **CO421** | **CO945** | **N14** | |
| **+VE CONTROL** | | 100 | 87 | 97 | 94.7 |
| **NAA 0** | 0 | 40.9 | 38.5 | 47.6 | 42.3 |
| | 2 | 50.0 | 34.8 | 47.8 | 44.2 |
| | 2.5 | 25.0 | 4.0 | 36.4 | 21.8 |
| | 3.0 | 47.4 | 45.5 | 47.4 | 46.8 |
| | 3.5 | 18.8 | 35.0 | 29.6 | 27.8 |
| | 4.0 | 35.0 | 26.1 | 50.0 | 37.0 |
| **Mean** | | **36.2** | **30.7** | **43.1** | **36.7** |
| **NAA 1** | 0 | 18.2 | 45.8 | 20.0 | 28.0 |
| | 2 | 40.9 | 21.1 | 40.0 | 34.0 |
| | 2.5 | 45.5 | 39.3 | 52.2 | 41.9 |
| | 3.0 | 44.5 | 48.3 | 32.0 | 41.6 |
| | 3.5 | 31.6 | 38.1 | 6.9 | 25.5 |
| | 4.0 | 33.3 | 36.4 | 52.2 | 40.6 |
| **Mean** | | **35.7** | **38.2** | **33.9** | **35.3** |
| **Grand Mean** | | **35.9** | **34.4** | **38.5** | **36.3** |

As shown in Table 4.1 it may be noted that except for application of 2, 4-D at 2.5 mg/L where the infectivity significantly increased, addition of NAA in the rooting media reduced percent infectivity. The reduction was significant in 2.0 $mgL^{-1}$ but not at 3.0 and 3.5 $mgL^{-1}$. Wang and Hu (1980) and Kartha (1986) reported the virus elimination through apical meristem from food crops, including *Brassica oleracea*,

*Pisum sativum*, infectivity increased at 4 mg/L; however, the increase was not significant.

## 4.4 Discussion

Amongst the viral diseases of sugarcane, SCMV is the most important virus disease. It is believed that SCMV has been distributed to all the sugarcane growing countries of the world to an extent that it is almost difficult to get single healthy sugarcane in the field (Mandehar, 1987). Yield losses due to SCMV were reported from almost 39-46% (Mandehar, 1987; Hema *et al*., 1997). The conventional methods to overcome the viral problem are already exhausted. However, for the last two decades *in vitro* techniques have been playing significant effective role in solving the problems of plant viral infection (Ahmad et al., 2007; Naz *et al.*, 2009). In vitro virus elimination technique has been successfully applied to wide range of horticulture plants and agricultural crops*Glycine max* and *Solanum tuberosum*

The phenomenon of virus elimination through apical meristem is based on the fact that apical meristems of infected plants are generally either free or carrying very low titer of the virus (Kartha, 1986; Parmessur et al., 2002; Reddy & Sreenvasulu, 2011). The reason proposed for absence of virus are lack of vascular system, high metabolic activity of meristematic cells, higher endogenous and exogenous level of hormones in cultured meristem cause inhibition to viral multiplication (Kartha, 1986). The present study revealed that that indirect somatic embryogenesis at various concentrations of auxin using young spindle leaf does not generation virus-free plants. The auxin 2, 4-D was applied at between 0 and 4mgl$^{-1}$ in callus formation media (exogenous) and that concentration does not therefore apparently inhibit SCMV multiplication in *in vitro* regenerated sugarcane.

Several investigators have worked on the effect of explant size on elimination of viruses in sugarcane (Chatenet *et al*. 2001; Fitch *et al*. 2001; Naz et al., 2009; Ramgareeb *et al*. 2010). Most of these workers are in agreement that for propagation of virus-free cells in sugarcane, a suitable meristem size likely to be free of virus is in the range of 0.2–1.5 mm in length. Ramgareeb *et al*. (2010) demonstrated that virus-

free sugarcane plants from material infected with ScYLV and SCMV can be obtained from 0.2 to 0.5 mm long meristems. The distribution of viruses in plants is uneven and the apical meristems of infected plants are either free or carry a very low concentration of viruses (Wang & Hu, 1980). The virus titre in the plant increases as the distance from the meristem-tip increases (Holms 1948; Kassannis 1957).

In the present study about 3 cm of the apical leaf spindle was used for callus initiation. The leaf spindles were therefore too long and would have included infected sections.

The difference in magnitude of SCMV infection among the three sugarcane genotypes was insignificant though the genotype N14 had slight higher infection than the other two genotypes. The genotypes CO421 and CO945 both originated from Coimbatore in India and could be genetically similar. The genotype N14 originated from Natal in South African and is probably genetically different from the other and hence the difference in infectivity.

### 4.5 Conclusions and recommendations

The investigation sought to determine whether callus culture at various concentration of 2, 4-D and NAA can eliminate sugarcane mosaic virus from *in vitro* regenerated materials of the three sugarcane varieties CO421, CO945 and N14. From the foregoing discussion it may be concluded that *callus* culture at the varying concentration of the hormones does not entirely eliminate SCMV from the sugarcane genotypes. It can therefore be concluded that callogenesis of the sugarcane varieties CO421, CO945 and N14 at the selected concentrations of 2, 4-D and NAA does not eliminate the SCMV. This conclusion is in conformity with findings by various authors (Naz *et al*., 2009; Parmessur *et al*., 2002) in related studies. Callogenesis is therefore not recommended for the multiplication of disease free sugarcane planting materials as it may lead to spread of SCMV.

# CHAPTER FIVE

# DIVERSITY AMONG SUGARCANE SOMACLONES GENERATED THROUGH CALLUS CULTURE


## Abstract

Callogenesis is one of the tools in sugarcane tissue culture for generating agronomically significant variation. Tissue culture derived variations are known as somaclonal variation. The study was carried out to investigate the effect of MS media supplemented with various concentrations of NAA and 2, 4-D on somaclonal variation in *in vitro* regenerated sugarcane genotype CO421, CO945 and N14. Screening of somaclonal variants was done on four months old field grown sugarcane plants that had originally been generated through *in vitro* culture. The morphological characters studied included; tillering capacity, diameter of the cane, internode length, leaf length and width. Analysis of variance was done using GenStat version 17.0 and pair wise comparison of means of phenotypic traits of all somaclones by calculating fisher's least significant difference (LSD) at $P \leq 0.05$. Pearson's correlation between all phenotypic traits and Tukey test of the selected clones were computed. Multivariate analysis of variance (MANOVA) revealed patterns of phenotypic diversity of quantitative traits studied. Means of each quantitative character were standardized before subjecting to the principal component analysis (PCA). The standardized data of the 5 quantitative traits were then used as an input for the PCA biplot loading and cluster analysis. An agglomerative, hierarchical cluster classification technique with Average linkage strategy was performed. The results of the analysis of variance for the differences in morphological traits indicated that genotype, 2, 4-D and the various interactions had significant effect on the various morphological traits. Application of 2, 4-D to CFM led to somaclonal variation irrespective of the sugarcane genotype used. The observed variation however had no correlation to the hormonal concentration supplemented in the CFM. The dendrogram demonstrated variation among the somaclones based on morphological traits, could be a valuable source for sugarcane improvement program.

## 5.1 Introduction

Sugarcane (Saccharum spp.) is an important industrial and cash crop in many tropical and sub-tropical countries. It is cultivated in about 74 countries between 40$^{o}$N and 32.5$^{o}$S, encompassing half the globe(Khan et al., 2007). Scientists call it photosynthetically efficient, in that it synthesis sucrose from sunlight, air, and water better than just about any other plant on the earth.

Sugarcane crop improvement in different countries relies mainly on conventional breeding (Dalvi *et al*. 2012; Rajeswari, 2009). Sugarcane improvement through conventional methods is time consuming (Cox & Smith, 2000) and is strictly dependent on the nature of flowering, viability of pollen, seed (Moore & Nuss, 1987; Khan *et al*. 2008) and the genomic complexity of the crop (Ingelbrecht *et al*., 1999).

An array of variations has been observed using tissue culture techniques in different crops (Nawaz *et al*., 2013). This variation is termed 'somaclonal variation'. Although somaclonal variation is undesirable for clonal propagation and genetic transformation efforts (Cerasela *et al*. 2012: Pandey *et al*. 2012), it may serve as a useful tool in some crop improvement programs (Brown & Thorpe, 1995; Tiwari *et al*., 2011). Thus far, for sugarcane, only a few improved variants have been released as cultivars after extensive efforts in different laboratories (Larkin & Scowcroft 1983; Krishnamurthi & Tlaskal, 1974). As most of the agronomic important traits are quantitatively inherited in sugarcane, the frequency of positive mutation in terms of high yield and increased sucrose content is very limited. Moreover, such variations are often unstable and infertile, which limits the ability of this phenomenon to be used as a tool for crop improvement in sugarcane (Irvine *et al*. 1991; Hoy *et al*. 2003; Matsuoka & Giglioti 2005).

Pre-existing variability among the cells may play a major role in the frequency of somaclonal variation (Brown & Thorpe, 1995; Hoy *et al*. 2003). Besides variations observed in sugarcane morphological characters such as stalk height, girth, stalk colour, leaf colour, foliar characters, auricle length, bud groove, bud missing, bud shape and size, flowering etc., variations were also observed in tillering, high silicate

deposits on leaf surface and differences in growth habits (Larkin & Scowcroft, 1983). The phenotypic variations are typically due to underlying genetic complexity from multiple interacting loci; with allelic effects that are sensitive to the environmental conditions each individual experiences (Lynch & Walsh, 1998). Phenotypic estimates are used to reveal the level of genetic relationship and therefore the similarity in phenotype characteristics may show genetic similarity of genotypes (Cox *et al*., 1985).

The major commercial sugarcane varieties grown in Kenya are of Indian and South African origin and include CO421, CO617, CO945 and N14 (Jamoza, 2005). These varieties were introduced into the country more than fifty years ago. The expansion of the sugarcane growing into diverse areas has increased the demand for new improved varieties. According Jamoza (2005) crossing or hybridization of sugarcane can only be done at the Kenyan coast where flowering occurs under natural conditions and varietal development takes 13-15 years. Considering the environmental conditions of Kenya where sugarcane breeding is limited due to non-viable fuzz (seeds) and the period it takes to develop new sugarcane varieties, somaclonal variation presents an alternative solution to overcome many difficulties in cane breeding (Shahid *et al*., 2011).

Comprehensive reviews of various distance measures are available in literature (Beaumont *et al*. 1998; Mohammadi & Prasanna, 2003). For molecular marker data, allele frequencies are calculated and used to generate a binary matrix for statistical analysis. The most commonly used measures of genetic distance-similarity using binary data to assess sugarcane diversity are: Nei and Li's coefficient, Jaccard's coefficient, and the simple matching coefficient (Henry & Kole, 2010). Alternatively, multivariate analytical techniques, which simultaneously analyze multiple measurements on each individual under investigation, are widely used in analysis of genetic diversity irrespective of the dataset (morphological, biochemical, or molecular marker data) (Mohammadi & Prasanna 2003). Among these algorithms, cluster analysis using the agglomerative hierarchical method UPGMA (Unweighted Paired Group Method using Arithmetic averages), principal component analysis

(PCA) and principal coordinate analysis (PCoA) are, at present, most commonly used and appear particularly useful (Aitken, *et al*, 2006).

The present study was conducted to evaluate the effect of sugarcane genotype, NAA and 2, 4-D concentrations on somaclonal variation in *in vitro* generated sugarcane clones.

## 5.2 Materials and Methods

### 5.2.1 Establishment of field experiments

The somaclones regenerated from immature leaf roll callus of sugarcane were evaluated for their yield and yield contributing characters at the Organic Farming Demonstration field of Jomo Kenyatta University of Agriculture and Technology using Split-Split Plot design in four (4) replications as shown in Appendix 3.

Field experiments were established using sugarcane seedlings from various treatments after one month hardening at the IBR tissue culture banana greenhouses. The seedlings were transplanted at the spacing of 90 X 60 cm. Diammonium Phosphate fertilizer was applied at 5 grams in each planting hole and thoroughly mixed with soil before the seedling was transplanted. The seedling was transplanted at the same level as it was in the polythene tube. The recommended agronomic practices including irrigation, weeding, fertilizer application and pests and disease management were carried out as recommended by Acland (1989).

### 5.2.2 Screening for somaclonal variants

Screening of somaclonal variants was done in four months old *in vitro* regenerated field growing plants in which plants were studied for and compared with parent plant for various morphological characters including; tillering capacity, diameter of the cane, internode length, leaf width and leaf length.

The average stem diameter was measured using a vernier caliper in the center of the internode located in the average length of the stem; 5 random readings were taken

per treatment. The average internode length, leaf width and length were measured using a tape measure from 5 random sugarcane plants. The total number of tillers was counted in the clumps analyzed.

Multivariate analysis of variance (MANOVA) was therefore conducted to reveal the patterns of phenotypic diversity of quantitative traits studied in set of treatments. Means of each quantitative character were standardized before subjecting to the principal component analysis (PCA) as suggested by Reddy *et al*. (2009). The standardized data of 5 quantitative traits were then used as an input for the PCA biplot loading and cluster analysis. An agglomerative, hierarchical cluster classification technique with Average linkage strategy was performed. Mead *et al*. (2002) indicated that the measures of similarity and dissimilarity were derived by calculating the Euclidean distance between pairs of objects. The Euclidean measure of distance was used for computing genetic distance among the populations as describe by Weir, (1996) where the genetic distance of phenotypic traits is expressed as:

$$d\,(i, j) = [(x_1 - y_1)^2 + (x_2 - y_2)^2 + \ldots (x_p - y_p)^2]^{1/2}$$

$$d(i,j)^2 = [(X_1 - y_1)^2 + (X_2 - y_2)^2 + \ldots (X_p - y_p)^2]^{1/2}$$

Where *i* and *j* is the Euclidean distance between two individuals having phenotypic traits (p) whereas x1, x2……xp is the traits for *i* individuals and y1, y2……yp is the traits for *j* individuals. Average linkage treats the distance between two clusters as the average distance between all pairs of items where one member of a pair belongs to each cluster (Kahraman *et al*., 2011). The Euclidean distance is a multivariate generalization of the Pythagorean Theorem. For the $r^{th}$ and $s^{th}$ objects measured on variable X1….XJ (Basnet *et al*., 2014), it is expressed as:

$$D_{rs} = \sqrt{\sum_j (X_{rj} - X_{sj})^2}$$

$$D_{rs} = \sqrt{\sum_i (X_{ri} - X_{si})^2}$$

The dissimilarity matrix so produced was useful in clustering of objects using hierarchical cluster analysis (HCA) method so as to obtain a dendrogram. All statistical analyses were employed using the appropriate procedure of statistical software GenStat version 17.0.

## 5.3 Results

### 5.3.1 Phenotyping

Attributes were collected four months after transplanting the *in vitro* regenerated sugarcane growing in the field (Plate 5.1). All the plants were of the same age. Plants grown in the field were analysed for morphological and agronomic characters like tiller number, leaf length, leaf length, internode length and cane diameter for primary screening of the plants.

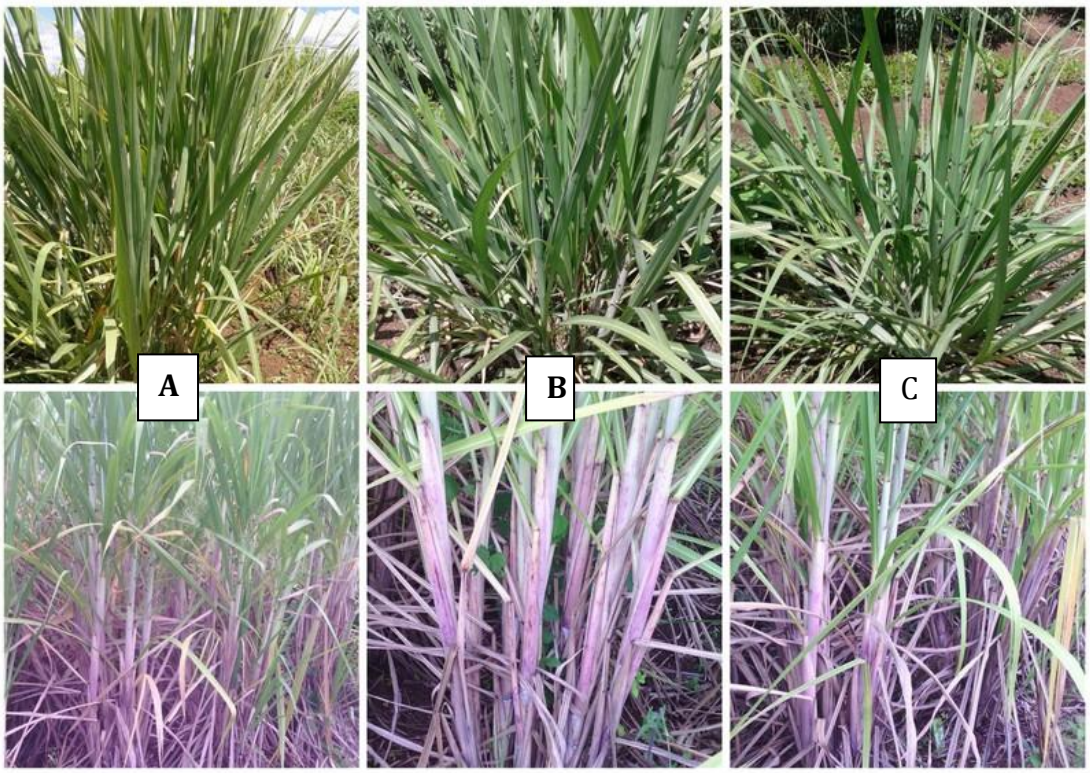


Plate 5.1: A field experiment of i*n vitro* developed and conventional sugarcane varieties (A – CO421, B – N13 C – CO945) growing under irrigation at the IBR Organic Farming Demonstration field at JKUAT, Juja. Notice the purple coloration of the leaf petiole and the cane attributed to somaclonal variation,

### 5.3.2 Analysis of Variance

Data was collected on morphological features that could indicate somaclonal variation in the i*n vitro* generated sugarcane genotypes CO421, CO945 and N14. These features included number of tillers per stool, leaf length (cm), leaf width (cm), cane diameter (cm), and internode length (cm). The analysis of variance showed significant treatment effects due to the three way interaction between genotype, NAA and 2, 4-D for all parameters under consideration (Appendix 7-10). The effect of 2, 4-D concentration was significant ($P<0.05$) for all parameters except leaf width for which there were no significant differences. NAA application had no significant effect ($P<0.05$) on all parameters under consideration. The effect of sugarcane genotype was only significant for number of tillers per stool and sugarcane leaf length.

The interaction between genotype and NAA application showed significant differences ($P<0.05$) for leaf length, leaf width, and number of tillers per stool. Genotype/2, 4-D concentration interaction effect was significant for all the parameters under consideration except for internode length and leaf width.

#### 5.3.2.1 Effect of sugarcane genotype on morphological and agronomic characteristics

As shown in Table 5.1 sugarcane genotype had a significant ($P<0.05$) effect on leaf length, and number of tillers per stool in the i*n vitro* regenerated sugarcane. However, it had no significant effect on the cane diameter, internode length and leaf width.

The sugarcane leaf length (cm) was significantly different among the three sugarcane genotypes. The sugarcane genotype CO421 had significantly shorter leaves (127 cm) than CO945 and N14 (134.95 and 137.77 cm respectively), which were not significantly different (Table 5.1). These differences in leaf length among the three sugarcane genotypes could be genetical.

Table 5.1: Mean effect of Sugarcane genotype on cane diameter (cm), internode length (cm), leaf width (cm), leaf length (cm) and number of tillers per stool

| **GENOTYPE** | Cane diameter (cm) | Internode length (cm) | Leaf width (cm) | Leaf length (cm) | Tiller per stool (number) |
|---|---|---|---|---|---|
| CO421 | 2.860 | 6.86 | 3.383 | 127.00 | 11.40 |
| CO945 | 2.828 | 6.22 | 3.388 | 134.95 | 12.78 |
| N14 | 2.852 | 7.54 | 3.613 | 137.77 | 14.78 |
| $LSD_{0.05}$ | 0.692 | 1.36 | 0.230 | 5.67 | 1.68 |
| CV | 11.30 | 13.50 | 4.50 | 2.90 | 8.80 |

A significant difference was noted in number of tillers per stool among the three sugarcane genotypes (Table 5.1). Tiller numbers were significantly different among the three sugarcane genotypes with sugarcane variety N14 having the highest mean of 14.78 and CO421 the lowest mean of 11.40. There was however no significant difference in tiller numbers between CO421 and CO945 sugarcane genotypes, though CO945 had comparatively more tillers than CO421. Sugarcane genotypes CO421 and CO945 have common ancestry in Coimbatore, India and could therefore be similar genetically. The differences in tiller numbers per stool among the three sugarcane genotypes could therefore be genetical but not treatment effect. No significant difference was noted among the three genotypes' cane diameter, internode length and leaf width at four months of age.

### 5.3.2.2 The influence of 2, 4-D concentration applied in callus formation media on morphological characteristics of selected sugarcane genotype

As shown in Table 5.2, except for leaf width all the other attributes were significantly ($P<0.05$) affected by treatment effects. The cane diameter, internode length, leaf length and tiller number were significantly greater than the controls.

Table 5.2: The influence of 2, 4-D concentration applied in callus formation media on morphological characteristics of sugarcane

| **2, 4-D concentration** | Cane diameter | Internode length | Leaf width | Leaf length | Tiller per stool |
|---|---|---|---|---|---|

| ($MgL^{-1}$) | (cm) | (cm) | (cm) | (cm) | (Number) |
|---|---|---|---|---|---|
| 0 | 2.24 | 5.30 | 3.34 | 10.27 | 127.27 |
| 2.0 mg/l | 2.94 | 7.39 | 3.39 | 15.07 | 131.13 |
| 2.5 mg/l | 2.90 | 7.11 | 3.43 | 14.63 | 136.33 |
| 3.0 mg/l | 2.85 | 6.95 | 3.57 | 14.10 | 138.57 |
| 3.5 mg/l | 2.99 | 7.52 | 3.58 | 11.53 | 131.23 |
| 4.0 mg/l | 3.16 | 6.97 | 3.45 | 12.33 | 135.10 |
| $LSD_{0.05}$ | 0.35 | 1.07 | 0.34 | 2.25 | 6.60 |
| CV | 8.4 | 13.5 | 4.5 | 7.10 | 3.90 |

The effect of 2, 4-D concentration on cane diameter, internode length, tillering capacity and length was significantly ($P<0.05$) higher than the control. However variation in 2, 4-D concentration had no significant effect on all the dependent variables except leaf length and number of tillers per stool. The mean number of tillers per stool was significantly ($P<0.05$) higher (15.07) at 2.0 mg/l of 2, 4-D and lowest at the control (10.27). However, the mean tiller number per stool was not significantly different at the various levels of 2, 4-D applied.

Application of 2, 4-D at various levels significantly increased the cane diameter compared to the control. However, there were no significant differences in cane diameter due to 2, 4-D levels applied implying that increase in 2, 4-D concentration may not be beneficial. The same maybe said about all the other attributes that showed significance except the number of tillers per stool and leaf length.

There was significant difference in leaf length in the i*n vitro* regenerated sugarcane when 2, 4-D was applied at various concentrations. However, there was no significant difference from the control when 2, 4-D was applied at 2.0 and 3.5 mg/L. The longest leaf length was observed at 3 mg/l 2, 4-D. This was however not significantly different from the mean leaf length at 2, 4-D concentration of 2.5 and 4.0 mg/l. Application of 2, 4-D at between 2.5 and 4.0 mg/l is therefore recommended for greater leaf length.

Application of 2, 4-D at various levels significantly increased the number of tillers

per stool compared to the controls. However, significantly greater number of tillers per stool was observed at 2.0 and 3.0 mg/l 2, 4-D than at higher application rates.

### 5.3.2.3 The influence of NAA concentration applied in callus formation media on morphological characteristics of selected sugarcane genotype

As shown in Figure 5.1 the effect of NAA concentration was not significant on any of the parameters were under consideration.

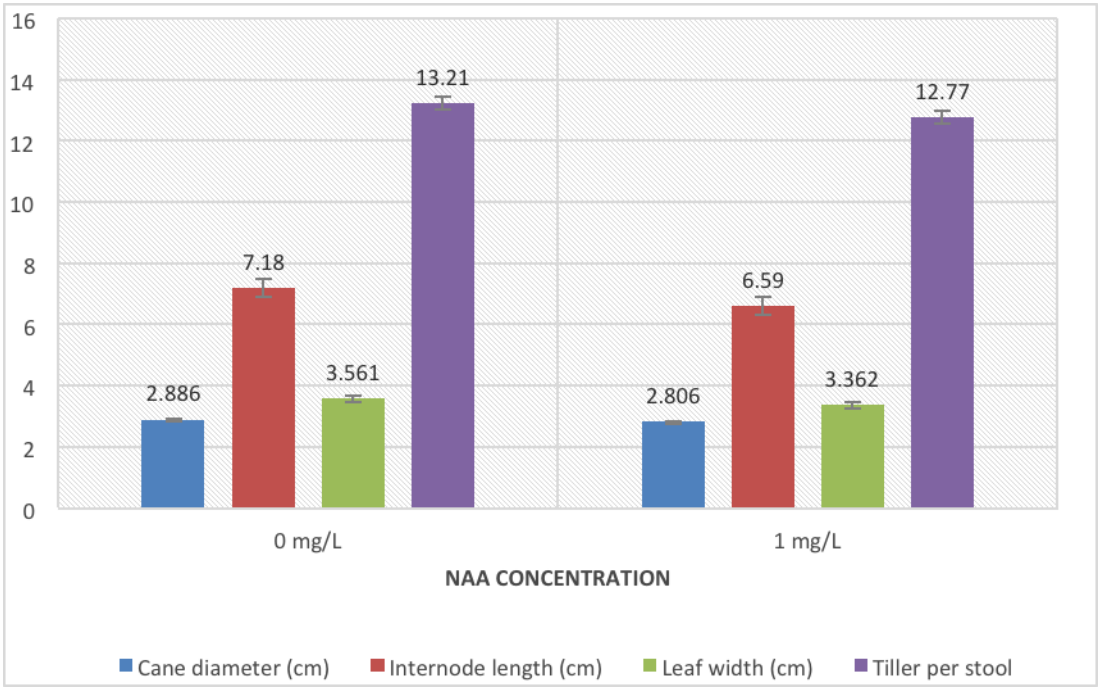


Figure 5.1: The influence of NAA concentration applied in callus formation media on morphological characteristics of sugarcane

Application of NAA in callus formation media had therefore no significant effect on somaclonal variation in the selected sugarcane genotypes.

### 5.3.2.4 The influence of 2, 4-D concentration applied in callus formation media on morphological characteristics of selected sugarcane genotype

The interaction effect between genotype and 2, 4-D concentration was significant for cane diameter, leaf length, and number of tillers per stool. The interaction was however not significant for internode length and leaf width.

As shown in Table 5.3 application of 2, 4-D generally led to a significant increase in cane diameter compared to the controls. Application of 4 mg/l 2, 4-D led to the highest cane diameter in CO421 and N14. However, application of 2, 4-D at the other levels was not significantly different from the controls for all the genotypes.

Application of 2, 4-D at between 2.0 and 4.0 mg/l led to significantly longer leaf length in i*n vitro* regenerated sugarcane genotypes than in the controls. This implies that application of the hormone at these concentrations led to variation in leaf length among the sugarcane genotypes.

The number of tillers per stool was significantly affected by the interaction between sugarcane genotype and 2, 4-D concentration. Significantly fewer tillers per stool were observed in CO945 when 2, 4-D was applied at 3.5 mg/l than in the controls for CO421 and N14. Significantly higher of tillers per stool were observed in N14 and CO945 with 2, 4-D applied at 2.5 and 2.0 mg/l respectfully. Higher numbers of tillers per stool for CO421 were observed at 2, 4-D application of 2.5 mg/l. This therefore implies that application of 2, 4-D at lower levels led to greater numbers of tillers per stool compared to higher concentration.

**Table 5.3: The influence of 2, 4-D concentration applied in callus formation media on morphological characteristics of selected sugarcane genotype**

| NAA/ 2, 4-D conc. (mg/l) | Cane diameter (cm) | Leaf length (cm) | Internode length (cm) | Leaf width (cm) | Tiller per stool (Number) |
|---|---|---|---|---|---|
| **CO421** | | | | | |
| 0.0 mg/L | 2.06 | 121.90 | 5.12 | 3.25 | 7.90 |
| 2.0 mg/L | 2.91 | 114.90 | 6.60 | 3.37 | 12.60 |
| 2.5 mg/L | 3.13 | 132.30 | 7.73 | 3.47 | 14.10 |
| 3.0 mg/L | 2.93 | 140.70 | 8.12 | 3.66 | 12.50 |
| 3.5 mg/L | 2.87 | 130.80 | 7.01 | 3.27 | 11.60 |
| 4.0 mg/L | 3.26 | 122.00 | 6.58 | 3.28 | 9.70 |
| **CO945** | | | | | |
| 0.0 mg/L | 2.36 | 137.40 | 4.89 | 3.22 | 13.80 |
| 2.0 mg/L | 3.07 | 145.00 | 7.38 | 3.40 | 17.60 |
| 2.5 mg/L | 2.80 | 137.00 | 5.73 | 3.34 | 10.10 |
| 3.0 mg/L | 2.95 | 128.50 | 6.31 | 3.44 | 14.70 |
| 3.5 mg/L | 3.13 | 122.80 | 7.41 | 3.62 | 7.70 |
| 4.0 mg/L | 2.66 | 139.00 | 5.61 | 3.31 | 12.80 |
| **N14** | | | | | |
| 0.0 mg/L | 2.29 | 122.50 | 5.90 | 3.56 | 9.10 |
| 2.0 mg/L | 2.84 | 133.50 | 8.19 | 3.40 | 15.00 |
| 2.5 mg/L | 2.79 | 139.70 | 7.88 | 3.48 | 19.70 |
| 3.0 mg/L | 2.66 | 146.50 | 6.43 | 3.62 | 15.10 |
| 3.5 mg/L | 2.98 | 140.10 | 8.13 | 3.85 | 15.30 |
| 4.0 mg/L | 3.55 | 144.30 | 8.73 | 3.77 | 14.50 |
| $LSD_{0.05}$ | 11.53 | 11.53 | 11.53 | 0.45 | 0.45 |
| CV | 8.40 | 8.40 | 8.40 | 3.20 | 3.20 |

### 5.3.2.5 The influence of NAA concentration applied in callus formation media on morphological characteristics of selected sugarcane genotype

The interaction between genotype and NAA application was significant for internode length, leaf width, and number of tillers per stool. The other parameters were not significantly affected by the treatment effect.

In general, except for the sugarcane genotype N14, application of NAA had no significant effect on the internode length (Table 5.4). However, application NAA significantly reduced the length of internodes in CO421 and CO945, while in N14 the contrast was true; the internode length was significantly increased. Further, the internode length where NAA was not applied (control) was not significantly different to NAA application in N14 indicating that NAA application was beneficial to N14 sugarcane genotype.

Table 5.4: The influence of NAA concentration applied in callus formation media on morphological characteristics of selected sugarcane genotype

| NAA concentration/ Genotype | Cane diameter (cm) | Internode length (cm) | Leaf length (cm) | Leaf width (cm) | Tillers per stool (Number) |
|---|---|---|---|---|---|
| **0 mg/l NAA** | | | | | |
| CO421 | 2.89 | 7.75 | 129.97 | 3.62 | 11.92 |
| CO945 | 3.00 | 6.91 | 136.80 | 3.49 | 14.37 |
| N14 | 2.76 | 6.89 | 136.83 | 3.58 | 13.33 |
| **1 mg/l NAA** | | | | | |
| CO421 | 2.82 | 5.97 | 124.23 | 3.15 | 10.87 |
| CO945 | 2.66 | 5.54 | 133.10 | 3.29 | 11.20 |
| N14 | 2.94 | 8.19 | 138.70 | 3.64 | 16.23 |
| $LSD_{0.05}$ | 0.500 | 2.76 | 7.053 | 0.61 | 5.410 |
| CV | 8.40 | 12.10 | 3.90 | 3.2 | 12.00 |

### 5.3.2.6 The influence of the interaction between NAA and 2, 4-D concentration applied in callus formation media on morphological characteristics of selected sugarcane genotype

The effect of the interaction between NAA and 2, 4-D application was significant for cane diameter, leaf length, and number of tillers per stool. The effect was not significant for internode length and leaf width (Appendix 3-7). As shown in Table 5.5 application of NAA at 1 mg/l led to significantly greater tiller numbers per stool in the sugarcane genotypes when 2, 4-D was applied at 2.0 $mgL^{-1}$.

Table 5.5: The influence of the interaction between NAA and 2, 4-D concentration applied in callus formation media on morphological characteristics of selected sugarcane genotype

| NAA/ 2, 4-D conc. (mg/l) | Cane diameter (cm) | Internode length (cm) | Leaf length (cm) | Leaf width (cm) | Tiller per stool (Number) |
|---|---|---|---|---|---|
| **0 mg/L NAA** | | | | | |
| 0.0 mg/L | 2.20 | 4.90 | 128.27 | 3.36 | 6.20 |
| 2.0 mg/L | 2.85 | 7.37 | 126.47 | 3.39 | 8.67 |
| 2.5 mg/L | 2.89 | 7.43 | 136.33 | 3.47 | 7.67 |
| 3.0 mg/L | 3.03 | 7.49 | 141.07 | 3.75 | 10.67 |
| 3.5 mg/L | 3.12 | 8.37 | 131.73 | 3.72 | 8.45 |
| 4.0 mg/L | 3.22 | 7.55 | 143.13 | 3.67 | 9.13 |
| **1 mg/L NAA** | | | | | |
| 0.0 mg/L | 2.27 | 5.71 | 126.27 | 3.33 | 5.33 |
| 2.0 mg/L | 2.03 | 7.41 | 135.80 | 3.39 | 8.60 |
| 2.5 mg/L | 2.92 | 6.80 | 136.13 | 3.38 | 10.67 |
| 3.0 mg/L | 2.66 | 6.41 | 136.07 | 3.39 | 7.67 |
| 3.5 mg/L | 2.87 | 6.67 | 130.73 | 3.44 | 6.27 |
| 4.0 mg/L | 3.09 | 6.39 | 127.07 | 3.23 | 7.13 |
| $LSD_{0.05}$ | 048 | 1.50 | 9.29 | 0.34 | 2.23 |
| CV | 8.40 | 12.10 | 3.90 | 3.20 | 16.80 |

The effect of the application of NAA was not significant between the control and 4.0 mg/l 2, 4-D. In general NAA application appears to be beneficial when 2, 4-D was applied at 2.0 or 2.5 mg/l. Application of 2, 4-D at between 2.0 and 4.0 mg/l led to

significantly greater tillers per stool without NAA application. This implies that at higher 2, 4-D application, NAA application at 1.0 mg/l may not lead to significant increase in number of tillers per stool.

In terms of leaf length, NAA controls gave significantly longer leaves in i*n vitro* regenerated sugarcane genotypes when 2, 4-D was applied at between 2.5 and 4.0 mg/l. 2, 4-D controls with NAA applied at 1.0 mg/l gave significantly lower leaf length. However, application of 2, 4-D at 2.0 mg/l in NAA controls was not significantly different from 2, 4-D controls with NAA application (Table 10).

The interaction effect of NAA and 2, 4-D was significant for cane diameter. Significantly larger cane diameter was observed in NAA controls with 2, 4-D applied at 3.5 mg/l and 4.0 mg/l (Table 5.5).

**5.3.2.8 The influence of the interaction between NAA and 2, 4-D concentration applied in callus formation media on morphological characteristics of selected sugarcane genotypes**

The three-way interaction effect between genotype, NAA and 2, 4-D was significant for all the parameters under consideration (Appendix 4-7).

**a) Internode length (cm) of selected sugarcane genotype as influenced by the interaction between NAA and 2, 4-D concentration applied in callus formation media**

In general the controls (NAA and 2, 4-D not applied) gave significantly lower cane diameter than the treatments in all genotypes.

Table 5.6: Cane diameter (cm) of selected sugarcane genotype as influenced by the interaction between NAA and 2, 4-D concentration applied in callus formation media

| NAA/2, 4-D mg/L | | Sugarcane genotype/ cane diameter (cm) | | |
|---|---|---|---|---|
| | | **CO421** | **CO945** | **N14** |
| **NAA 0** | 0.0 | $1.64^{a}$ | $2.06^{abc}$ | $2.90^{cdefghijk}$ |
| | 2.0 | $2.86^{cdefghijk}$ | $3.28^{fghijkl}$ | $2.40^{abcdef}$ |
| | 2.5 | $3.22^{fghijkl}$ | $2.70^{cdefghijk}$ | $2.76^{cdefghijk}$ |
| | 3.0 | $2.94^{cdefghijk}$ | $3.40^{ijkl}$ | $2.76^{cdefghijk}$ |
| | 3.5 | $3.40^{hijkl}$ | $3.40^{ijkl}$ | $2.56^{cdefghij}$ |
| | 4.0 | $3.30^{fghijkl}$ | $3.16^{efghijkl}$ | $3.20^{efghijkl}$ |
| **NAA 1** | 0.0 | $2.48^{abcdefg}$ | $2.66^{cdefghijk}$ | $1.68^{ab}$ |
| | 2.0 | $2.96^{cdefghijk}$ | $2.86^{cdefghijk}$ | $3.28^{ghijkl}$ |
| | 2.5 | $3.04^{defghijkl}$ | $2.90^{defghijk}$ | $2.82^{cdefghijk}$ |
| | 3.0 | $2.92^{cdefghijk}$ | $2.50^{abcdefgh}$ | $2.56^{cdefghi}$ |
| | 3.5 | $2.34^{abcde}$ | $2.86^{cdefghijk}$ | $3.40^{hijkl}$ |
| | 4.0 | $3.22^{fghijkl}$ | $2.16^{abcd}$ | $3.90^{l}$ |

The interaction effect was not significant in all cases on the cane diameter except for N14 with NAA applied at 1.0 $mgL^{-1}$ and 4.0 $mgL^{-1}$ 2, 4-D that gave significantly higher cane diameter than the other treatments (Table 5.2).

As shown in Table 5.6 at 0.0 mg/l CO945 gave significantly greater cane diameter when 2, 4-D was applied at 2.0, 3.0 and 4.0 $mgL^{-1}$. The cane diameter of N14 was significantly higher at the controls than for the other genotypes. At 1 mg/l NAA application N14 had significantly greater cane diameter at 2.0, 3.5 and 4.0 mg/l 2, 4-D. The cane diameter of CO421 was significantly greater than the other genotypes at 3.0 mg/l with 1 mg/l of NAA applied. At 2, 4-D control (0 mg/l 2, 4-D) CO421 and CO945 gave significantly greater cane diameter than N14.

**a) Internode length (cm) of selected sugarcane genotype as influenced by the interaction between NAA and 2, 4-D concentration applied in callus formation media**

The interaction effect was significant on the internode length of the *in vitro* generated sugarcane with the longest internodes being observed in N14 with NAA applied at 1.0 mg/l and 2, 4-D at 4.0 mg/l (Table 5.7).

Table 5.7: Internode length (cm) of selected sugarcane genotype as influenced by the interaction between NAA and 2, 4-D concentration applied in callus formation media

| NAA/2, 4-D mg/L | | Sugarcane genotype/ Internode length (cm) | | |
|---|---|---|---|---|
| | | **CO421** | **CO945** | **N14** |
| **NAA 0** | 0.0 | $3.88^{ab}$ | $3.88^{ab}$ | $6.94^{cdefgh}$ |
| | 2.0 | $8.02^{ghij}$ | $7.98^{ghij}$ | $6.10^{abcdefg}$ |
| | 2.5 | $7.64^{efghij}$ | $6.74^{cdefgh}$ | $7.72^{efghij}$ |
| | 3.0 | $8.30^{ghijk}$ | $7.48^{defghi}$ | $6.70^{cdefgh}$ |
| | 3.5 | $10.14^{ijk}$ | $7.72^{efghij}$ | $7.24^{cdefgh}$ |
| | 4.0 | $8.36^{ghijk}$ | $7.64^{efghij}$ | $6.66^{cdefgh}$ |
| **NAA 1** | 0.0 | $6.36^{abcdefgh}$ | $5.90^{abcdefg}$ | $4.86^{abcd}$ |
| | 2.0 | $5.18^{abcdef}$ | $6.78^{cdefgh}$ | $10.28^{jk}$ |
| | 2.5 | $7.64^{efghij}$ | $4.72^{abc}$ | $8.04^{ghij}$ |
| | 3.0 | $7.94^{ghij}$ | $5.14^{abcde}$ | $6.16^{abcdefg}$ |
| | 3.5 | $3.88^{ab}$ | $7.10^{cdefgh}$ | $9.02^{hijk}$ |
| | 4.0 | $4.80^{abcd}$ | $3.58^{a}$ | $10.80^{k}$ |

Sugarcane genotype N14 generally gave longer internodes at 2.5 mg/l and 1.0 mg/l NAA, while CO421 seem to thrive at between 2.0 and 4.0 mg/l 2, 4-D with NAA applied. CO945 gave significantly longer internode length at 2.0 mg/l in the absence of NAA.

However 2.5, 3.5 and 4.0 mg/l 2, 4-D gave similar results in the absence of NAA. It can therefore be inferred that for longer internode length a combination of 1.0 mg/l

NAA and any level of 2, 4-D is essential. In contrast, CO421 and CO945 will only require application of 2, 4-D for greater internode length.

**b) Leaf length (cm) of selected sugarcane genotype as influenced by NAA concentration applied callus formation media**

As shown in Table 5.8 significantly longer leaves were observed in i*n vitro* generated sugarcane with only 2, 4-D applied in the callus formation media. The genotype CO945 had significantly longer leaves at 2.0 mg/l 2, 4-D without NAA.

Table 5.8: Leaf length (cm) of selected sugarcane genotype as influenced by the NAA and 2, 4-D concentration applied in callus formation media

| NAA/2, 4-D mg/L | | Sugarcane genotype/ leaf length (cm) | | |
|---|---|---|---|---|
| | | **CO421** | **CO945** | **N14** |
| **NAA 0** | 0.0 | $117.00^{abc}$ | $131.80^{cdefgh}$ | $136.00^{defghi}$ |
| | 2.0 | $109.40^{ab}$ | $152.20^{j}$ | $117.80^{abc}$ |
| | 2.5 | $127.60^{bcdefg}$ | $143.00^{fghij}$ | $139.00^{efghij}$ |
| | 3.0 | $143.60^{ghij}$ | $130.20^{cdefgh}$ | $149.40^{ij}$ |
| | 3.5 | $141.40^{efghij}$ | $118.40^{abc}$ | $135.40^{defghi}$ |
| | 4.0 | $140.80^{efghij}$ | $145.20^{hij}$ | $143.40^{ghij}$ |
| **NAA 1** | 0.0 | $126.80^{cde}$ | $143.00^{fghij}$ | $109.00^{ab}$ |
| | 2.0 | $120.40^{bcd}$ | $137.80^{efghij}$ | $149.20^{ij}$ |
| | 2.5 | $137.00^{efghij}$ | $131.00^{cdefg}$ | $140.40^{ghij}$ |
| | 3.0 | $137.80^{efghij}$ | $126.80^{cde}$ | $145.20^{hij}$ |
| | 3.5 | $120.20^{bcd}$ | $127.20^{cdef}$ | $143.60^{hij}$ |
| | 4.0 | $103.20^{a}$ | $132.80^{cdefgh}$ | $144.80^{hij}$ |

Significantly longer leaves were observed in CO421 and N14 at 2.0 without NAA and with NAA application at all levels of 2, 4-D respectfully. The experiment demonstrated the fact that application of NAA at 1.0 mg/l enhanced leaf length in sugarcane genotypes generally especially in N14.

c) **Leaf width (cm) of selected sugarcane genotype as influenced by the interaction between NAA and 2, 4-D concentration applied in callus formation media**

As shown in Table 5.9 significantly wider leaves were observed in i*n vitro* generated sugarcane variety N14 with 2, 4-D and NAA applied in the callus formation media. The leaf width in N14 increased with increase in 2, 4-D concentration with NAA applied at 1 $mgL^{-1}$ with the widest leaves observed at 3.5 and 4 $mgL^{-1}$ 2, 4-D. The genotype CO421 had significantly wider leaves at 3.0 and 3.5 $mgL^{-1}$ 2, 4-D without NAA. The experiment demonstrated the fact that application of NAA at 1.0 $mgL^{-1}$ enhanced leaf width in sugarcane genotypes generally especially in N14.

Table 5.9: Leaf width (cm) of selected sugarcane genotype as influenced by the interaction between NAA and 2, 4-D concentration applied in callus formation media.

| NAA/2, 4-D mg/L | | **Sugarcane genotype/ leaf width (cm)** | | |
|---|---|---|---|---|
| | | **CO421** | **CO945** | **N14** |
| **NAA 0** | 0.0 | $3.00^{abcde}$ | $3.18^{abcdef}$ | $3.90^{ij}$ |
| | 2.0 | $3.78^{fghij}$ | $3.44^{bcdefghij}$ | $2.94^{abc}$ |
| | 2.5 | $3.62^{fghij}$ | $3.34^{abcdefghi}$ | $3.46^{bcdefghij}$ |
| | 3.0 | $3.98^{j}$ | $3.60^{efghij}$ | $3.68^{fghij}$ |
| | 3.5 | $3.78^{fghij}$ | $3.66^{efghij}$ | $3.80^{ghij}$ |
| | 4.0 | $3.54^{cdefghij}$ | $3.76^{fghij}$ | $3.72^{fghij}$ |
| **NAA 1** | 0.0 | $3.50^{cdefghij}$ | $3.26^{abcdefgh}$ | $3.22^{abcdefg}$ |
| | 2.0 | $2.96^{abcd}$ | $3.36^{abcdefghi}$ | $3.86^{hij}$ |
| | 2.5 | $3.32^{abcdefghi}$ | $3.34^{abcdefghi}$ | $3.50^{cdefghij}$ |
| | 3.0 | $3.34^{abcdefghi}$ | $3.28^{abcdefgh}$ | $3.56^{defghij}$ |
| | 3.5 | $2.76^{a}$ | $3.66^{fghij}$ | $3.90^{ij}$ |
| | 4.0 | $3.02^{abcde}$ | $2.86^{ab}$ | $3.82^{ghij}$ |

d) **The number of tillers per stool in sugarcane as influenced by the interaction between NAA and 2,4-D applied at various concentrations in callus formation media**

AS shown in Table 5.10 significantly higher numbers of tillers per stool was observed in CO945 at 2.0 mg/l 2, 4-D without NAA and in N14 at 2.5 mg/l 2, 4-D with 1.0 mg/l NAA applied. CO945 seem to have performed comparatively better than the other genotypes at 2.0, 3.0 and 4.0 mg/l 2, 4-D without NAA application.

The genotype N14 performed comparatively better than the others at virtually all levels of 2, 4-D application with 1.0 mg/l of NAA applied. CO421 performed significantly better at 2.5 mg/l 2, 4-D when NAA was applied at 1.0 mg/l. Except for the genotype C945, NAA application at 1.0 mg/l NAA in conjunction with 2, 4-D at between 2.0-3.0 mg/l enhances tillering in sugarcane. NAA is shoot multiplication hormone and enhances shooting in plants.

Table 5.10: The effect of the interaction between genotype, NAA and 2, 4-D concentration applied in callus formation media on number of tillers per stool in field grown sugarcane

| NAA/2, 4-D mg/L | | **Sugarcane genotype/ tillers per stool** | | |
|---|---|---|---|---|
| | | **CO421** | **CO945** | **N14** |
| **NAA 0** | 0.0 | $9.20^{abcdef}$ | $13.80^{efghijklm}$ | $9.00^{abcde}$ |
| | 2.0 | $10.40^{bcdefghijk}$ | $21.00^{no}$ | $12.20^{cdefghijkl}$ |
| | 2.5 | $10.00^{abcdefghi}$ | $10.20^{abcdefghi}$ | $17.60^{lmno}$ |
| | 3.0 | $13.80^{efghijklm}$ | $17.40^{lmno}$ | $12.40^{cdefghijkl}$ |
| | 3.5 | $13.60^{efghijklm}$ | $8.00^{abcd}$ | $15.40^{ijklm}$ |
| | 4.0 | $14.60^{fghijklm}$ | $15.80^{klmn}$ | $13.20^{defghijklm}$ |
| **NAA 1** | 0.0 | $6.60^{ab}$ | $13.80^{efghijklm}$ | $9.20^{abcdef}$ |
| | 2.0 | $14.80^{ghijklm}$ | $14.20e^{fghijklm}$ | $17.80^{mno}$ |
| | 2.5 | $18.20^{mno}$ | $10.00^{abcdefghi}$ | $21.80^{o}$ |
| | 3.0 | $11.20^{bcdefghijk}$ | $12.00^{bcdefghijk}$ | $17.80^{mno}$ |
| | 3.5 | $9.60^{abcdefg}$ | $7.40^{abc}$ | $15.20^{ijklm}$ |
| | 4.0 | $4.80^{a}$ | $9.80^{abcdefgh}$ | $15.60^{jklmn}$ |

### 5.3.3 Principal component analysis

The first two principal components (PC 1 and PC II) accounted for 68.88 % of the total variation (Table 5.11). The Eigenvectors decreased significantly from principal component III from 15.77 % to 4.76 % (Table 5.6). Quantitative traits which contributed more to the first principal component (PC) accounted for 49.52 % of the total variation, mainly due to variation in the leaf length, internode length, leaf width, number of tillers per stool and cane diameter. Quantitative traits which contributed more to the second principal component (PC) accounted for 19.36 % of the total variation, mainly due to variation in the internode length and leaf width. Tillers per stool and leaf length had negative contribution towards PC II. The third, fourth and fifth principal component (PC) explained 15.77 %, 10.59 % and 4.76 %, respectively, with both negative and positive contributions by the various traits.

The first PC with a total variation of 49.52 % revealed the most variation among the populations, showing a high degree of correlation among the traits studied. Overall, the PCA analysis under this study shows that phenotypic markers are useful in segregating *in vitro* regenerated sugarcane populations from parent materials since the observed traits were quite outstanding.

Table 5.11: Principal components of analysis of various morphological traits in *in vitro* regenerated sugarcane genotypes through callus induction at various concentrations of 2, 4-D and NAA

| | **PC 1** | **PC II** | **PC III** | **PC IV** | **PCV** |
|---|---|---|---|---|---|
| Eigen value | 2..50 | 1.00 | 0.79 | 0.53 | 0.24 |
| % Total variance | 49.52 | 19.36 | 15.77 | 10.59 | 4.76 |
| Cumulative variance | 49.52 | 68.88 | 84.65 | 95.24 | 100.00 |
| **Factor loading by various traits** | | | | | |
| Cane diameter | 0.37 | 0.29 | -0.79 | 0.38 | 0.02 |
| Internode length | 0.40 | 0.44 | 0.59 | 0.48 | 0.26 |
| Leaf length | 0.52 | -0.40 | -0.06 | -0.36 | 0.66 |
| Leaf width | 0.49 | 0.41 | 0.07 | -0.61 | -0.46 |
| Tillers per stool | 0.44 | -0.63 | 0.13 | 0.34 | -0.53 |

### 5.3.4 Principal components biplot

High phenotypic diversity observed among the populations from treatments (Appendix 2) under present study was also shown by the PCA biplot (Figure 5.2). The biplot display pattern (Figure 5.2) is explained by the similarities in length of internode, leaf length, leaf width and tillers per stool of the studied populations. Nonetheless, populations 4, 5, 6, 10, 14, 28, 29, 30, 34 and 54 are found in the positive right quadrant. They show a close genetic relationship. Similarly 13, 19, 20, and 33 sugarcane populations clustered together in the lower but right quadrant.

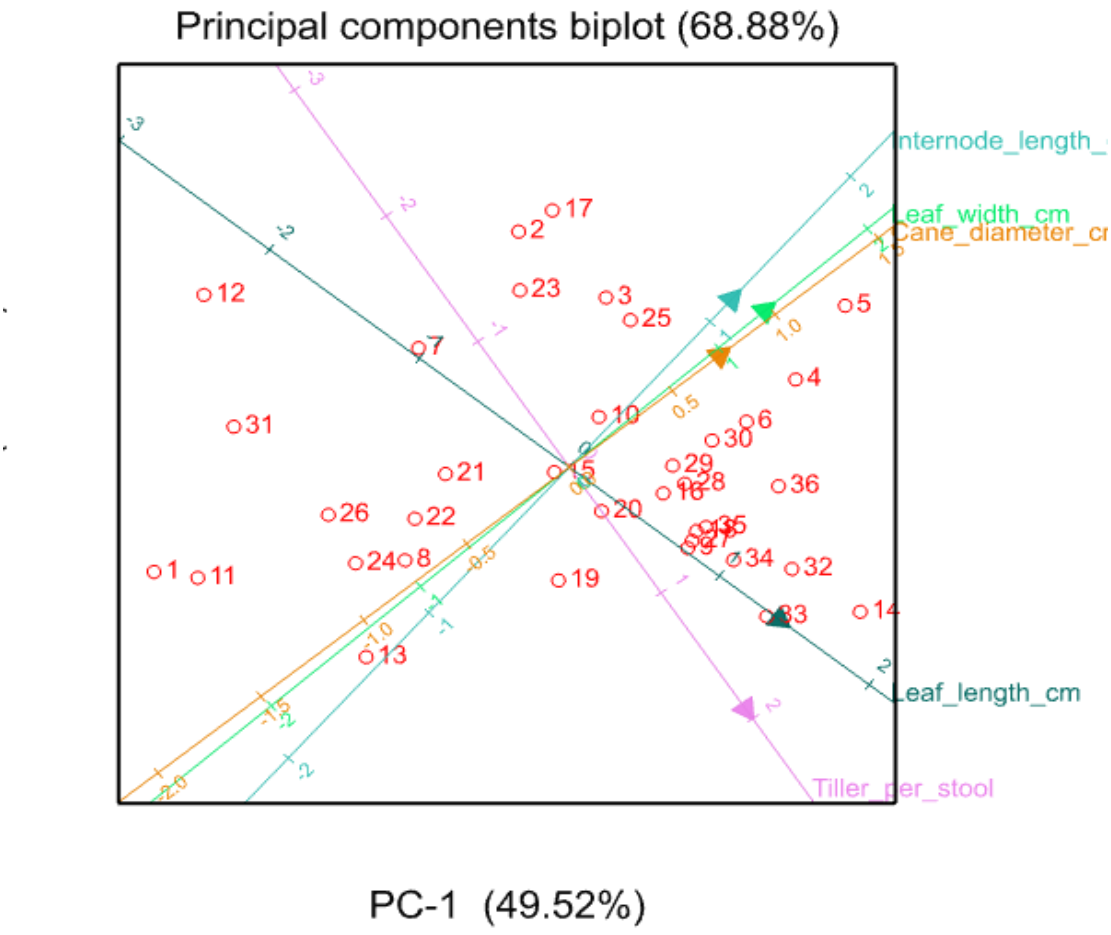


Figure 5.2: Principal component analysis biplot (68%) for Mean effect of genotype and 2, 4-D in concentration ($mgL^{-1}$) applied on callus formation media on cane diameter (cm), internode length (cm), leaf width (cm) and number of tillers per stool

The cluster shows a close relationship with regard to phenotypic traits. Somaclones 1, 7, 8, 11, 12, 15, 21, 22, 24, 26 and 31 revealed a high divergence from other sugarcane somaclones. The loading biplot (Figure 5.2) showed that some quantitative traits were significantly positively correlated for instance leaf length, cane diameter and leaf width. The close association between leaf length and leaf width could significantly influence the cane yield per stool. Therefore, the PCA biplot display revealed the existence of wider phenotypic diversity among the 36 somaclones or treatments (Appendix 2) studied.

### 5.3.5 Hierarchical Cluster analysis

Standardization of data was done before carrying out multivariate analysis so as get uniform units for the 16 quantitative traits studied. The standardized data of the 5 quantitative traits was subjected to cluster analysis. An agglomerative, hierarchical classification technique with variance-covariance matrix sorting strategy and Euclidean distance measure was used for clustering the 36 sugarcane populations or regenerates form callus formation media (Appendix 2).

Cluster analysis dendrogram based on phenotypic traits showed a clear demarcation between populations in terms of genetic distances (Figure 5.3). The percentage similarity ranged from 70 to 100. The dendrogram produced divided the 36 sugarcane populations into three main clusters with the first main cluster comprising 10 populations. The second cluster had 23 populations while the last cluster had only 3 populations. The first cluster mainly consisted of populations originally derived from the sugarcane genotype CO421 through callogenesis. Cluster II consisted of populations mainly derived from CO945 and N14 sugarcane genotypes.

The genetic variation was mainly due to high scores in number of tillers and high grain yield per panicle. The wide genetic distance existing between this population and the populations in other clusters could be exploited for gene mining to improve the crop cane yield and sucrose content.

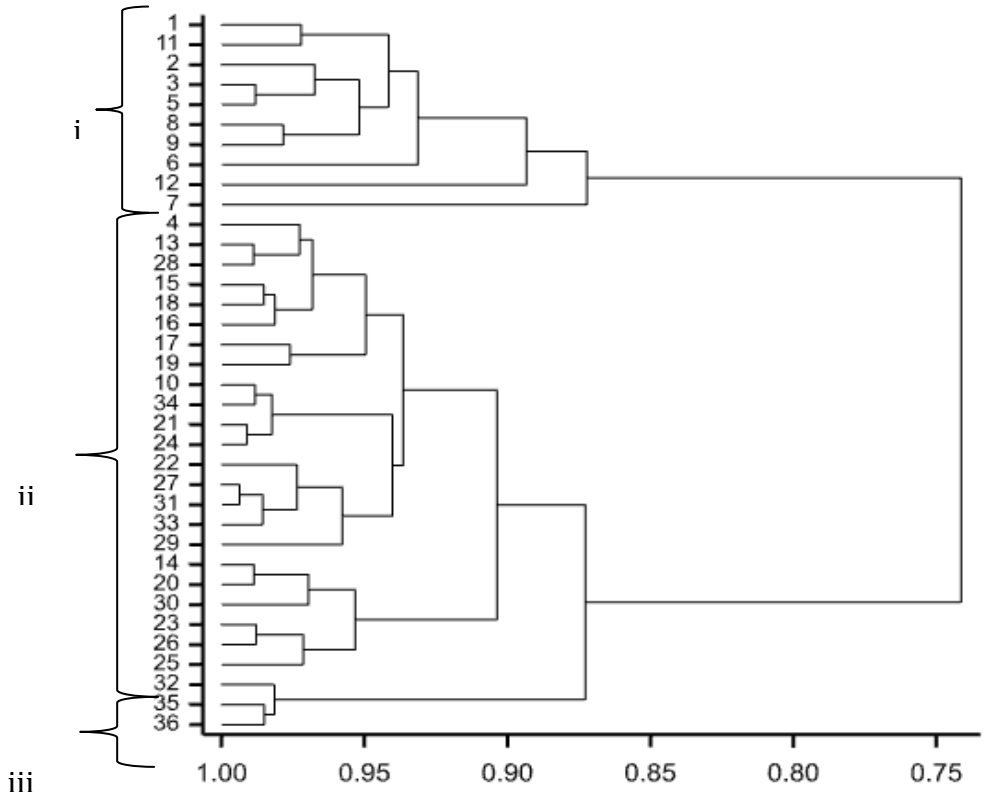


Figure 5.3: Dendrogram of mean effect of genotype and 2, 4-D concentration (mg/L) applied in callus formation media on cane diameter (cm), internode length (cm), leaf width (cm) and number of tillers per stool

**Key**: The somaclones number 1 to 36 indicated in the dendrogram correspond to the treatments as indicated in Appendix 2.

The larger genetic distance between clusters explains that such groups are highly divergent populations for the traits studied. The summary of the cluster means (Table 5.11) of studied populations could help in identification of traits describing the genetic divergence among populations.

### 5.3.5 Correlation analysis of morphological markers

As shown in Table 5.12 sugarcane diameter was very significantly and positively correlated (r=0.433, <0.01) with leaf length. Further, the number of tillers per stool was very significantly and positively correlated (r=0.660, <0.01) with the sugarcane leaf length.

Table 5.12: Spearman Rank Correlation between morphological markers

| | Cane diameter (cm) | Internode length (cm) | Leaf length (cm) | Leaf width (cm) | Tiller per stool (number) |
|---|---|---|---|---|---|
| Cane diameter (cm) | 1.000 | .323$^{ns}$ | .433$^{**}$ | .298$^{ns}$ | .298$^{ns}$ |
| Internode length (cm) | .323$^{ns}$ | 1.000 | .294$^{ns}$ | .385$^{*}$ | .292$^{ns}$ |
| Leaf length (cm) | .433$^{**}$ | .294$^{ns}$ | 1.000 | .348$^{*}$ | .660$^{**}$ |
| Leaf width (cm) | .298$^{ns}$ | .385$^{*}$ | .348$^{*}$ | 1.000 | .073$^{ns}$ |
| Tiller per stool (No) | .298$^{ns}$ | .292$^{ns}$ | .660$^{**}$ | .073$^{ns}$ | 1.000 |

*Ns- Not significant **. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).*

The other parameters that were significantly and positively correlated were leaf width with internode length (r=0.385, <0.05) and leaf width with leaf length (r=348, <0.05).

Cane diameter was equally positively correlated with the internode length, leaf width and number of tiller per stool though the correlation was not significant. Further, it was noted that internode length, leaf width and tillers per stool were positively correlated to each other though the correlation was equally insignificant in all cases.

## 5.4 Discussion

The analysis of variance showed significant (P<0.05) treatment effects due to the three way interaction between genotype, NAA and 2, 4-D for all parameters under consideration (Appendix 7-10). The effect of 2, 4-D concentration was significant (P<0.05) for all parameters except leaf width for which there were no significant differences. NAA application had no significant effect on all parameters under

consideration. The effect of sugarcane genotype was only significant for number of tillers per stool and sugarcane leaf length.

The interaction between genotype and NAA application showed significant differences ($P<0.05$) for leaf length, leaf width, and number of tillers per stool. Genotype/2, 4-D concentration interaction effect was significant for all the parameters under consideration except for internode length and leaf width.

### 5.4.1 Effect of sugarcane genotype on morphological and agronomic characters

Sugarcane genotype had a significant effect on leaf length, and number of tillers per stool in the i*n vitro* regenerated sugarcane. However, it had no effect on the cane diameter, internode length and leaf width.

The sugarcane leaf length (cm) was significantly different among the three sugarcane genotypes. The sugarcane genotype CO421 had significantly shorter leaves (127 cm) than CO945 and N14 (134.95 and 137.77 cm respectively), which were not significantly different (Table 5.1). These differences in leaf length among the three sugarcane genotypes could probably be due to genotypic differences.

A significant difference was noted in number of tillers per stool among the three sugarcane genotypes. Tiller numbers were significantly different among the three sugarcane genotypes with sugarcane variety N14 having the highest mean of 14.78 and CO421 the lowest mean of 11.40. There was however no significant difference in tiller numbers between CO421 and CO945 sugarcane genotypes, though CO945 had comparatively more tillers than CO421. Sugarcane genotypes CO421 and CO945 have common ancestry in Coimbatore, India and could therefore be similar genetically. The differences in tiller numbers per stool among the three sugarcane genotypes could therefore be due to treatment effect.

According to Brown and Thorpe (1995) and Hoy *et al.* (2003) pre-existing variability among cells may play a major role in frequency of somaclonal variation. This explains the similarities among the varieties CO421 and CO945. Besides variations observed in sugarcane morphological characters such as stalk height, girth, stalk

colour, leaf colour. Foliage characteristics, auricle length and flowering among others, variations have been observed tillering among other traits. Variation in tillering among the various varieties of sugarcane could therefore be attributed to somaclonal variation in *in vitro* culture.

### 5.4.2 Effect of 2, 4-D concentration applied in callus formation media on the sugarcane morphological and agronomic attributes

As shown in Table 5.2, except for leaf width all the other attributes were significantly affected by treatment effects. The cane diameter, internode length, leaf length and tiller number were significantly greater than the controls.

The effect of 2, 4-D concentration on cane diameter, internode length, tillering capacity and length was significantly higher than the control. However variation in 2, 4-D concentration had no significant effect on all the dependent variables except leaf length and number of tillers per stool. The mean number of tillers per stool was significantly higher (15.07) at 2.0 mg/l of 2, 4-D and lowest at the control (10.27). However, the mean tiller number per stool was not significantly different at the various levels of 2, 4-D applied.

Application of 2, 4-D had various levels significantly increased the cane diameter compared to the control. However, there were no significant differences in cane diameter due to 2, 4-D levels applied implying that increase in 2, 4-D concentration may not be beneficial. The same maybe said about all the other attributes that showed significance except the number of tillers per stool and leaf length.

There was significant difference in leaf length in the i*n vitro* regenerated sugarcane when 2, 4-D was applied various concentrations. However, there was no significant difference from the control when 2, 4-D was applied at 2.0 and 3.5 mg/L. The longest leaf length was observed at 3 mg/l 2, 4-D. This was however not significantly different from the mean leaf length at 2, 4-D concentration of 2.5 and 4.0 mg/l. Application of 2, 4-D at between 2.5 and 4.0 mg/l is therefore recommended for greater leaf length.

Application of 2, 4-D at various levels significantly increased the number of tillers per stool compared to the controls. However, significantly greater number of tillers per stool was observed at 2.0 and 3.0 mg/l 2, 4-D than at higher application rates.

The findings are therefore in agreement with various reports of somaclonal variation induced by culture media (exposure to growth regulators and length of time in culture) that resulted in variations in traits in sugarcane (Sengar 2010; Rajeswari *et al*. 2009; Patade and Suprasanna 2008; Singh *et al*. 2008; Khan *et al*. 2007a; Gandonou *et al*. 2006; Doule 2006; Abo-Elwafa 2004; Zambrano *et al*. 2003; Zambrano *et al*. 1999; Peros *et al*. 1994; Krishnamurthi and Tlaskal 1974; Heinz 1973; Heinz and Mee 1969). In related studies the synthetic auxin (2, 4-D) has been shown to increase the frequency of blue to pink mutation in the *Tradescantia* stamen hair system (Dolezal and Novak, 1984). Application of 2, 4-D therefore induces somaclonal variation in sugarcane.

### 5.4.3 Effect of NAA concentration (mg/L) applied in callus formation media on sugarcane morphological and agronomic attributes

As shown in Figure 5.1 the effect of NAA concentration was not significant on any of the parameters were under consideration. Application of NAA in callus formation media had therefore no significant effect on somaclonal variation in the selected sugarcane genotypes. NAA does not induce callus formation and therefore it is application in media probably cannot lead to somaclonal variation. However, no research evidence exists in support of the finding.

### 5.4.4 Effect of 2, 4-D concentration (mg/L) applied in callus formation media on morphological and agronomic attributes of sugarcane

The interaction effect between genotype and 2, 4-D concentration was significant for cane diameter, leaf length, and number of tillers per stool. The interaction was however not significant for internode length and leaf width.

As shown in Table 5.3 application of 2, 4-D generally led to a significant increase in cane diameter compared to the controls. Application of 4 mg/l 2, 4-D led to the highest cane diameter in CO421 and N14. However, application of 2, 4-D at the

other levels was not significantly different from the controls for all the genotypes. Application of the hormone at higher concentration appears to increase cane diameter in the two genotypes but not CO945.

Application of 2, 4-D at between 2.0 and 4.0 $mgL^{-1}$ led to significantly longer leaf length in i*n vitro* regenerated sugarcane genotypes than in the controls. This implies that application of the hormone at these concentrations led to variation in leaf length among the sugarcane genotypes.

The number of tillers per stool was significantly affected by the interaction sugarcane genotype and 2, 4-D concentration. Significantly fewer tillers per stool were observed in CO945 when 2, 4-D was applied at 3.5 $mgL^{-1}$ than in the controls for CO421 and N14. Significantly higher of tillers per stool were observed in N14 and CO945 with 2, 4-D applied at 2.5 and 2.0 mg/l respectfully. Higher numbers of tillers per stool for CO421 were observed at 2, 4-D application of 2.5 $mgL^{-1}$. This therefore implies that application of 2, 4-D at lower levels in N14 and CO945 led to greater numbers of tillers per stool compared to higher concentration.

### 5.4.5 Effect of NAA concentration ($mgL^{-1}$) applied in callus formation media on morphological attributes selected sugarcane genotypes

The interaction between genotype and NAA application was significant for internode length, leaf width, and number of tillers per stool. The other parameters were not significantly affected by the treatment effect.

In general, except for the sugarcane genotype N14, application of NAA had no significant effect on the internode length (Table 5.4). However, application NAA significantly reduced the length of internodes in CO421 and CO945, while in N14 the contrast was true; the internode length was significantly increased. Further, the internode length where NAA was not applied (control) was not significantly different to NAA application in N14 indicating that NAA application was beneficial to N14 sugarcane genotype.

### 5.4.6 The effect of 2, 4-D and NAA concentration ($mgL^{-1}$) applied in callus formation media on sugarcane morphological attributes

The effect of the interaction between NAA and 2, 4-D application was significant for cane diameter, leaf length, and number of tillers per stool. The effect was not significant for internode length and leaf width (Appendix 3-7). As shown in Table 5-5 application of NAA at 1 $mgL^{-1}$ led to significantly greater tiller numbers per stool in the sugarcane genotypes when 2, 4-D was applied at 2.0 $mgL^{-1}$.

The effect of the application of NAA led to no significantly between difference the control 4.0 $mgL^{-1}$ 2, 4-D. In general NAA application appears to be beneficial to when 2, 4-D was applied at 2.0 or 2.5 $mgL^{-1}$. Application of 2, 4-D at between 2.0 and 4.0 $mgL^{-1}$ led to significantly greater tillers per stool without NAA application. This implies that at higher 2, 4-D application, NAA application at 1.0 $mgL^{-1}$ may not lead to significant increase in number of tillers per stool.

In terms of leaf length, NAA controls gave significantly longer leaves in i*n vitro* regenerated sugarcane genotypes when 2, 4-D was applied at between 2.5 and 4.0 $mgL^{-1}$. 2, 4-D controls with NAA applied at 1.0 $mgL^{-1}$ gave significantly lower leaf length. However, application of 2, 4-D at 2.0 $mgL^{-1}$ in NAA controls was not significantly different from 2, 4-D controls with NAA application (Table 10).

The interaction effect of NAA and 2, 4-D was significant for cane diameter. Significantly larger cane diameter was observed in NAA controls with 2, 4-D applied at 3.5 $mgL^{-1}$ and 4.0 $mgL^{-1}$ (Table 5.5).

### 5.4.8 The effect of the interaction between sugarcane genotype and NAA and 2, 4-D concentration applied in callus formation media on morphological features

The three-way interaction effect between genotype, NAA and 2, 4-D was significant for all the parameters under consideration (Appendix 4-7).

In general the controls (NAA and 2, 4-D not applied) gave significantly lower cane diameter than the treatments in all genotypes. The interaction effect was not

significant in all cases on the cane diameter except for N14 with NAA applied at 1.0 $mgL^{-1}$ and 4.0 $mgL^{-1}$ 2, 4-D that gave significantly higher cane diameter than the other treatments (Table 5.6). This indicated that the interaction had no effect somaclonal variation in the sugarcane genotypes.

As shown in Table 5.6 at 0.0 $mgL^{-1}$ NAA CO945 gave significantly greater cane diameter when 2, 4-D was applied at 2.0, 3.0 and 4.0 $mgL^{-1}$. The cane diameter of N14 was significantly higher at the controls than for the other genotypes. At 1 mg/l NAA application N14 had significantly greater cane diameter at 2.0, 3.5 and 4.0 $mgL^{-1}$ 2, 4-D. The cane diameter of CO421 was significantly greater than the other genotypes at 3.0 $mgL^{-1}$ with 1 $mgL^{-1}$ of NAA applied. At 2, 4-D control (0 $mgL^{-1}$ 2, 4-D) CO421 and CO945 gave significantly greater cane diameter than N14.

The interaction effect was significant on the internode length of the *in vitro* generated sugarcane with the longest internodes being observed in N14 with NAA applied at 1.0 $mgL^{-1}$ and 2, 4-D at 4.0 $mgL^{-1}$ (Table 5.7).

Sugarcane genotype N14 generally gave longer internodes at 2.5 $mgL^{-1}$ and 1.0 $mgL^{-1}$ NAA, while CO421 seem to thrive at between 2.0 and 4.0 $mgL^{-1}$ 2, 4-D with NAA applied. CO945 gave significantly longer internode length at 2.0 $mgL^{-1}$ in the absence of NAA.

However 2.5, 3.5 and 4.0 $mgL^{-1}$ 2, 4-D gave similar results in the absence of NAA. It can therefore be inferred that for longer internode length a combination of 1.0 $mgL^{-1}$ NAA and any level of 2, 4-D is essential. In contrast, CO421 and CO945 will only require application of 2, 4-D for greater internode length.

As shown in Table 5.8 significantly longer leaves were observed in i*n vitro* generated sugarcane with only 2, 4-D applied in the callus formation media. The genotype CO945 had significantly longer leaves at 2.0 mg/l 2, 4-D without NAA. Significantly longer leaves were observed in CO421 and N14 at 3.0 and 4.0 $mgL^{-1}$ 2, 4-D without NAA. The experiment demonstrated the fact that application of NAA at 1.0 $mgL^{-1}$ enhanced leaf length in sugarcane genotypes generally especially in N14.

As shown in Table 5.9 significantly wider leaves were observed in i*n vitro* generated sugarcane variety N14 with 2, 4-D and NAA applied in the callus formation media. The leaf width in N14 increased with increase in 2, 4-D concentration with NAA applied at 1 $mgL^{-1}$ with the widest leaves observed at 3.5 and 4 $mgL^{-1}$ 2, 4-D. The genotype CO421 had significantly wider leaves at 3.0 and 3.5 $mgL^{-1}$ 2, 4-D without NAA. The experiment demonstrated the fact that application of NAA at 1.0 mg/l enhanced leaf width in sugarcane genotypes generally especially in N14.

AS shown in Table 5.10 significantly higher numbers of tillers per stool was observed in CO945 at 2.0 $mgL^{-1}$ 2, 4-D without NAA and in N14 at 2.5 $mgL^{-1}$ 2, 4-D with 1.0 $mgL^{-1}$ NAA applied. CO945 seem to have performed comparatively better than the other genotypes at 2.0, 3.0 and 4.0 $mgL^{-1}$ 2, 4-D without NAA application.

The genotype N14 performed comparatively better than the others at virtually all levels of 2, 4-D application with 1.0 $mgL^{-1}$ of NAA applied. CO421 performed significantly better at 2.5 $mgL^{-1}$ 2, 4-D when NAA was applied at 1.0 $mgL^{-1}$. Except for the genotype C945, NAA application at 1.0 mg/l NAA in conjunction with 2, 4-D at between 2.0-3.0 $mgL^{-1}$ enhances tillering in sugarcane. NAA is shoot multiplication hormone and enhances shooting in plants.

### 5.4.9 Principal component analysis

The first two principal components (PC 1 and PC II) accounted for 68.88 % of the total variation (Table 5.11). According to Chatfield and Collins (1980), components with an eigenvalue of <1 should be eliminated so that fewer components are dealt with. Moreover, eigenvalues greater than one is considered significant. The Eigenvectors decreased significantly from principal component III from 15.77 % to 4.76 % (Table 5.11). This suggests that after principal component II more principal components did not describe much variation. Thus, only the first two eigenvalues were considered. Quantitative traits which contributed more to the first principal component (PC) accounted for 49.52 % of the total variation, mainly due to variation in the leaf length, internode length, and leaf width, number of tillers per stool and cane diameter. Quantitative traits which contributed more to the second

principal component (PC) accounted for 19.36 % of the total variation, mainly due to variation in the internode length and leaf width. Tillers per stool and leaf length had negative contribution towards PC II. The third, fourth and fifth principal component (PC) explained 15.77 %, 10.59 % and 4.76 %, respectively, with both negative and positive contributions by the various traits.

The first PC with a total variation of 49.52 % revealed the most variation among the populations, showing a high degree of correlation among the traits studied. Overall, the PCA analysis under this study shows that phenotypic markers are useful in segregating *in vitro* regenerated sugarcane populations from parent materials since the observed traits were quite outstanding.

### 5.4.10 Principal components biplot

High phenotypic diversity observed among the populations under present study was also shown by the PCA biplot (Figure 5.2). Biplot analysis of a population by phenotypic traits not only provides the selection criteria but also enables the researcher to consider the population under study as part of an integrated system comprising other components such as selection objectives. The biplot display pattern (Figure 5.7) is explained by the similarities in length of internode, leaf length, leaf width and tillers per stool of the studied populations. Nonetheless, populations 4, 5, 6, 10, 14, 28, 29, 30, 34 and 54 are found in the positive right quadrant. They show a close genetic relationship. Similarly 13, 19, 20, and 33 sugarcane populations clustered together in the lower but right quadrant.

The cluster shows a close relationship with regard to phenotypic traits. Somaclones 1, 7, 8, 11, 12, 15, 21, 22, 24, 26 and 31 revealed a high divergence from other sugarcane somaclones. The loading biplot (Figure 5.2) showed that some quantitative traits were significantly positively correlated for instance leaf length, cane diameter and leaf width. The close association between leaf length and leaf width could significantly influence the cane yield per stool. Therefore, the PCA biplot display revealed the existence of wider phenotypic diversity among the 36 somaclones studied.

### 5.4.11 Hierarchical Cluster analysis

Cluster analysis dendrograms based on phenotypic traits showed a clear demarcation between populations in terms of genetic distances (Figure 5.3). The percentage similarity ranged from 70 to 100. The dendrogram produced divided the 36 sugarcane populations into three main clusters with the first main cluster comprising 10 populations. The second cluster had 23 populations while the last cluster had only 3 populations. The first cluster mainly consisted of populations originally derived from the sugarcane genotype CO421 through callogenesis. Cluster II consisted of populations mainly derived from CO945 and N14 sugarcane genotypes.

The genetic variation was mainly due to high scores in number of tillers and high grain yield per panicle. The wide genetic distance existing between this population and the populations in other clusters could be exploited for gene mining to improve the crop cane yield and sucrose content.

The larger genetic distance between clusters explains that such groups are highly divergent populations for the traits studied. The genetic distance is very useful for population geneticists as it explains genetic variations of crop phenotypic traits in relation to evolutionally history. Therefore the large genetic distance between members of first and second clusters indicates high divergence between these populations hence could be exploited by crossing them to obtain heterozygotes. The summary of the cluster means (Table 5.5) of studied populations could help in identification of traits describing the genetic divergence among populations. The results from this analysis show a wide genetic diversity between and among members of different clusters. Thus, cluster analysis was appropriate for population discrimination through the use of phenotypic markers in population genetics studies.

### 5.4.12 Correlation analysis of morphological markers

As shown in Table 5.12 sugarcane diameter was very significantly and positively correlated (r=0.433, <0.01) with leaf length. Further, the number of tillers per stool was very significantly and positively correlated (r=0.660, <0.01) with the sugarcane

leaf length. This simply implies that an increase in leaf length led to very significant increase in cane diameter and number of tillers per stool.

Whereas leaf surface area trait could contribute to quantity of food synthesized by the plant during photosynthesis, the cane diameter could serve well in storage of water and translocated food from the aerial part of the plant. More food could probably lead to more profuse production of tillers.

The other parameters that were significantly and positively correlated were leaf width with internode length ($r=0.385$, $<0.05$) and leaf width with leaf length ($r=348$, $<0.05$). This implied that the wide the leaves the long the internodes. Again wide leaves imply larger leaf area and hence for food production and bigger storage organs (cane).

Cane diameter was equally positively correlated with the internode length, leaf width and number of tiller per stool though the correlation was not significant. Further, it was noted that internode length, leaf width and tillers per stool were positively correlated to each other though the correlation was equally insignificant in all cases.

Overall, the observed strong and positive correlations among some of the studied quantitative traits indicate similar genetic backgrounds as they reveal gene linkage and pleitropic effects. The findings of the present study reveal the suitability of phenotypic markers in determining the genetic divergence of the studied populations.

## 5.5 Conclusion and Recommendation

In this study a set of sugarcane somaclones derived indirectly through callus culture at various concentration of 2, 4-D and NAA using selected sugarcane genotypes were at four months after transplanting to the field observed for various yield components consisting of internode length, cane diameter, leaf length, number of tillers per stool and leaf width. The data was then subjected to analysis of variance and MANOVA.

- The results of the analysis of variance for the differences indicated that genotype, 2, 4-D and the various interactions had significant effect on the morphological traits.
- The dendrogram demonstrated variation among the somaclones based on morphological traits, could be a valuable source for sugarcane improvement programmes in Western Kenya.
- Application of 2, 4-D to CFM therefore leads to somaclonal variation irrespective of the sugarcane genotype used. The observed variation however has no correlation to the hormonal concentration supplemented in the CFM.
- NAA application in CFM has no effect on somaclonal variation irrespective of 2, 4-D concentration and sugarcane genotype.

Many authors have concluded that *in vitro* culture can be applied as a complementary system to conventional breeding to improve its efficiency, create variants and increase variations within sub-clonal populations (Doule *et al*., 2008; Rajeswari et al., 2009). These somaclonal variations can be exploited by researchers in Kenya for rapid sugarcane improvement programs.

# CHAPTER SIX

# GENERAL DISCUSSION

## 6.1 Effect of 2, 4-D and NAA concentrations on callogenesis and organogenesis in sugarcane

It is evident from the study that 2, 4-D concentration of between 2.0 and 3.5 $mgL^{-1}$ gave the highest percent young leaf spindle disks explants forming primary *callus,* embryogenic *callus* and shoots (Table 3.1). The *callus* produced for the three sugarcane varieties was generally white to creamy-white, compact, dry and nodular (Plate. 3.1)

The results are consistent with reports by several researchers; Pandey *et al* (2011); Ather *et al.* (2009); Mamun *et al.* (2004); Smiullah *et al.* (2012). Smiullah *et al.* (2012) for example in observed that 3 mg L-1 2, 4-D was the most potent concentration with an average of 3.2 *callus* score. Eldessoky *et al.* (2011) obtained the best embryogenic calli at 4 mg L-1 2, 4-D. The three sugarcane varieties under investigation produced poor *callus* with lower regeneration at the controls and lower concentration of 2, 4-D supplementation in MS basal media. The studies showed that the three sugarcane varieties require higher concentration of 2, 4-D for *callus* induction and shoot formation.

As shown in Table 3.3 no significant differences were noted in percent primary *callus* and embryogenic *callus* formation among the sugarcane genotypes. These findings are consistent with those of Sani and Mustapha (2010); Raza *et al.* (2010) and Gandonou *et al.* (2005). Sani and Mustapha for instance in a study on the response of the sugarcane varieties to *callus* induction and embryogenic *callus* production noted that optimum response to *callus* induction was recorded in the sugarcane hybrids; M1176/77 (65.00%), B47419 (61.00%) and M2119/88 (60.75%) and were significantly higher than SP726180 (45.75%). Genotypic response to embryogenic *callus* production demonstrated that, M2119/88, B47419 and M1176/77 exhibited optimum embryogenic *callus*

production of 50.50%, 49.00% and 51.50% respectively, while SP726180 exhibited a significantly lower embryogenic *callus* production of 32.75%. From these results it can be noted that sugarcane varieties that are closely related respond in the same way to 2, 4-D supplementation in MS media. Khan *et al.* (2009) observed non-significant difference in shoot induction from three different sugarcane cultivars. In these cases, highly embryogenic young explants, such as immature leaf roll discs and pre-emergent inflorescences, are the explants of choice (Snyman *et al*. 2006).

As observed in Table 3.4, application of 1 $mgL^{-1}$ NAA to *callus* formation media tended to significantly depress *callus* formation and shooting in the sugarcane genotype N14. However, the three varieties showed no significant differences in primary *callus* formation, embryogenic *callus* formation and shoot formation in either the controls or the 1.0 $mg/^{L-1}$ NAA application. This indicates that NAA treatment in *callus* formation media mighty not be necessary for regeneration of the three sugarcane varieties through callogenesis. These findings are consistent with those of Khattak *et al*, (2014) and Behera and Sahoo (2009). In studies on the effect of different media on callogenesis in sugarcane Khattak *et al* (2014) observed that the addition of NAA at the concentration of 2.0 and 3.0 mg $L^{-1}$ led to production of small amount of *callus* that was grayish globular and hardy in nature. This *callus* turned non-regenerable in subsequent sub-culture. However, in the present study on second sub-culture the *callus* NAA tended to induce early rooting instead of shooting in the explants which is undesirable. This corroborate findings in related studies that in some sugarcane varieties, NAA and kinetin stimulate root formation (Aftab and Iqbal 1999;Gill *et al*. 2002).

Application of 1.0 $mgL^{-1}$ NAA to the media had no significant effect on all the parameters measured as compared to the control (Figure 4-4). However, application of 2, 4-D at between 2.0 and 3.5 $mgL^{-1}$ led to significantly higher percent primary *callus* formation and embryogenic *Callus* formation in both the NAA controls and treatments. A significantly higher percent shoot formation was observed in 2, 4-D treatments in both cases of NAA treatment with 3.0 $mg/L^{-1}$ 2, 4-D giving higher percent shoot formation than in the other levels. NAA treatment in *callus* formation

media is therefore not necessary for sugarcane callogenesis and regeneration. However, application of 2, 4-D at between 2.0 and 3.5 $mgL^{-1}$ is essential.

Similar results were reported by Khattak *et al,* (2014); Sani and Mustapha (2010). For instance in an evaluation of a range of 2, 4-D concentrations (2.5-4.0mg L-1) for *callus* induction and embryogenic *callus* production Sani and Mustapha (2010) noted that for all sugarcane varieties, highest percentage of explants forming *callus* was recorded with 3.5mg L-1 of 2, 4-D and *callus* formation slightly decreased when 2, 4-D was increase to 4.0mg L-1 (83.7%) and progressively decreases with decrease in the 2, 4-D concentration.

According to Sani and Mustapha (2010) the high specificity for 2, 4-D for callogenesis in sugarcane could be attributed to the presence of putative 2, 4-D receptors (auxin-binding protein) present on the surface of cell membrane of the explant. It is believed that 2, 4-D plays an important pivotal role in the dedifferentiation of somatic cells into embryogenic *callus* cells. Michalczuk *et al,* (1992) reported that culture of explant in 2, 4-D containing medium, increases the endogenous auxin levels in the explants. Polar transport of the endogenous auxins (IAA) is essential for the establishment of bilateral symmetry during embryogenesis in monocot (Fischer and Neuhaus, 1996). The efficacy of 2.0 to 3.5 mg $L^{-1}$ 2, 4-D in the induction of *callus* in sugarcane in the study proved that these concentrations are optimum for stimulating considerable increase in the IAA levels in the cultured explant.

This response of sugarcane varieties to i*n vitro* callogenesis could perhaps be attributed to the physiological factors, particularly the endogenous hormones levels. Endogenous hormones levels were perhaps causes of the difference between varieties with various grades of embryogenic competence in sugarcane.

The study showed that there was significant positive correlation between *callus* induction percentage and embryogenic *callus* production percentage, *callus* induction percentage and shoot formation percentage and embryogenic *callus* formation percentage and shoot formation percentage. The high correlation observed indicate

that *callus* induction and embryogenic *callus* production constitute a good index for the ability of sugarcane leaf disk explants to regenerate plants after several weeks of *in vitro* culture. These findings are in agreement with those of Gandonou *et al*. (2005) in a similar study in which high correlation was observed between the ability of sugarcane cultivars to produce embryogenic *callus* and their capacity for plant regeneration. They concluded that embryogenic *callus* percentage constitute a good index for *callus* ability to regenerate later on plantlets. In contrast, they observed no correlation between *callus* induction percentage and embryogenic *callus* percentage and between *callus* induction percentage and plant regeneration percentage indicating that *callus* induction and regeneration capacity may have been controlled by different mechanisms in the study. In the current study this attributes were therefore controlled by the same mechanism as evidenced by the positive correlations.

## 6.2 Elimination of Sugarcane Mosaic Virus through in Vitro Indirect Regeneration

Amongst the viral diseases of sugarcane, SCMV is the most important. It is believed that sugarcane mosaic virus is distributed to all the sugarcane growing countries of the world to an extent that it is almost difficult to get single healthy sugarcane in the field (Naz *et al*., 2009). Yield losses due to SCMV have been reported from almost 39-46% (Hema *et al*., 1997). The conventional methods to overcome the viral problem have been exhausted. However, for the last two decades i*n vitro* techniques have been playing significant effective role in solving the problems of plant viral infection (Ahmad *et al*., 2007). Several authors (Dean, 1983; Kartha 1986; Chatenet *et al*., 2001; Naz *et al*., 2009 ) have reported virus elimination through apical meristem from food crops, including sugarcane, *Brassica oleracea, Pisum sativum, Glycine max* and *Solanum tuberosum*. However, only Dean (1983) and Naz *et al*., (2009) have reported elimination of SCMV in sugarcane through callus culture of young spindle leaves. However, Naz *et al*. (2009) observed that the size of meristem played a pivotal role in elimination of virus in micro-propagated plants. They noted that Plants regenerated from meristems of size 0.2-3.0 mm were all free of SCMV

symptoms, while one plant derived from 4.0 mm and two from 5.0 mm size meristems showed SCMV symptoms. This indicated that the size of the meristem was also a determining factor in elimination of the virus. The larger apices were more prone to retaining virus compared to smaller one.

For Sugarcane Mosaic Virus Indexation, most of the authors used infectivity tests and commonly used *Sorghum bicolor* and other *gramineae* members as indicator plants (Dean, 1983; Lockhart *et al*., 1992; Naz *et al*., 2009; Reddy and Sreenvasulu, 2011). This test is considered as relatively sensitive and extensively in use for virus detection (Kartha, 1986; Rao *et al*., 2001).

The present study revealed the successful regeneration of virus-free plants through callus culture of young spindle leaf; however the virus was not completely eliminated.

The distribution of viruses in plants is uneven and the apical meristems of infected plants are either free or carry a very low concentration of viruses (Bhojwani and Dantu, 2013). The same authors further state that virus titre in the plant increases as the distance from the meristem-tip increases. Several reasons have been proposed for the lack of viruses in the meristem-tip (Bhojwani and Dantu, 2013): virus multiplication is dependent on the metabolism of the host plant. High metabolic activity in the actively dividing meristematic cells does not allow virus replication; the rapid spread of viruses in the plant is through the vascular system which is absent in the meristem. Those viruses invading non-vascular regions move from cell to cell via the plasmodesmatal connections, which is rather slow to keep pace with rapidly growing tip region, ; a high endogenous auxin level in the shoot tips may be inhibitory to the viruses, and the meristem is probably protected by certain 'virus inactivating systems'.

The phenomenon of virus elimination through apical meristem is based on the fact that apical meristems of infected plants are generally either free or carrying very low titer of the virus (Kartha 1986; Naz *et al*. 2009a). The reasons proposed for absence of virus were lack of vascular system, high metabolic activity of meristimatic cells,

and higher endogenous and exogenous level of hormones in cultured meristem which cause inhibition to viral multiplication (Kartha, 1986). In the present study about 3 cm of the apical leaf spindle was used for callus initiation. Most of the materials were fairly mature hence likely to have the vascular systems and hence viral particles. The presence of SCMV could therefore be probably due to this reason. The observation confirms findings by Dean (1982), Naz *et al*. (2009). As observed by Kartha (1986) use of higher concentration of the plant growth hormones (3.5 mg/L 2, 4-D and 1 mg/L NAA) would have led to the reduction in virus infection.

The magnitude of SCMV infection among the three sugarcane genotypes was insignificant though the genotype N14 had slight higher infection than the other genotypes. The genotypes CO421 and CO945 both originated from Coimbatore in India and could be genetically similar. The genotype N14 originated from Natal in South African and is probably genetically different from the other and hence the difference in infectivity.

### 6.3 Effect of 2, 4-D and NAA concentration on morphological and agronomic characters of sugarcane

The investigation revealed that sugarcane genotype had an effect on leaf length, and number of tillers per stool in the i*n vitro* regenerated sugarcane. The sugarcane leaf length (cm) was significantly different among the three sugarcane genotypes. The sugarcane genotype CO421 had significantly shorter leaves (127 cm) than the other two genotypes, CO945 and N14 (134.95 and 137.77 cm respectively), which were not significantly different (Table 5.1). These differences in leaf length among the three sugarcane genotypes could be genetical.

A significant difference was noted in number of tillers per stool among the three sugarcane genotypes. Tiller numbers were significantly different among the three sugarcane genotypes with sugarcane variety N14 having the highest mean of 14.78 and CO421 the lowest mean of 11.40. There was however no significant difference in tiller numbers between Co421 and CO945 sugarcane genotypes, though CO945 had comparatively more tillers than CO421. Sugarcane genotypes CO421 and

CO945 have common ancestry in Coimbatore, India and could therefore be similar genetically. The differences in tiller numbers per stool among the three sugarcane genotypes could therefore be genetical but not due treatment effect. No significant difference was noted among the three genotypes' in cane diameter, internode length and leaf width at four months of age.

As shown in Table 5.2, except for leaf width all the other attributes were significantly affected by the treatments. The cane diameter, internode length, leaf length and tiller number were significantly greater than the controls. The effect of 2, 4-D concentration on cane diameter, internode length, tillering capacity and length was significantly higher than the control. However variation in 2, 4-D concentration had no significant effect on all the dependent variables except leaf length and number of tillers per stool. The mean number of tillers per stool was significantly higher (15.07) at 2.0 mg/l of 2, 4-D and lowest at the control (10.27). However, the mean tiller number per stool was not significantly different at the various levels of 2, 4-D applied.

Application of 2, 4-D had various levels significantly increased the cane diameter compared to the control. However, there were no significant differences in cane diameter due to 2, 4-D levels applied implying that increase in 2, 4-D concentration may not be beneficial. The same maybe said about all the other attributes that showed significance except the number of tillers per stool and leaf length.

There was significant difference in leaf length in the i*n vitro* regenerated sugarcane when 2, 4-D was applied various concentrations. However, there was no significant difference from the control when 2, 4-D was applied at 2.0 and 3.5 mg/L. The longest leaf length was observed at 3 mg/l 2, 4-D. This was however not significantly different from the mean leaf length at 2, 4-D concentration of 2.5 and 4.0 mg/l. Application of 2, 4-D at between 2.5 and 4.0 mg/l is therefore recommended for greater leaf length.

Application of 2, 4-D at various levels significantly increased the number of tillers per stool compared to the controls. However, significantly greater number of tillers

per stool was observed at 2.0 and 3.0 mg/l 2, 4-D than at higher application rates.

As shown in Figure 5.1 the effect of NAA concentration was not significant on any of the parameters were under consideration. Application of NAA in callus formation media had therefore no significant effect on somaclonal variation in the selected sugarcane genotypes. The effect between genotype and 2, 4-D concentration was significant for cane diameter, leaf length, and number of tillers per stool. The interaction was however not significant for internode length and leaf width.

As in Table 5.2 application of 2, 4-D generally led to a significant increase in cane diameter compared to the controls. Application of 4 mg/l 2, 4-D led to the highest cane diameter in CO421 and N14. However, application of 2, 4-D at the other levels was not significantly different from the controls for all the genotypes. Application of 2, 4-D at between 2.0 and 4.0 mg/l led to significantly longer leaf length in i*n vitro* regenerated sugarcane genotypes than in the controls. This implies that application of the hormone at these concentrations led to variation in leaf length among the sugarcane genotypes.

The number of tillers per stool was significantly affected by the interaction sugarcane genotype and 2, 4-D concentration. Significantly fewer tillers per stool were observed in CO945 when 2, 4-D was applied at 3.5 mg/l than in the controls for CO421 and N14. Significantly higher of tillers per stool were observed in N14 and CO945 with 2, 4-D applied at 2.5 and 2.0 mg/l respectfully. Higher numbers of tillers per stool for CO421 were observed at 2, 4-D application of 2.5 mg/l. This therefore implies that application of 2, 4-D at lower levels led to greater numbers of tillers per stool compared to higher concentration.

The interaction between genotype and NAA application was significant for internode length, leaf width, and number of tillers per stool. In general, except for the sugarcane genotype N14, application of NAA had no significant effect on the internode length (Table 5.4). However, application NAA significantly reduced the length of internodes in CO421 and CO945, while in N14 the contrast was true; the internode length was significantly increased. Further, the internode length where

NAA was not applied (control) was not significantly different to NAA application in N14 indicating that NAA application was beneficial to N14 sugarcane genotype.

The effect of the interaction between NAA and 2, 4-D application was significant for cane diameter, leaf length, and number of tillers per stool. The effect was not significant for internode length and leaf width (Appendix 3.7). As shown in Table 5.5 application of NAA at 1 mg/l led to significantly greater tiller numbers per stool in the sugarcane genotypes when 2, 4-D was applied at 2.0 mg/l.

The effect of the application of NAA led to no significantly between difference the control 4.0 mg/l 2, 4-D. In general NAA application appears to be beneficial to when 2, 4-D was applied at either 2.0 or 2.5 mg/l. Application of 2, 4-D at between 2.0 and 4.0 mg/l led to significantly greater tillers per stool without NAA application. This implies that at higher 2, 4-D application, NAA application at 1.0 mg/l may not lead to significant increase in number tillers per stool.

In terms of leaf length, NAA controls gave significantly longer leaves in i*n vitro* regenerated sugarcane genotypes when 2, 4-D was applied at between 2.5 and 4.0 mg/l. 2, 4-D controls with NAA applied at 1.0 mg/l gave significantly lower leaf length. However, application of 2, 4-D at 2.0 mg/l in NAA controls was not significantly different from 2, 4-D controls with NAA application (Table 5.5).

The interaction effect of NAA and 2, 4-D was significant for cane diameter. Significantly larger cane diameter was observed in NAA controls with 2, 4-D applied at 3.5 mg/l and 4.0 mg/l (Table 5.5).

In general the controls (NAA and 2, 4-D not applied) gave significantly lower cane diameter than the treatments in all genotypes. The interaction effect was not significant in all cases on the cane diameter except for N14 with NAA applied at 1.0 mg/l and 4.0 mg/l 2, 4-D that gave significantly higher cane diameter than the other treatments (Table 5.6).

As shown in Table 5.6 at 0.0 mg/l CO945 gave significantly greater cane diameter when 2, 4-D was applied at 2.0, 3.0 and 4.0 mg/l. The cane diameter of N14 was

significantly higher at the controls than for the other genotypes. At 1 mg/l NAA application N14 had significantly greater cane diameter at 2.0, 3.5 and 4.0 mg/l 2, 4-D. The cane diameter of CO421 was significantly greater than the other genotypes at 3.0 mg/l with 1 mg/l of NAA applied. At 2, 4-D control (0 mg/l 2, 4-D) CO421 and CO945 gave significantly greater cane diameter than N14.

The interaction effect was significant on the internode length of the i*n vitro* generated sugarcane with the longest internodes being observed in N14 with NAA applied at 1.0 mg/l and 2, 4-D at 4.0 mg/l (Table 5.7). Sugarcane genotype N14 generally gave longer internodes at 2.5 mg/l and 1.0 mg/l NAA, while CO421 seem to thrive at between 2.0 and 4.0 mg/l 2, 4-D with NAA applied. CO945 gave significantly longer internode length at 2.0 mg/l in the absence of NAA.

However 2.5, 3.5 and 4.0 mg/l 2, 4-D gave similar results in the absence of NAA. It can therefore be inferred that for longer internode length a combination of 1.0 mg/l NAA and any level of 2, 4-D is essential. In contrast, CO421 and CO945 will only require application of 2, 4-D for greater internode length.

As shown in Table 5.8 significantly longer leaves were observed in *in vitro* generated sugarcane with only 2, 4-D applied in the callus formation media. The genotype CO945 had significantly the longest leaves at 2.0 mg/l 2, 4-D without NAA. Significantly longer leaves were observed in CO421 and N14 at 3.0 and 4.0 mg/l 2, 4-D without NAA. Application of NAA at 1.0 mg/l enhanced leaf length in sugarcane genotypes generally especially in N14.

AS shown in Table 5.10 significantly higher number of stools per stool was observed in CO945 at 2.0 mg/l 2, 4-D without NAA and in N14 at 2.5 mg/l 2, 4-D with 1.0 mg/l NAA applied. CO945 seem to have performed comparatively better than the other genotypes at 2.0, 3.0 and 4.0 mg/l 2, 4-D without NAA application.

The genotype N14 performed comparatively better than the others at virtually all levels of 2, 4-D application with 1.0 mg/l of NAA applied. CO421 performed significantly better at 2.5 mg/l 2, 4-D when NAA was applied at 1.0 mg/l. Except for

the genotype C945, NAA application at 1.0 mg/l NAA in conjunction with 2, 4-D at between 2.0-3.0 mg/l enhances tillering in sugarcane. NAA is shoot multiplication hormone and enhances shooting in plants.

The first two principal components (PC 1 and PC II) accounted for 68.88 % of the total variation (Table 5.11). According to Chatfield and Collins (1980), components with an eigenvalue of <1 should be eliminated so that fewer components are dealt with. Moreover, eigenvalues greater than one is considered significant. The Eigenvectors decreased significantly from principal component III from 15.77 % to 4.76 % (Table 5.11). This suggests that after principal component II more principal components did not describe much variation. Thus, only the first two eigenvalues were considered. Quantitative traits which contributed more to the first principal component (PC) accounted for 49.52 % of the total variation, mainly due to variation in the leaf length, internode length, leaf width, number of tillers per stool and cane diameter. Quantitative traits which contributed more to the second principal component (PC) accounted for 19.36 % of the total variation, mainly due to variation in the internode length and leaf width. Tillers per stool and leaf length had negative contribution towards PC II. The third, fourth and fifth principal component (PC) explained 15.77 %, 10.59 % and 4.76 %, respectively, with both negative and positive contributions by the various traits.

The first PC with a total variation of 49.52 % revealed the most variation among the populations, showing a high degree of correlation among the traits studied. Overall, the PCA analysis under this study shows that phenotypic markers are useful in segregating *in vitro* regenerated sugarcane populations from parent materials since the observed traits were quite outstanding.

High phenotypic diversity observed among the populations under present study was also shown by the PCA biplot (Figure 5.2). Biplot analysis of a population by phenotypic traits not only provides the selection criteria but also enables the researcher to consider the population under study as part of an integrated system comprising other components such as selection objectives. The biplot display pattern

(Figure 5.2) is explained by the similarities in length of internode, leaf length, leaf width and tillers per stool of the studied populations. Nonetheless, populations 4, 5, 6, 10, 14, 28, 29, 30, 34 and 54 are found in the positive right quadrant. They show a close genetic relationship. Similarly 13, 19, 20, and 33 sugarcane populations clustered together in the lower but right quadrant.

The cluster shows a close relationship with regard to phenotypic traits. Somaclones 1, 7, 8, 11, 12, 15, 21, 22, 24, 26 and 31 revealed a high divergence from other sugarcane somaclones. The loading biplot (Figure 5.2) showed that some quantitative traits were significantly positively correlated for instance leaf length, cane diameter and leaf width. The close association between leaf length and leaf width could significantly influence the cane yield per stool. Therefore, the PCA biplot display revealed the existence of wider phenotypic diversity among the 36 somaclones studied.

Standardization of data was done before carrying out multivariate analysis so as get uniform units for the 16 quantitative traits studied. The standardized data of the 5 quantitative traits was subjected to cluster analysis. An agglomerative, hierarchical classification technique with variance-covariance matrix sorting strategy and Euclidean distance measure was used for clustering the 36 populations.

Cluster analysis dendrograms based on phenotypic traits showed a clear demarcation between populations in terms of genetic distances (Figure 5.3). The percentage similarity ranged from 70 to 100. The dendrogram produced divided the 36 sugarcane populations into three main clusters with the first main cluster comprising 10 populations. The second cluster had 23 populations while the last cluster had only 3 populations. The first cluster mainly consisted of populations originally derived from the sugarcane genotype CO421 through callogenesis. Cluster II consisted of populations mainly derived from CO945 and N14 sugarcane genotypes.

The genetic variation was mainly due to high scores in number of tillers and high grain yield per panicle. The wide genetic distance existing between this population

and the populations in other clusters could be exploited for gene mining to improve the crop cane yield and sucrose content.

The larger genetic distance between clusters explains that such groups are highly divergent populations for the traits studied. The genetic distance is very useful for population geneticists as it explains genetic variations of crop phenotypic traits in relation to evolutionally history. Therefore the large genetic distance between members of first and second clusters indicates high divergence between these populations hence could be exploited by crossing them to obtain heterozygotes. The summary of the cluster means (Table 5.11) of studied populations could help in identification of traits describing the genetic divergence among populations. The results from this analysis show a wide genetic diversity between and among members of different clusters. Thus, cluster analysis proves to be appropriate for population discrimination through the use of phenotypic markers in population genetics studies.

As shown in Table 5.12 sugarcane diameter was very significantly and positively correlated ($r=0.433$, $<0.01$) with leaf length. Further, the number of tillers per stool was very significantly and positively correlated ($r=0.660$, $<0.01$) with the sugarcane leaf length. This simply implies that an increase in leaf length led to very significant increase in cane diameter and number of tillers per stool. Whereas leaf surface area trait could contribute to quantity of food synthesized by the plant during photosynthesis, the cane diameter could serve well in storage of water and translocated food from the aerial part of the plant. More food could probably lead to more profuse production of tillers.

The other parameters that were significantly and positively correlated were leaf width with internode length ($r=0.385$, $<0.05$) and leaf width with leaf length ($r=348$, $<0.05$). This implied that the wide the leaves the long the internodes. Again wide leaves imply larger leaf area and hence for food production and bigger storage organs (cane).

Cane diameter was equally positively correlated with the internode length, leaf width and number of tiller per stool though the correlation was not significant. Further, it

was noted that internode length, leaf width and tillers per stool were positively correlated to each other though the correlation was equally insignificant in all cases.

Overall, the observed strong and positive correlations among some of the studied quantitative traits indicate similar genetic backgrounds as they reveal gene linkage and pleitropic effects. The findings of the present study reveal the suitability of phenotypic markers in determining the genetic divergence of the studied populations.

# CHAPTER SEVEN

# CONCLUSION AND RECOMMENDATION

In the present study we have tried to optimize and improve the mass propagation protocol of the three sugarcane varieties and studied their regeneration ability in relation to the concentration of 2, 4-D and NAA supplemented in the MS callus formation media. The study revealed that *Callus* can be induced from totipotent tissues of plants including sugarcane young leaf spindle disks. The callogenic response from dissected tissues of the young leaf roll confirmed that leaf sections beyond apical meristematic region could be used for *callus* induction and subsequent regeneration into plants. It is evident that *callus* induction was triggered in all concentration of the hormones. However, the best *callus* induction and regeneration was observed on MS medium supplemented with between 2.0 and 3.5 mg $L^{-1}$ of 2, 4-D in the three sugarcane varieties (CO421, CO945 and N14). It is worth noting that from the study NAA application may not be essential for sugarcane callogenesis.

The investigation sought to determine whether callus culture at various concentration of 2, 4-D and NAA can eliminate sugarcane mosaic virus from i*n vitro* regenerated materials of the three sugarcane varieties CO421, CO945 and N14. From the foregoing discussion it may be concluded that callus culture at the various concentration of the hormones does not entirely eliminate SCMV from the sugarcane genotypes. It can therefore be concluded that callogenesis of the sugarcane varieties CO421, CO945 and N14 at various concentrations of 2, 4-D and NAA does not eliminate the SCMV. This conclusion is in conformity with findings by various authors (Naz *et al*., 2009; Parmessur *et al*. 2002) in related studies. Callogenesis is therefore not recommended for the multiplication of disease free sugarcane planting materials as it may lead to spread of SCMV.

Further research should be undertaken on the effect of the location of the leaf spindle relative the actively growing on elimination of the disease.

In this study a set of sugarcane somaclones derived indirectly through callus culture at various concentration of 2, 4-D and NAA using selected sugarcane genotypes were at four months after transplanting to the field observed for various yield components consisting of internode length, cane diameter, leaf length, number of tillers per stool and leaf width. The data was then subjected to analysis of variance and MANOVA.

- The results of the analysis of variance for the differences indicated that genotype, 2, 4-D and the various interactions had significant effect on the various morphological traits.
- The dendrogram demonstrated variation among the somaclones based on morphological traits, could be a valuable source for sugarcane improvement programmes in Western Kenya.
- Application of 2, 4-D to callus formation media therefore leads to somaclonal variation irrespective of the sugarcane genotype used. The observed variation however has no correlation to the hormonal concentration supplemented in the callus formation media.
- NAA application in callus formation media has no effect on somaclonal variation irrespective of 2, 4-D concentration and sugarcane genotype.

It is hoped that these findings will benefit Kenyan researchers and other stakeholders by providing relevant information and adding more to the already existing knowledge on sugarcane *in vitro* culture. It may also assist policy makers in the Ministry of Agriculture in evaluation of its policy on biotechnology in order to make it more effective in developing suitable programmers to address the problems in agriculture. The gaps that may emerge may serve as an eye opener for further research in this area.

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## APPENDICES

Appendix 1**:** Composition of Murashige and Skoog (1962) Medium

| Constituent | Quantity mg per liter of media |
|---|---|
| A. Stock 1: Macronutrients | |
| **NH4NO3** | 33000 |
| **KNO3** | **38000** |
| **CaCl2.2H2O** | 8800 |
| **MgSO4.7H2O** | 7400 |
| **KH2PO4** | 3400 |
| B. Stock II: Micronutrients | |
| **H3BO3** | **1240** |
| **MnSO4.4H2O** | 4460 |
| **ZnSO4.7H2O** | 1720 |
| **KI** | 166 |
| **Na2MoO4.2H2O** | 50 |
| **CoCl2.6H2O** | 5 |
| **CuSO4.7H2O** | 5 |
| C. STOCK III[b] | |
| **NaEDTA.2H2O** | 7460 |
| **FeSO4.7H2O** | 5560 |
| C. Stock IV: Vitamins | |
| **MYOINOSITOL** | 20000 |
| **GLYCINE** | 400 |
| **NICOTINIC ACID** | 100 |
| **PYRIDOXINE HCL** | 100 |
| **THIAMINE HCL** | 20 |

**Source:** (Information, 1994)

Appendix 2: Laboratory experiment treatment table

| S/NO | TREATMENT | S/NO | TREATMENT | S/NO | TREATMENT |
|---|---|---|---|---|---|
| 1 | V1H0N0 | 13 | V2H0N0 | 25 | V3H0N0 |
| 2 | V1H1N0 | 14 | V2H1N0 | 26 | V3H1N0 |
| 3 | V1H2N0 | 15 | V2H2N0 | 27 | V3H2N0 |
| 4 | V1H3N0 | 16 | V2H3N0 | 28 | V3H3N0 |
| 5 | V1H4N0 | 17 | V2H4N0 | 29 | V3H4N0 |
| 6 | V1H5N0 | 18 | V2H5N0 | 30 | V3H5N0 |
| 7 | V1H0N1 | 19 | V2H0N1 | 31 | V3H0N1 |
| 8 | V1H1N1 | 20 | V2H1N1 | 32 | V3H1N1 |
| 9 | V1H2N1 | 21 | V2H2N1 | 33 | V3H2N1 |
| 10 | V1H3N1 | 22 | V2H3N1 | 34 | V3H3N1 |
| 11 | V1H4N1 | 23 | V2H4N1 | 35 | V3H4N1 |
| 12 | V1H5N1 | 24 | V2H5N1 | 36 | V3H5N1 |

**KEY**

V1 = Sugarcane variety Co 421

V2 = Sugarcane variety Co 945

V3 = Sugarcane variety N 14

H1 = 0.0 mg/L 2, 4 –D

H2 = 2.0 mg/L 2, 4 –D

H3 = 2.5 mg/L 2, 4 -D

H4 = 3.0 mg/L 2, 4 -D

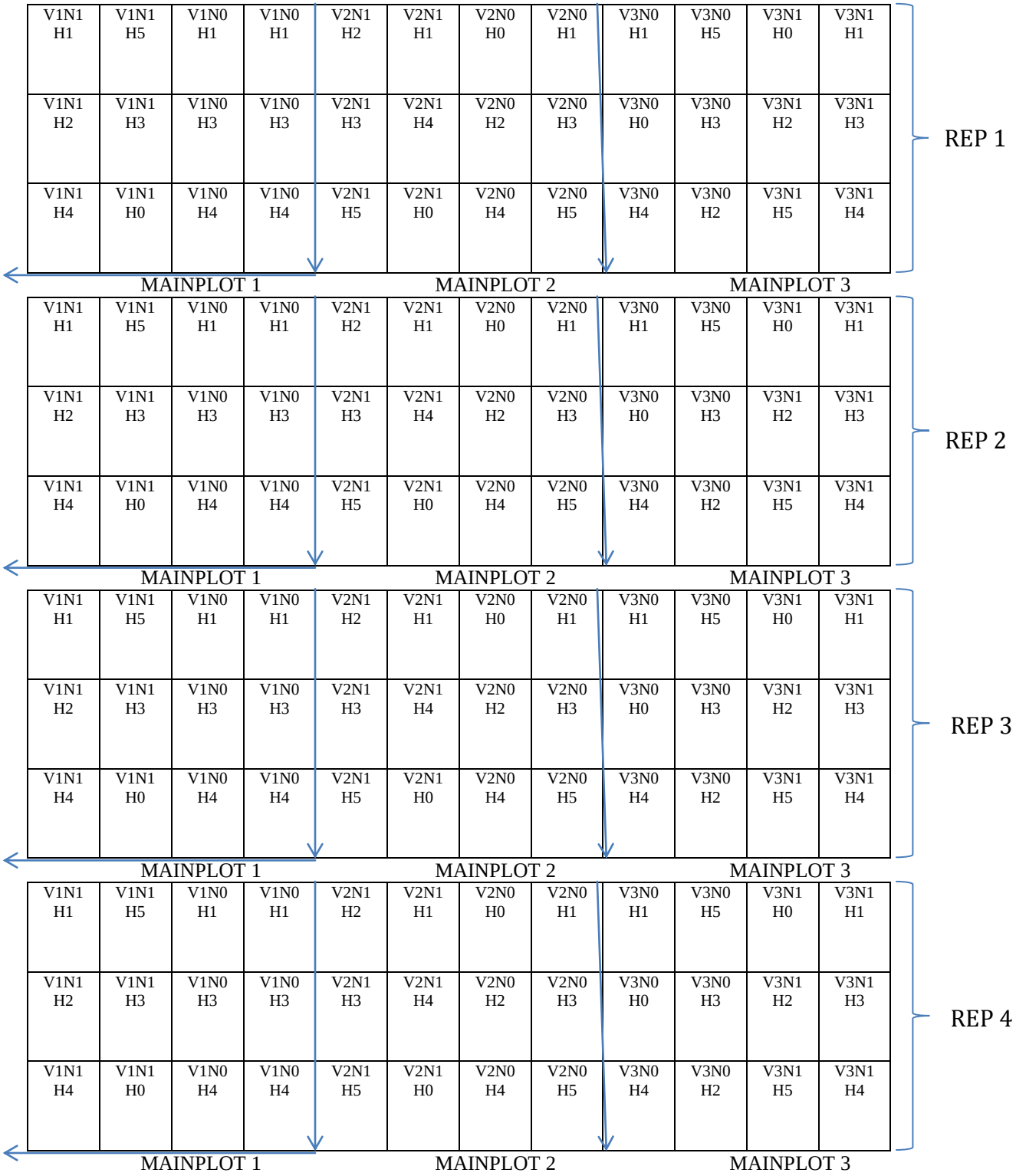
H5 = 3.5 mg/L 2, 4-D

H6 = 4.0 mg/L 2, 4-D

N1 = 0.0 mg/L NAA

N2 = 1.0 mg/L NAA

Appendix 3: Field experiment layout in split-split plot design




**KEY**: MAINPLOT FACTOR = SUGARCANE VARIETIES (V); SUBPLOT FACTOR = NAA (N); SUB-SUBPLOT FACTOR = 2, 4-D (H)

Appendix 4: Analysis of variance table of the effect of sugarcane genotype, NAA and 2, 4-D concentration on *callus* formation

| Source | DF | SS | MS | F value | Pr > F |
|---|---|---|---|---|---|
| GENOTYPE | 2 | 8.6 | 4.3 | 0.03 | 0.966ns |
| NAA | 1 | 38.1 | 38.1 | 0.30 | 0.582ns |
| 2_4_D | 5 | 50779.9 | 10156.0 | 81.32 | <.001** |
| GENOTYPE.NAA | 2 | 751.7 | 375.8 | 3.01 | 0.052* |
| GENOTYPE.%2_4_D | 10 | 752.9 | 75.3 | 0.60 | 0.810ns |
| NAA.2_4_D | 5 | 757.5 | 151.5 | 1.21 | 0.306ns |
| GENOTYPE.NAA.%2_4_D | 10 | 957.3 | 95.7 | 0.77 | 0.661ns |
| Residual | 144 | 17983.8 | 124.9 | | |
| Total | 179 | 72029.7 | | | |

*ns – Not significant, * ---Significant at 5% ** ---Significant at 1%*

Appendix 5: Analysis of variance table of the effect of sugarcane genotype, NAA and 2, 4-D concentration on embryogenic *callus* induction

| Source | DF | SS | MS | F value | Pr > F |
|---|---|---|---|---|---|
| GENOTYPE | 2 | 393.1 | 196.6 | 1.21 | 0.302ns |
| NAA | 1 | 4.9 | 4.9 | 0.03 | 0.863ns |
| 2_4_D | 5 | 24562.1 | 4912.4 | 30.13 | <.001** |
| GENOTYPE.NAA | 2 | 383.5 | 191.7 | 1.18 | 0.311ns |
| GENOTYPE.2_4_D | 10 | 1004.4 | 100.4 | 0.62 | 0.798ns |
| NAA.2_4_D | 5 | 2158.6 | 431.7 | 2.65 | 0.025* |
| GENOTYPE.NAA.2_4_D | 10 | 2133.6 | 213.4 | 1.31 | 0.231ns |
| Residual | 144 | 23476.6 | 163.0 | | |
| Total | 179 | 54116.9 | | | |

*ns – Not significant, * ---Significant at 5%, ** ---Significant at 1%*

Appendix 6: Analysis of variance table of the effect of sugarcane genotype, NAA and 2, 4-D concentration on % shoot induction

| **Source** | **df** | **SS** | **MS** | **Fvalue** | **Pr>F** |
|---|---|---|---|---|---|
| GENOTYPE | 2 | 3592.1 | 1796.0 | 7.91 | <.001** |
| NAA | 1 | 65.6 | 65.6 | 0.29 | 0.592ns |
| 2_4_D | 5 | 15116.9 | 3023.4 | 13.32 | <.001** |
| GENOTYPE.NAA | 2 | 2186.6 | 1093.3 | 4.82 | 0.009** |
| GENOTYPE.2_4_D | 10 | 2909.6 | 291.0 | 1.28 | 0.246ns |
| NAA.2_4_D | 5 | 1502.6 | 300.5 | 1.32 | 0.257ns |
| GENOTYPE.NAA.2_4_D | 10 | 1444.2 | 144.4 | 0.64 | 0.781ns |
| Residual | 144 | 32677.5 | 226.9 | | |
| Total | 179 | 59495.1 | | | |

*ns – Not significant, * ---Significant at 5%, ** ---Significant at 1%*

Appendix 7: Analysis of variance table of the effect of sugarcane genotype, NAA and 2, 4-D concentration on *number of days to rooting*

| Source | DF | SS | MS | F value | Pr > F |
|---|---|---|---|---|---|
| GENOTYPE | 2 | 375.644 | 87.822 | 73.98 | <.001** |
| NAA | 1 | 619.756 | 19.756 | 44.11 | <.001** |
| 2_4_D | 5 | 39.711 | 7.942 | 3.13 | 0.010** |
| GENOTYPE.NAA | 2 | 22.578 | 11.289 | 4.45 | 0.013** |
| GENOTYPE.%2_4_D | 10 | 151.689 | 15.169 | 5.97 | <.001** |
| NAA.2_4_D | 5 | 253.978 | 50.796 | 20.01 | <.001** |
| GENOTYPE.NAA.%2_4_D | 10 | 135.289 | 13.529 | 5.33 | <.001** |
| Residual | 144 | 365.600 | 2.539 | | |
| Total | 179 | 1964.244 | | | |

*ns – Not significant, * ---Significant at 5% ** ---Significant at 1%*

Appendix 8: Analysis of variance table of the effect of sugarcane genotype, NAA and 2, 4-D concentration on *number of length of shoots*

| Source | DF | SS | MS | F value | Pr > F |
|---|---|---|---|---|---|
| GENOTYPE | 2 | 28.544 | 14.272 | 4.93 | 0.008 |
| NAA | 1 | 0.356 | 0.356 | 0.12 | 0.726 |
| 2_4_D | 5 | 0.711 | 0.142 | 0.05 | 0.999 |
| GENOTYPE.NAA | 2 | 9.811 | 4.906 | 1.69 | 0.187 |
| GENOTYPE.%2_4_D | 10 | 13.056 | 1.306 | 0.45 | 0.918 |
| NAA.2_4_D | 5 | 5.778 | 1.156 | 0.40 | 0.849 |
| GENOTYPE.NAA.%2_4_D | 10 | 8.856 | 0.886 | 0.31 | 0.979 |
| Residual | 144 | 416.800 | 2.894 | | |
| Total | 179 | 483.911 | | | |

*ns – Not significant, * ---Significant at 5% ** ---Significant at 1%*

Appendix 9: Analysis of variance table of the effect of sugarcane genotype, NAA and 2, 4-D concentration on *number of roots per shoots*

| Source | DF | SS | MS | F value | Pr > F |
|---|---|---|---|---|---|
| GENOTYPE | 2 | 55.21 | 27.61 | 2.52 | 0.084 |
| NAA | 1 | 4.05 | 4.05 | 0.37 | 0.544 |
| 2_4_D | 5 | 366.56 | 73.31 | 6.69 | <.001 |
| GENOTYPE.NAA | 2 | 7.43 | 3.72 | 0.34 | 0.713 |
| GENOTYPE.%2_4_D | 10 | 38.26 | 3.83 | 0.35 | 0.966 |
| NAA.2_4_D | 5 | 6.52 | 1.30 | 0.12 | 0.988 |
| GENOTYPE.NAA.%2_4_D | 10 | 38.30 | 3.83 | 0.35 | 0.965 |
| Residual | 144 | 1578.00 | 10.96 | | |
| Total | 179 | 2094.33 | | | |

*ns – Not significant, * ---Significant at 5% ** ---Significant at 1%*

Appendix 10: Analysis of variance table of the effect of sugarcane genotype, NAA and 2, 4-D concentration on percentage shoots with roots

| Source | DF | SS | MS | F value | Pr > F |
|---|---|---|---|---|---|
| GENOTYPE | 2 | 1466.0 | 733.0 | 5.30 | 0.006 |
| NAA | 1 | 19923.8 | 9923.8 | 44.19 | <.001 |
| 2_4_D | 5 | 1665.2 | 333.0 | 2.41 | 0.039 |
| GENOTYPE.NAA | 2 | 1031.9 | 515.9 | 3.73 | 0.026 |
| GENOTYPE.%2_4_D | 10 | 2361.8 | 236.2 | 1.71 | 0.084 |
| NAA.2_4_D | 5 | 1673.1 | 334.6 | 2.42 | 0.038 |
| GENOTYPE.NAA.%2_4_D | 10 | 3489.9 | 349.0 | 2.53 | 0.008 |
| Residual | 144 | 19897.7 | 138.2 | | |
| Total | 179 | 51509.4 | | | |

Appendix 11: Analysis of variance of the effect of sugarcane genotype, NAA and 2, 4-D concentration on % leaf length (cm)

| **Source** | **df** | **SS** | **MS** | **Fvalue** | **Pr>F** |
|---|---|---|---|---|---|
| BLOCK | 4 | 231.2 | 57.8 | 0.32 | |
| GENOTYPE | 2 | 3666.7 | 1833.3 | 10.12 | 0.006** |
| RESIDUE | 8 | 1448.7 | 181.1 | 1.14 | |
| NAA | 1 | 286.3 | 286.3 | 1.80 | 0.204* |
| NAA.GENOTYPE | 2 | 464.4 | 232.2 | 1.46 | 0.270* |
| RESIDUE | 12 | 1903.9 | 158.7 | 0.95 | |
| 2,4-D | 5 | 2566.2 | 513.2 | 3.08 | 0.012** |
| 2,_4-D*.GENOTYPE | 10 | 8672.1 | 867.2 | 5.21 | <0.001*** |
| 2,_4-D*.NAA | 5 | 2529.30 | 505.9 | 3.04 | 0.013** |
| 2,_4-D*.GENOTYPE*.NAA | 10 | 8629.9 | 863.0 | 5.18 | <0.001*** |
| RESIDUE | 120 | 19993.1 | 166.6 | | |
| TOTAL | 179 | 50391.7 | | | |

*ns – Not significant, * ---Significant at 5% level of significance*

Appendix 12: Analysis of variance of the effect of sugarcane genotype, NAA and 2, 4-D concentration on cane diameter (cm)

| **Source** | **df** | **SS** | **MS** | **Fvalue** | **Pr>F** |
|---|---|---|---|---|---|
| BLOCK | 4 | 8.3852 | 2.0963 | 1.68 | |
| GENOTYPE | 2 | 0.0323 | 0.0162 | 0.01 | 0.987ns |
| RESIDUE | 8 | 9.9688 | 1.2461 | 3.62 | |
| NAA | 1 | 0.2722 | 0.2722 | 0.79 | 0.391ns |
| NAA.GENOTYPE | 2 | 2.0308 | 1.0154 | 2.95 | 0.091** |
| RESIDUE | 12 | 4.1253 | 0.3438 | 0.74 | |
| 2,4-D | 5 | 15.0607 | 3.0121 | 6.49 | <0.001** |
| 2,_4-D*.GENOTYPE | 10 | 6.4730 | 0.6473 | 1.39 | 0.191* |
| 2,_4-D*.NAA | 5 | 1.6818 | 0.3364 | 0.72 | 0.607* |
| 2,_4-D*.GENOTYPE*.NAA | 10 | 16.1612 | 1.6161 | 3.48 | <0.001** |
| RESIDUE | 120 | 19993.1 | 166.6 | | |
| TOTAL | 179 | 119.9280 | | | |

*ns – Not significant, * ---Significant at 5% level of significance*

Appendix 13: Analysis of variance of the effect of sugarcane genotype, NAA and 2, 4-D concentration on number of tillers per stool

| **Source** | **df** | **SS** | **MS** | **Fvalue** | **Pr>F** |
|---|---|---|---|---|---|
| BLOCK | 4 | 122.98 | 30.74 | 1.94 | |
| GENOTYPE | 2 | 347.21 | 173.61 | 10.97 | 0.005** |
| RESIDUE | 8 | 126.62 | 15.83 | 1.09 | |
| NAA | 1 | 8.89 | 8.89 | 0.61 | 0.450* |
| NAA.GENOTYPE | 2 | 284.74 | 142.37 | 9.79 | 0.003** |
| RESIDUE | 12 | 174.53 | 14.54 | 0.75 | |
| 2,4-D | 5 | 546.44 | 109.29 | 5.63 | <0.001** |
| 2,_4-D*.GENOTYPE | 10 | 883.92 | 88.39 | 4.55 | <0.001** |
| 2,_4-D*.NAA | 5 | 307.44 | 61.49 | 3.17 | 0.010** |
| 2,_4-D*.GENOTYPE*.NAA | 10 | 416.52 | 41.65 | 2.14 | 0.026** |
| RESIDUE | 120 | 2330.67 | 19.42 | | |
| TOTAL | 179 | 5549.98 | | | |

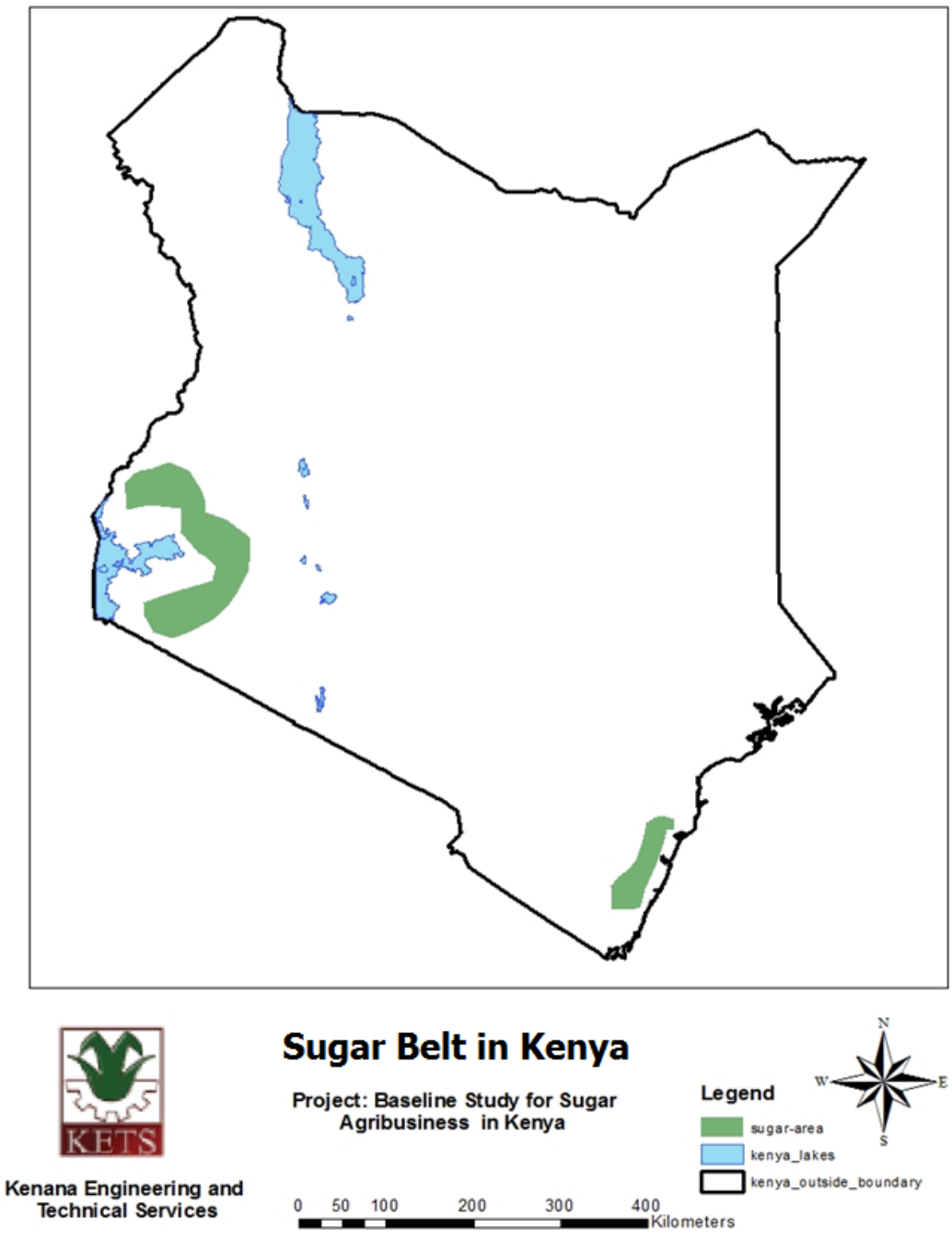
*ns – Not significant, * ---Significant at 5% level of significance*

Appendix 14: Analysis of variance of the effect of sugarcane genotype, NAA and 2, 4-D concentration on internode length (cm)

| **Source** | **df** | **SS** | **MS** | **Fvalue** | **Pr>F** |
|---|---|---|---|---|---|
| BLOCK | 4 | 86.023 | 21.506 | 2.07 | |
| GENOTYPE | 2 | 52.424 | 26.212 | 2.52 | 0.141** |
| RESIDUE | 8 | 83.061 | 10.383 | 2.51 | |
| NAA | 1 | 17.236 | 17.236 | 4.16 | 0.064** |
| NAA.GENOTYPE | 2 | 84.150 | 42.075 | 10.16 | 0.003** |
| RESIDUE | 12 | 49.698 | 4.141 | 0.94 | |
| 2,4-D | 5 | 49.698 | 19.318 | 4.41 | 0.001** |
| 2,_4-D*.GENOTYPE | 10 | 72.563 | 7.256 | 1.65 | 0.099** |
| 2,_4-D*.NAA | 5 | 31.121 | 6.224 | 1.42 | 0.222** |
| 2,_4-D*.GENOTYPE*.NAA | 10 | 219.204 | 21.920 | 5.00 | <0.001** |
| RESIDUE | 120 | 526.246 | 4.385 | | |
| TOTAL | 179 | 1318.318 | | | |

*ns – Not significant, * ---Significant at 5% level of significance*

Appendix 15: Sugarcane growing zones in Kenya

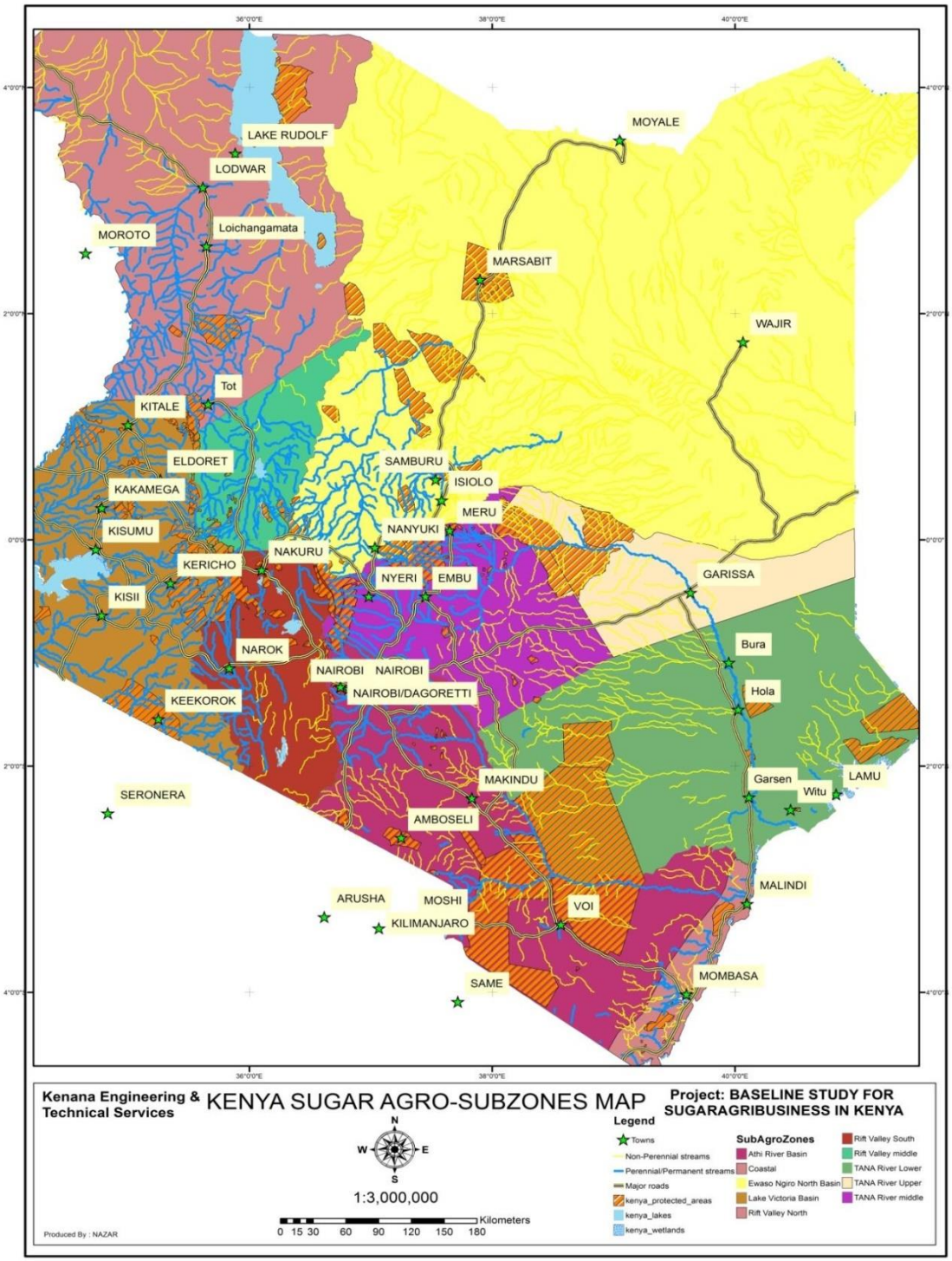



**Source:** Kenani Engineering and Technical Services, 2013

**Appendix 16**: **Map of Kakamega County**

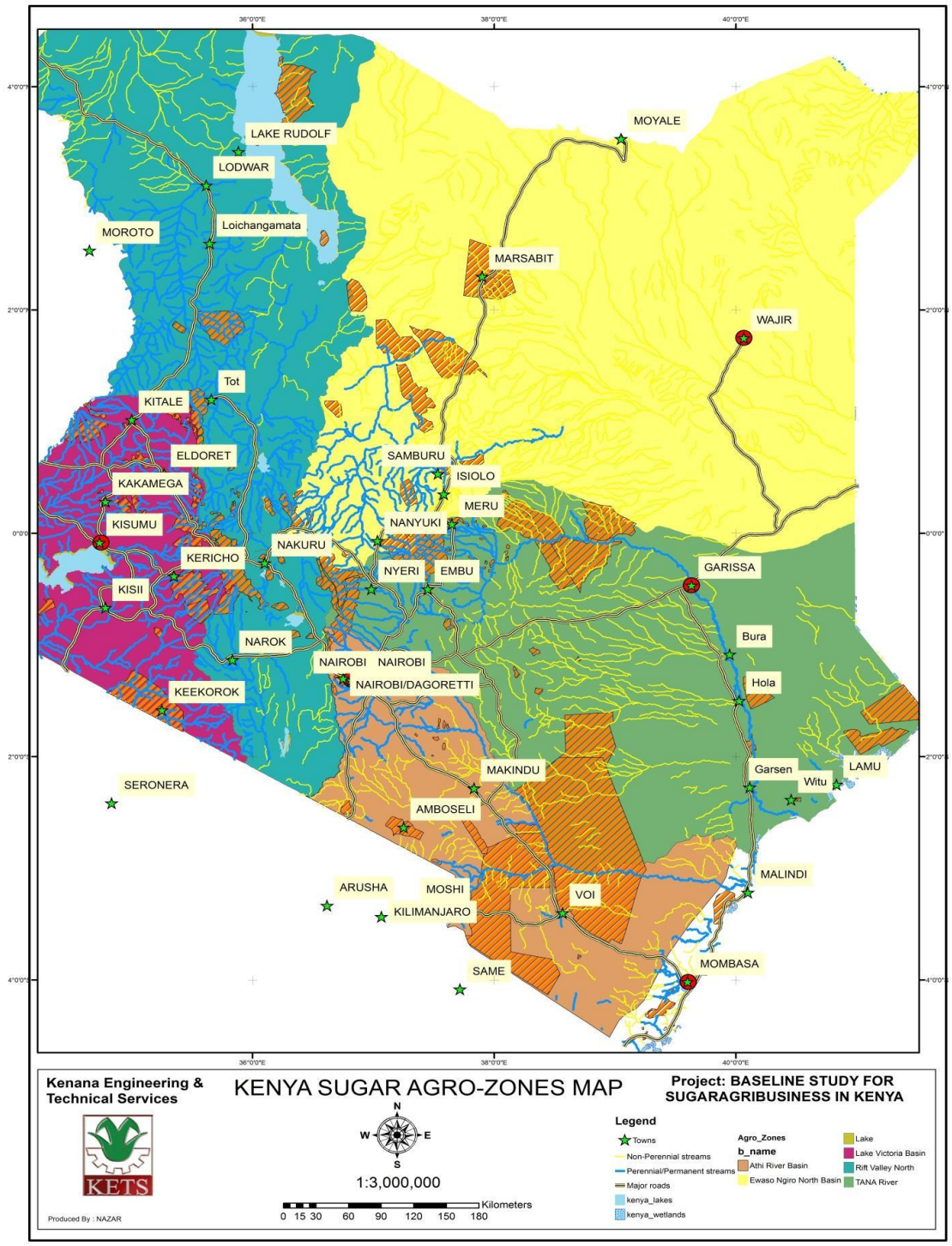

Kakamega County consists of A) Mumias; B) Butere; C) Kakamega Central; D) Kakamega South; E) Kakamega North; F) Kakamega East; and G) Lugari Sub-Counties.

Appendix 17**:** Kenya Sugarcane growing Agro sub-zones




**Source: Kenani Engineering and Technical Services, 2013**

Appendix 18: Kenya Sugarcane Agro-zones




Source: Kenani Engineering and Technical Services, 2013