RESPONSE OF EMBRYOGENIC CALLI TO DIFFERENT COLCHICINE CONCENTRATIONS TO INDUCE POLYPLOIDY FOR ENHANCED PERFORMANCE IN *CENCHRUS PURPUREUS* SCHUM

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A Thesis submitted for Examination in partial fulfillment of the Requirements for the Degree of Master of Science in Genetics and Plant Breeding, Masinde Muliro University of Science and Technology (MMUST).

NOVEMBER 2025

DECLARATION

I certify that this thesis is my original work and has not been previously submitted for the award of a degree in any other university.

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PLAGIARISM

I hereby declare that this thesis is my original work and has been carried out in partial fulfillment of the requirements for the award of the Master of Science in Genetics and Plant Breeding at Masinde Muliro University of Science and Technology (MMUST). This work has not been submitted for any academic award at any other institution.

All sources of information used in this thesis have been appropriately acknowledged. I fully understand that plagiarism is a serious academic offense and confirm that this thesis has been subjected to plagiarism detection to ensure academic integrity and originality.

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DEDICATION

To my mother Annelita Barasa Shikuku and my father Leonard Shikuku Wafula for inculcating the values of diligence and commitment in every sincere pursuit, alongside their unwavering love, constant prayers, continuous support, enthusiastic education, and unshakeable belief in my academic pursuit.

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God bless you family

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ACRONYMS AND ABBREVIATION

ASAL	Arid and semi-arid lands
BAP	Best Agronomic Practices
CP	Crude protein

DM	Dry Matter
FAO	Food Agricultural Organization
GOK	Government of Kenya
KNBS	Kenya Bureau of Statistics
KALRO	Agriculture and Livestock Research Organization
IVDMD	In-vitro dry matter digestibility
NARC	National Agriculture Research Center
PGRs	Plant growth regulators
2,4-D	2,4- dichlorophenoxyacetic acid

ABSTRACT

Napier grass *(Cenchrus purpureus schumach)* is an important forage crop and livestock feed. However, its yield and quality in Kenya are often limited by Napier grass headsmut and stunt disease. Napier grass genetic improvements through mutation breeding and selection could avail cultivars with increased forage. This study investigated the response of embryogenic calli to different levels of colchicine in inducing polyploidy in the two germplasms of Napier grass; *South africa* and *Bana grass*. The experiments were carried out in a factorial experiment laid in a completely randomized design (CRD). The colchicine concentrations used were 0, 0.05, 0.1, and 0.2%, and the exposure durations were 24, 48, and 72 h. During the shoot regeneration stage, culturing explants on an MS medium (Murashige and Skoog) supplemented with 0.2 mg L^{-1} Benzyl Adenine (BAP), 0.1 mg L^{-1} dichlorophenoxyacetic acid (2, 4-D), and 0.1 mg L^{-1} indole-3-butyric acid (IBA) was the most suitable for shoot regeneration. Chromosome doubling was confirmed by genomic DNA and, the stomata size and number. Culturing explants on an MS medium supplemented with 1 mg L^{-1} IBA, 1 mg L^{-1} 2, 4-D, and 0.5 mg L^{-1} BAP was most suitable in inducing embryogenic calli in both genotypes. Polyploidy results revealed that a 0.1% concentration of colchicine with two days of treatment resulted in the maximum number of octoploid plantlets induced in vitro, while a 0.2% concentration was very toxic. The stomata size and number of derived octoploid plantlets were bigger with a lower density, a shorter plant height, and a smaller stem diameter, and despite being the first to produce tillers, they were significantly higher than their progenitors. Induced mutants also had a significantly higher number of chromosomes and showed different band patterns and distances during gel electrophoresis. However, we recommend the use of flow cytometry to confirm the ploidy level. The superior mutant plantlets can be selected and recommended for characterization across representative agro-ecologies for large-scale production and used in *Cenchrus purpureus* breeding programs in Kenya and its environments.

Keywords: napier grass, colchicine, induced mutation, polyploidy, genotype improvement

CHAPTER ONE:

INTRODUCTION

1.1 Background to the study

Livestock is a significant resource of benefit to the society in the form of food, income, employment, insurance and clothing (Herrero et al., 2013). Globally by 2050, the total demand for meat and milk, is projected to almost double in the developing world due to population growth, urbanization, income increase, and change in dietary preferences—the "livestock revolution" (Alexandratos and Bruinsma, 2012). Kenya is one of Africa's largest livestock producers with an estimated 80% beef production and 50% dairy production from smallholder farmers, while the remaining percent is from ranchers and dairy farms (Omore and Muriuki, 2009). The main production takes place in ASAL and highland areas (Government of Kenya Session Paper No. 8 of 2012). When compared to Eastern Africa countries, Kenya has the highest number of improved dairy breeds that comprise of Friesian, Guernsey, Aryshire, Jersey and their crosses, which are mainly kept for milk production (Kibiengo et al., 2015; Mburu and Kariuki 2020).

Kenya produces an estimate of 3.43 billion liters annually (RoK, 2015) where average milk produced per lactating cow is 2,850 liters, which translates to 7.9 liters of milk produced per day (KDB report May 2021). This represents 18% of the 4% global contribution of sub-Saharan Africa (The international initiative for impact evaluation (3ie) 2015) which was a rapidly expanding dairy subsection with over 85% of the dairy cattle population mainly coming from Kenya-Eastern Africa (ILRI 2000 and IGAD LPI working paper No 03-11 September 21 2011). The highland regions like Central, Rift valley, and Western, smallholder dairy farmers own over 80% dairy cattle producing over 56% of total milk, while the remaining 20% comes from large dairy farms and indigenous herds (RoK, 2015). The dairy animals in the smallholder farms are kept under intensive and semi-intensive system of livestock production. The estimated milk and milk products (e.g. butter, yoghurt etc.) at 198 liters per person per year (Kenya National Bureau of Statistics (2020). This shows that the country does not produce enough milk to meet her demand. Furthermore, these estimates will continue to rise due to improved life style and increase in human population.

In Kenya, like many Eastern Africa countries, majority (80%) of famers practice mixed crop-livestock systems and mainly rely on rain-fed agriculture. Because of the small farm sizes, farmers predominantly practice intensive and semi intensive system of livestock production (Muia et al., 2011). Upgrading and intensification of smallholder dairy development is seen as a viable poverty alleviation strategy. It can provide opportunities for daily income throughout the year, contrary to crop income that is bound to harvest seasons. Milk has even been coined “white gold” for its potential of income generation (Makoni et al., 2013). Notably, commercial livestock production in the country is also expanding due to income increase that is generated from the sale of livestock and livestock products, rapid urbanization and changing dietary habits (Agriconsortium 2003).

Livestock (ruminants) have the capacity to convert roughages, concentrates and other by- products from the human food industry, into valuable products like milk and meat. Forage feed accounts for approximately 70% of the total cost of milk production (Ouema and Obare.2004), however, the major constraint to smallholder dairy farming systems is inadequate quality and availability throughout the year. Cultivation of fodder is the only effective way to increase livestock productivity and provide an opportunity of livelihood diversification by selling surplus fodder. The key challenges to fodder production include the competing needs of small pieces of land for production of feed, food and cash crops; recurrent droughts which are becoming frequent due to climate change and low yields (Ouema and Obare.2004).

Napier grass *(Pennisetum purpureum* Schumach) is the major livestock feed for most farmers keeping dairy and beef cattle in Kenya (Muyekho et al., 2003; Lukuyu et al., 2011) and still the major feed for the cut-and –carry zero grazing dairy and semi-intensive dairy and beef system in Kenya. Generally, napier grass compared to *Chloris gayana and Bachiaria ruziziensis* has the highest leaf to stem ratio (3.18), and also nutritive value of (9 to 10% crude protein (CP); 66% in-vitro dry matter digestibility (IVDMD) and 56% in-vitro organic matter digestibility (IVOMD) (Muyekho et al., 2003;Lukuyu et al 2011.) Napier grass is able to withstand repeated cutting of between 4 to 6, depending on ecological conditions, variety and good agronomic practices (BAP), they can yield a Dry matter (DM) of 15 to 22 tons/ha/year (Muyekho et al., 2003,2008; Mula et al., 2010; Munyasi et al., 2015).

Cultivation has intensified due to small-scale dairy farming shift from extensive to zero-grazing systems owing to population pressure, this constituted up to 80% of forage for small scale livestock farmers (Staal etal., 1987). Farmers who kept no livestock or have surplus fodder sold bundles of Napier grass to other farmers in need of fodder, making the crop a valuable source of income to farm households (Kabirizi et al., 2015). However, continued contribution of napier grass to the livelihoods of the small-scale farmers is threatened by low feed value, low growth vigor, low biomass due to inferior germplasm and partly as a result of biotic and abiotic stressors (Jones et al., 2004). Furthermore, the selection of napier germplasm has resulted to less productive cultivars or those that produce yields similar to farmer preferred. These varieties are susceptible to disease and have low biomass, hence do not meet forage demands of farmers (Muyekho et al., 2003; Mulaa et al. 2010) Consequently, it is crucial to develop climate resilient *Cenchrus purpureus* germplasms that generate high biomass, high feed value, increase growth vigor and tolerance to diseases. This will enhance livestock feed production and alleviate the severe shortage of livestock feed.

There is an urgent need to enhance genetic diversity of pasture and forage to meet growing demand. This has been proven to be key strategy for improving food crops resulting to enhanced plant performance and adaptability (Ardabili et al., 2015). Induced mutation breeding, although less studied in forage crops, is a breeding technique that is extensively used in food crops and ornamental crops (Mutlu et al., 2015). This results in changes in gene dosage that cause chromosomal rearrangements, epigenetic remodeling, and reunion of divergent gene regulatory that brings about variations (Kannan et al., 2013). It is a widely adopted method in breeding programs to generate genetic diversity and select new mutants with favorable agronomic traits like drought tolerance, pest and diseases tolerance, increase feed value maturity time, size of organ and increased biomass (Mishra et al., 2010). Mutation can be induced *in vitro* by exposing plant tissue to proper dose of Gramma irradiation, X-Rays (physical) or mutagens colchicine, oryzalin or trifluralin (chemical) which induces polyploidy in plants (Zhang et al. (2018). In addition to the type and concentration of the mutagen, *in vitro* chromosome doubling depends on culture media explant type and treatment duration (Dhooghe *et al.* 2010).

Plant growth regulators (PGRs) play a pivotal role in embryogenic callus induction, shoot regeneration and subsequent rooting regeneration. Studies by Unami et al. (2016), Fabio et al. (2016), and Gondo et al. (2017) demonstrated successful induction of embryogenic callus from shoot tips of tillers on MS medium supplemented with 2 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP. In the same studies, shoot regeneration was achieved on MS medium containing 0.1 mg L^{-1} 2,4-D and 2 mg L^{-1} BAP, while rooting was induced on MS medium supplemented with 1 mg L^{-1} NAA and 0.1 mg L^{-1} 2,4-D or 0.1 mg L^{-1} IBA. Similarly, Yu et al. (2009), in their work on chromosome doubling of the bioenergy crop Miscanthus × giganteus, reported successful establishment of callus cultures on MS medium containing 13.6 µM 2,4-D and 0.44 µM BAP. Shoot regeneration was promoted on MS medium supplemented with 1.3 µM NAA and 22 µM BAP, followed by rooting on MS medium containing 1 mg L^{-1} NAA and 0.1 mg L^{-1} IBA. Exposing this plant tissues to mutagen like colchicine, leads to chromosomal disjunction that results in the presence of extra sets of chromosomes per nucleus in plants. Furthermore, Colchicine induces a higher frequency of point mutations compared to physical mutagenes like gamma rays (Van Harten, 1998). However, the most critical factor in inducing point mutation is selecting the optimal dosage of the mutagen, which involves determining the appropriate concentration and treatment duration. As Alam and Razaq (2015) explain, polyploid plants have the ability to adapt to a wide range of environments and survive in adverse ecological conditions, which makes them more effective than their diploid ancestors due to the presence of additional alleles that increase their heterozygosity.

Furthermore, Eng (2019) noted that artificial polyploidization yields greater phenotypic variations than single gene mutation. Sattler et al. (2016) described the most important result of polyploidization as an increase in cell size. Despite the increased sterility of induced tetraploids, Nagahatenna et al. (2008)

explained that polyploidization is a key tool in breeding forage crops as vegetative propagation remains the major propagation system. Therefore inducing polyploidy in Napier grass is a promising approach to improve its productivity and resilience (Murthy et al., 2014). However, information on the use of chemical mutation on *Cenchrus purpureus* species in Sub Sahara Africa and Kenya is limited. In that case, inducing polyploidy in napier grass is a promising approach to improve its performance and make them more adaptable to changing environmental conditions. The purpose of this study is to investigate the response of embryogenic calli to different colchicine concentration in inducing polyploidy for regeneration of napier grass mutants.

1.2 Statement of the problem

Napier grass plays a vital role in livestock production, providing 9 to 10% crude protein depending on the cultivar and crop management (Lukuyu et al 2011, Tittonell et al., 2015). However, the current tetraploid Napier grass with 28 number of chromosomes cultivars yield and quality are often limited by biotic and abiotic stressors. This made them more susceptible to pest, diseases and drought while the tolerant ones yielded low herbage dry matter thereby not meeting forage demands of farmers (Ayele et al., 2012, Tittonell et al., 2015). Therefore, there is a need to enhance genetic diversity of Napier grass to meet growing demand (Ardabili et al., 2015). This can be done through inducing polyploidy *in viro* in napier grass plant tissues. Although less studied in forage crops, it is a breeding technique that is extensively used in food crops and ornamental crops (Ardabili et al., 2015). However, for *in vitro* chromosome doubling to be successful, it depends on culture media, animitottic agent concentration and treatment duration (Dhooghe *et al.* 2010).

Polyploidization results in changes in gene dosage that causes chromosomal rearrangements, epigenetic remodeling, and reunion of divergent gene regulation that brings about variations (Sattler et al., 2016). This leads to a new Octoploid (2n = 8x = 56) Napier grass germplasm with large leaves, thicker stems, increased biomass and increased tolerance to biotic and abiotic stressors. Colchicine treatment is a commonly used method for inducing polyploidy in plants (Nagahatenna et al., 2008. Although embryogenic callus induction and polyploidy techniques have shown promise in related grass species (Yu et al., 2009 and Chae et al., 2012). However, the optimal plant growth regulator combinations for Napier grass, as well as their interaction with colchicine during polyploidy induction, remain poorly understood. This knowledge gap limits the successful generation of high-quality callus, compromises survival during chromosome doubling, and ultimately constrains the development of improved polyploid Napier grass lines with superior yield, resilience, and agronomic performance.

1.3 General Objective

To evaluate the response of embryogenic calli of Napier grass to different colchicine concentration for the induction of polyploidy aimed at enhancing agronomic performance.

1.3.1 Specific objectives

This study's specific objectives were to:

1. Determine the most suitable plant growth regulators for the growth of napier explants
2. Determine the effect of different colchicine concentration on survival of napier explants
3. Evaluate the effect of polyploidy on induced napier mutants two months after regeneration

1.4 Hypotheses of the study

The research hypothesis is a null statistical mode.

H_{01}: There is no significant difference in the growth of explants and callus induction among different plant growth regulators

H_{02}: Different colchicine concentrations have no significant effect on the survival rate of explants.

H_{03}: There are no significant differences in morphological or physiological traits between polyploid and diploid regenerants two months after regeneration.

1.5 Justification and Significance of the study

Cenchrus purpureus, is a very important forage crop with high potential for use as feed, but its yield and quality are often limited by biotic and abiotic stresses. Therefore, developing new napier grass germplasms through inducing polyploidy could be a promising approach to improve its performance and make them more adaptable to changing environmental conditions (Ardabili et al., 2015). Increased napier grass forage is imperative, for increased milk and beef yield at farm level and ultimately, reduced cost of production (Tittonell et al., 2015). In addition, high yielding varieties if patented will generate income through sale of cane seed. To this end, advanced plant breeding methods like polyploidization in plants is an indispensable tool. Polyploidy induction using colchicine has been shown to be an effective strategy for enhancing plant performance including increased biomass yield, faster regrowth after grazing, increased plant height, increased feed value and production of disease tolerant germplasms (Ardabili and Zakaria.2015

The success of polyploidy induction is highly dependent on the quality of embryogenic callus (Fabio et al., 2016) and the responsiveness of explants to specific plant growth regulator combinations (Unami et al., 2022). By identifying optimal plant growth regulators and evaluating how different colchicine concentrations influence survival and polyploid formation, this study provides a necessary foundation for efficient development of improved Napier grass varieties. Ultimately, this study is essential for advancing modern forage breeding in Kenya and supporting sustainable livestock production.

1.6 Scope of the study

This study was limited to evaluating the response of Napier grass explants and embryogenic calli to plant growth regulators and colchicine under controlled *in vitro* conditions. The study focused on three main aspects:

1. Determining the most suitable plant growth regulators for the growth of explants
2. Determining the effect of different colchicine concentration on survival of explants
3. Evaluating the effect of polyploidy on induced mutants two months after regeneration

The research was confined to selected Napier grass genotypes maintained at KALRO Kakamega Tissue Culture Laboratory, where all experiments involving callus induction, colchicine treatment, regeneration, and ploidy assessment were conducted. The study did not extend to field evaluation, long-term agronomic performance, nutritional quality, or multi-environment testing of induced mutants. Only colchicine was used as the mutagenic agent, and only early-stage morphological traits and cytological indicators were considered in determining polyploidy.

CHAPTER TWO

LITERATURE REVIEW

2.0 Introduction

2.1 Brief overview

Due to technology and innovation in agriculture, plant breeders are coming up with ways of breeding both aneuploid and Euploid plants (induced polyploidy) to come up with new germplasm that are better performers than the current. This can result to production of more and better-quality pasture, and fodder through an attempt to produce a properly balanced diet at the least cost to the farmer that give them an optimum income from livestock enterprise. Advancement of technology in agriculture, like use of colchicine to enhance chromosome doubling in plants propagated by seed and vegetative, has been put into practice.

2.2 Importance of Livestock

2.2.1 Globally and in Kenya

Livestock were domesticated 6,000 to 10,000 years ago (Speller et al., 2010) and have contributed 40% of the global value of agriculture output that support the livelihoods and food security of the world population (FAO, 2011). Products from livestock are of high-value particularly when compared to crops. According to FAO, the total global price of a tonne of red meat is 10 times higher than the price of Bean and Maize, while milk is estimated to be 70% higher (FAOSTAT, 2011) which makes meat and milk to be ranked as agricultural commodities with the highest gross value of production (GVOP) in the developing world (FAOSTAT, 2011).

Consequently, a study conducted by FAO showed that in South Asia, approximately 1 billion people are living on less than 2 dollars a day, over 80% of less fortune in Africans kept livestock, while in India and Bangladesh it has been reported that between 40% and 66% of poor people practice small scale livestock production (FAO, 2009). Keeping of livestock by the less fortunate across the developing world has played a numerous role by supporting 68% of households through earning income from the sale of animals and their products hence improving their livelihoods (Davis et al., 2007).

In Kenya, livestock sector plays a major role in food security and creation of employment, which contributed to 12% of Kenya GDP and 50% of the agriculture labour force (RoK, 2015). Keeping of livestock enables this people to earn income and increase their crop production (FAO, 2009). This is achieved by selling of animals, collection of manure to be used as fertilizer in crop fields and animal power (for ploughing and transporting goods to markets), store wealth and to feed their families hence helping household to improve livelihoods and reduce poverty (Staal et al., 2009). Despite the significant contribution of livestock to global and Kenyan food security, income generation, and poverty reduction, there remains a critical gap in ensuring sustainable and sufficient high-quality forage supply such as Napier grass to support livestock productivity, particularly for smallholder farmers in developing regions.

2.2.2 Cattle statistics on milk, beef production and consumption in Kenyan

Kenya is estimated to have a total livestock (dairy and beef) population of 62.3 milion that comprises of Cattle, goats and sheep found in Highland and, Arid and Semi-aid (ASAL) region (GoK Census 2009) where ASAL Counties are estimated to be hosting 70% of the country's Livestock herd. These ASAL Counties include Marsabit, Laikipia, Narok, Turkana etc and Laikipia County being one of the leading producers of beef (Government of Kenya. Household Report; Laikipia County ASDSP, Ministry of Agriculture, Livestock and Fisheries, Government Press; Nairobi-Kenya 2014). Animals kept are highly adapted indigenous East Africa Zebu and Boran, also goats and sheep. Exotic beef breeds for example, Simmental, Angus, Hereford and their crosses are also kept by commercial ranchers which constituted to a major source of Kenyas meat (Kibiengo et al., 2015, Mugambi et al 2015).

In Kenyan highland regions like Central, Rift valley, and Western, smallholder dairy farmers own over 80% dairy cattle producing 2.74 billion liters of milk which translates to over 80% of total milk while the remaining 686.210 million liters which amount to 20% from large dairy farms and indigenous herds (RoK, 2015).The country relies heavily on dairy and Beef sector where total milk produced is estimated to be 5.788 billion liters (Highland: 4.781billion liters, Semi-arid: 637.102 million liters and Arid: 370.600 million liters). Estimated milk consumption or conversion into dairy products like Butter,Yoghurt etc for consumption is 198 liters per person per year (IGAD LPI working paper No 03-11 September 21 2011), while total Beef produced is estimated to be 2.243 million heads of offtake [63% from domestic

Pastoralists/Ranchers, 22% pastoral imports and 15% from Dairy sector (IGAD LPI working paper No 03-11)].

The estimated consumption of beef locally per year is 588,991MT (IGAD LPI working paper No 03-11) and export volume to Middle Eastern Market and Sub-Saharan African Market is 2500MT (Muthee et al., 2006, KRA 2012). Besides Milk and Beef as major products produced by livestock, they also produce hides and skin for leather and footwear, blood for blood meal and bones for bone meal. Those products are used locally, also they are exported to China, Pakistan, Turkey, India etc which contributed to about 2% of the total export (KRA, 2012). Despite Kenya's large and diverse livestock population and substantial contributions of milk, beef, and other products to the economy, there remains a critical gap in the availability of high-quality, reliable forage such as Napier grass particularly in both highland and ASAL regions, which limits livestock productivity and the full potential of the dairy and beef sectors.

2.2.3 Current constraints and opportunities

Livestock production plays a significant role in the national economy of Kenya, as noted in the report by the Republic of Kenya (RoK, 2015). However, this sector is facing several challenges, including inadequate and quality animal feed and nutrition due to poor cultivars, susceptibility of specific cultivars to pests and diseases such as Napier grass stunt and smut disease, poor genetic potential, market problems, inefficiency of livestock development services with respect to credit, extension, marketing, infrastructure, and most importantly, low quality and quantity of animal feed (Ayele, 2012; CAIS, 2000; Jones et al., 2004).

Forage production, the main source of animal feeds in Kenya and Africa at large, that provide herbage to over 90% of livestock feed requirement through grazing and in cut-and-carry for animals kept under zero grazing, according to studies by Bebe et al. (2008) and Muyekho et al. (2008). However, poor quality feeds resulting from low nutritive value of pasture-forage and inadequate supply of feed biomass from grazing-land and forage species are among the factors contributing to low output in livestock production, (Denbela and Ayele,2017); (Muyekho et al.,2003). Inadequate feed supply is further worsened by the low breeding vigor of specific cultivars, leading to insufficient feed (Ayele et al., 2012; Muyekho et al., 2003).

To mitigate these constraints, numerous opportunities have emerged, and one of the most promising is improvement of cultivars through breeding of new germplasm that are high yielding, nutritious, and shows tolerance to biotic and abiotic stress (Ayele et al., 2012; Tittonell et al., 2015). This will increase productivity of forage, leading to better quality and quantity of animal feed and reduced methane emissions from cows as a result of feeding on high-quality forages (Peters et al. 2013). In contrast, Kenya's livestock sector continues to face low productivity largely due to poor-quality and inadequate forage especially disease-susceptible, low feed value and low growth vigor cultivars like Napier grass, highlighting a critical need for improved, high-yielding, nutritious, and stress-tolerant germplasm through advanced breeding interventions.

2.2.4 Breeding of forage crops in Kenya

Development of forage resources in Kenya was felt as early as the 20th century up to 1980, with initial efforts made to introduce exotic forages to increase biomass yields and the nutritive value of livestock diets (Said et al. 1985) Institutions were established to carry out research on forages for ecological adaptability, mainly influenced by agricultural production systems and technologies to address problems of large European settler farmers, where the main activities on forages were the determination of herbage species and strains suitable to various areas, development of intensive grazing, and improvement of natural pastures (Said et al., 1985). In the 1950s, the importance of research in grasses increased, which culminated in the establishment of more research stations on a regional basis. Molo, Ol Joro Orok, and Kitale were established to cater to wetter highlands, Marigat and Katumani for medium altitude areas, with an aim to serve different agro-ecologies and provide information to African farmers (Said, 1985).

Research stations were also established in the coastal region in 1960 at Mtwapa and Maariakani with an objective of developing suitable pasture for coastal lowlands and its environs. Consequently, from 1930 to date, research in pastures has focused on the characterization and field screening of both indigenous and exotic grasses and legumes for adaptability and nutritive value (Ball, 1936; Muyekho et al., 2003; Muyekho et al., 2014). Among the useful indigenous grass genera collected and evaluated were Cenchrus, Chloris, Setaria, Panicum, Cynodon, and Eragrostis (Rossiter and Ndegwa, 1974). Furthermore, in the 1960s and 1970s, research conducted was directed towards breeding, which led to the release of a number of grass varieties belonging to the genera Chloris and Setaria at the Grassland Research Station, Kitale, through cross-pollination and single-plant mass recurrent selection for three generations (Bogdan, 1965). Moreover, studies conducted in the central region of Kenya and Kenya at large on Napier grass (*Cenchrus purpureus* Schumach.) found that over 80% of farmers relied on Napier grass as the main feed for livestock (Maher et al., 1936; Mwangi, 1995).

Napier grass breeding and selection was initiated at the National Agricultural Research Centre, Kitale, in 1973 (Van Gastel, 1978) where several Napier grasses were collected locally and introduced from other African countries. Selection for superior clones was based on disease resistance, palatability, and morphological characteristics such as smoothness of leaves, tillering ability, and density of clumps (Boonman, 1997). Based on the above criteria of selection, cvv. Bana and French Cameroon were selected but later became susceptible to the fungal snow mold disease (Cowdria sphaeroidea) caused by Beniowskia sphaeroidea (Kalchbr & Cke) Mason. A further selection identified Clone 13 from the highly susceptible French Cameroon (Van Gastel, 1978). In addition, Pakistan Napier hybrid, a drought-tolerant variety from a cross between Napier grass and bullrush millet, was introduced for regions receiving less than 1000 mm of rainfall (Muyekho et al., 2003).

Consequently, with increase in human population and land fragmentation, significant interest persists in further investigating the selection of high-yielding Napiers, as their importance was undeniable; however, only a few lines proved to be as productive as cv. Bana. In a recent field study conducted in northwestern Kenya, the performance of 57 accessions from local collections and germplasm maintained by ILRI was examined (Nyambati et al., 2007). The study encompassed the evaluation of their morphological characteristics, herbage quality, and productivity. The research revealed variations in agro-morphological traits among the 12 cultivars, with Clone 13 exhibiting the highest productivity at 18.6 t/ha. Notably, Kakamega 1 and II, as well as Uganda L14, also demonstrated considerably high yields. Since 1950s to date a lot of work in forage has been on characterization and field screening of both indigenous and exotic grasses and legumes for adaptability and nutritive value of various forage cultivars (Ball, 1936; Muyekho et al., 2003; Muyekho et al., 2014; Munyasi et al., 2015). Importantly, forage breeding has also been done although it has received less attention. According to Bogdan (1965) and Nyambati et al. (2007), cross pollination and single-plant mass recurrent selection for three generations was mainly used as breeding methods in forage crops. However, although Napier grass has been widely characterized and selected in Kenya, there is still a critical gap in the development and assessment of colchicine-induced polyploid lines, whose potential to enhance yield, stress tolerance, and disease resistance remains largely unexplored.

2.2.5 Methods of hybridization in forage crops

i. Selection

This involved the process that favors adaptation and survival that promote propagation of plants having more desired traits than the rest. Direct selection of forage from natural diversity based on phenotypic traits of cultivars from most tropical grasses (Hacker and Jank, 1998) where breeding procedure for improvement of forage species is mass selection, harvesting and bulking seed without progeny evaluation. Along with those numerous breeding methods employed in development of varieties and superior cultivars in large germplasm and identification of greater concurrences, was given importance and guaranteed 80 to 130 % productivity in leaf, 26 % increase in digestibility (Burton, 1989; Burton et al., 1993). Resistance to many disease and insect pests is based on additive genes, dominant gene or some degree of dominance.

This breeding method has the following advantages; Mass selection retains considerable genetic variability and maintains the original adaptation of a variety because many plants are selected, whereas pureline selection provides uniformity that simplifies the identification and selection of desirable varieties. However, mass selection produces non-uniform populations and does not allow determination of homozygosity without progeny testing, while pureline selection, although offering uniformity, is more time-consuming and expensive to conduct. In general, despite the long use of mass and pureline selection in improving tropical forage grasses, there remains a significant gap in integrating modern biotechnological tools such as polyploidy induction through somatic embyogenesis to overcome the limitations of conventional selection methods and accelerate the development of superior, uniform, high-yielding Napier grass cultivars.

ii. Hybridization

This entailed crossing of two plant or lines of dissimilar genotype in plants where pollen grains from the male genotype is deposited on the stigma of flower of the other female genotype, the female parent mostly in asexual plants. This breeding technique is widely used in forage improvement programs to enhance characteristics such as yield, quality, disease resistance, and adaptation to specific environmental conditions. By combining the genetic diversity of different parental plants, hybridization allows the creation of new hybrids that exhibits improved performance and agronomic attributes compared to their parent species.

A study by Acharya et al. (2016) focused on hybridization of alfalfa (*Medicago sativa*), a widely cultivated forage crop. The researchers employed hybridization techniques to create interspecific hybrids between two closely related species, *M. sativa* and *M. falcata*. The resulting hybrids exhibited enhanced biomass production, higher forage quality, and improved persistence under abiotic stresses, such as drought and salinity, compared to the parental species. These findings highlighted the potential of hybridization in developing alfalfa varieties with improved performance and adaptability to challenging environments. In another study, Ercoli et al. (2020) investigated hybridization in Italian ryegrass (*Lolium multiflorum*), a valuable forage grass species. The researchers crossed different varieties of Italian ryegrass with other closely related Lolium species to create hybrids with improved agronomic traits. The hybrid progeny displayed increased biomass production, better nutritive value, and enhanced persistence under grazing compared to the parent species. The study demonstrated that hybridization is also an effective method that could be used in breeding forage to enhance the performance and quality of forage.

Generally, although hybridization has effectively improved yield, quality, and stress tolerance in many forage species, its application in Napier grass remains limited mostly due to sterility, leaving a critical gap in exploiting advanced methods such as colchicine-induced polyploidy to generate superior, resilient cultivars beyond what conventional hybridization alone can achieve.

iii. Interspecific and polyploidy breeding

Polyploidy, a phenomenon where a plant possesses more than two sets of chromosomes that act as an important mechanism for plant evolution (Ardabili and Zakaria. 2015) A study by Zhang et al. (2018) focused on interspecific polyploid breeding in Brassica species, specifically the creation of a new hybrid species by crossing *B. napus* (canola) and *B. rapa* (Chinese cabbage). The resulting polyploid offspring, known as intertribal hybrid Brassica polyploids (IBP), exhibited enhanced resistance to blackleg disease compared to the parental species. Moreover, the IBP plants demonstrated improved yield potential and broader ecological adaptation, highlighting the potential benefits of interspecific polyploid breeding in creating new plant varieties with enhanced agronomic traits.

In another research article by Chen et al. (2019), interspecific polyploid breeding was employed to develop new *turfgrass* varieties with improved qualities. The study involved crossing two different species, *Zoysia* *japonica* and *Zoysia matrella,* resulting in interspecific polyploid hybrids. The polyploid hybrids displayed increased growth vigor, improved tolerance to drought and low temperatures, and enhanced resistance to diseases compared to their parental species. For instance, Polyploidy increases heterosis, enhances plant vigor, provides gene redundancy that protects against harmful mutations, and can stimulate new gene expression, but it may also disrupt nuclear and cell functions leading to gene silencing, and its epigenetic instability can cause non-additive or transgressive gene regulation.

Importantly, despite evidence that polyploidy enhances vigor, stress tolerance, and disease resistance in many crops, its application in Napier grass remains insufficiently explored, leaving a gap in understanding how induced polyploidy particularly through colchicine can be used to develop superior, high-performing forage cultivars.

2.2.6 Effect of plant growth Regulators on embryogenic callus formation and Shoot regeneration

A study on callus induction by Unami et al 2016; Fabio et al 2016 and Gondo et al 2017 successfully induced embryogenic callus from shoot tips of shoot tillers on MS media containing 2mg L-1 2,4-D and 0.5 mg L-1 BAP. In the same study, shoots were able to be regenerated on MS media containing 0.1mg L-1 2,4-D and 2mg L-1 BAP while rooting was induced on MS supplemented with 1mg L-1 NAA and 0.1mg L-1 2,4-D or 0.1Mg L-1 IBA . In another study by Yu et al 2009 on chromosome doubling of the bioenergy crop *Miscanthus X giganteus* reported a success in establishment of callus culture on MS medium containing 2,4-D 13.6 um and BAP 0.44 um, shooting regeneration media supplemented with NAA 1.3 um and BAP 22um. Consequently, rooting was induced on MS media containing NAA 1mg L-1 and 0.1mg L-1 IBA.

Despite successful reports of embryogenic callus induction and shoot regeneration in various grass species using specific plant growth regulator combinations, there remains a significant gap in optimizing these protocols for Napier grass particularly in relation to how plant growth regulators influence callus responsiveness during colchicine-induced polyploidy.

2.2.7 Effect of Colchicine concentration on callus induction

Colchicine concentration used in callus induction affect the success of polyploid induction and callus induction rate. For instance, a study by Wang et al. (2020) found that a higher concentration of colchicine led to a higher frequency of polyploid in tobacco callus. However, this also resulted in death of calli and decreased callus induction rates. The study concluded that the optimal colchicine concentration for inducing polyploidy in tobacco callus was 0.1%. Another study by Wang et al. (2019) investigated the effects of colchicine on callus induction and plant regeneration of sweet potatoes. The researcher found that a low concentration of colchicine (0.05%) led to higher callus induction rate and a higher frequency of polyploidy, while a high concentration of colchicine (0.2%) led to lower callus induction rates and a lower frequency of polyploidization.

Consequently, colchicine concentration used in callus induction significantly affected polyploidy induction success and callus induction rates (Ardabili and Zakaria. 2015). Similarly, several studies also investigated effects of colchicine on callus survival and regeneration rate. For example, in a study on the medicinal plant *Scutellaria baicalensis*, it was found that a colchicine concentration of 0.05% resulted in the highest callus survival rate, while higher concentrations of colchicine (0.1% and 0.2%) resulted in lower survival rates (Li et al., 2015. Consequently, in another study on the ornamental plants *Chrysanthemum morifolium,* it was found out that a colchicine concentration of 0.1% resulted in the highest callus induction rate while higher concentration of colchicine (0.2% and 0.3%) resulted in lower callus induction rate and decreased shoot regeneration capacity (Wang et al. 2019)

Furthermore, a higher concentration of 0.2% with a duration of 8hr was found to be toxic in *Freesia refracta* plant cell and tissue (Liu et al., 2016). In another study conducted on *Lupinus angustifolius* plants showed that increasing colchicine concentration from 0.1% to 0.2% with longer treatment duration led to higher ploidy induction rate and increased the percentage of chromosome-doubled, but the highest concentration tested (0.3%) led to plant death (Palczynska et al., 2008). Despite extensive studies on colchicine effects in various plant species, the optimal colchicine concentrations and treatment conditions required to efficiently induce polyploidy in embryogenic calli of Napier grass without compromising callus survival and regeneration capacity.

2.2.7 Effect of colchicine on ploidy level

Polyploidization can be achieved by use of colchicine obtained from *Colchicum autumnale* that had an inhibitory effect on the spindle formation. This resulted in failure at anaphase disjunction during mitosis, hence chromosome doubling which change plants' characteristics and increased genetic diversity (Dhooghe and Van Laere., 2011). Treatment with colchicine results either to pure (Mixoploid) polyploidy plants or Impure (aneuploidy) depending on colchicine concentration, duration of exposure and plant tissues development stage (Venial and Mendonça., 2020). For example, a study conducted by Kang et al. (2013) found that treatment of wheat embryos with 0.1% colchicine resulted in a polyploidization frequency of 1.98% while Chen et al. (2014) reported a polyploidization frequency of 1.72% in hexaploid wheat treated with 0.05% colchicine concentration.

Furthermore, another research finding showed that culturing of callus in tissue culture media mixed with a concentration of 0.1% colchicine in vitro led to octaploid plantlets in auto-alloctaploid C. *arabica* coffee plant (Venial and Mendonça 2020). Based on research findings, a colchicine concentration of 0.015% in tissue culture was recommended to be effective in polysomic tetraploidy of *Lolium perenne* (Dabkevičienė and Statkevičiūtė., 2016). Consequently, in sorghum a concentration of 0.1% colchicine treatment recorded a higher number of *in vitro* induced tetraploid plantlets (Ardabili and Zakaria., 2015). Generally, the concentration of colchicine treatment affected the degree of polyploidy, and the resulting polyploid types. For example, low concentrations of colchicine typically induce triploid in citrus fruit trees (Albrigo and Bowman, 2005; Hao et al., 2019). Moderate concentrations of colchicine were found to induce tetraploidy in Alfalfa (Bouton, 2007; Zhuang et al., 2020), while higher concentrations of colchicine resulted in induced hexaploidy in Cotton (Li et al., 2018) and Oats (Slamovic et at., 2002). In some cases, colchicine treatment also led to mixoploidy in *Tragopogon miscellus*, which is the presence of cells with different chromosome numbers within the same organism.

The concentration of colchicine used in inducing polyploidy influenced the degree and type of polyploidy results. Triploids, tetraploids, hexaploids and mexoploid can exhibit different genetic and phenotypic characteristics, which can contribute to enhanced plant performance. (Zhuang et al., 2020). Similarly, several studies have also been conducted to investigate the effects of colchicine on chromosome number in different plant species using Karyotyping to analyze chromosome number which help to determine ploidy level of germplasm. A study by Ma et al. (2017) investigated the effects of colchicine treatment on the chromosome number of a forage grass species, *Festuca arundinacea.* The researcher treated the seeds with different concentrations of colchicine (ranging from 0.1% to 0.5%), and then analyzed the chromosome number in the root tips and young leaf of treated seedlings using karyotyping. The study found that colchicine treatment caused an increase in the chromosome number, resulting in polyploidy in some treated plants.

Furthermore, Chen et al. (2017) employed karyotyping to determine chromosome number of a medicinal plant *Artemisia annua* after collecting shoot tips from seedlings that had been treated with different colchicine concentrations, for chromosome visualization. Furthermore, Gondo et al., (2017) was able to evaluate ploidy levels of putative polyploids using genomic DNA where the occurrence of the GUS gene in the genomic DNA of a transgenic *Pennisetum purpureum* line was confirmed by DNA gel blot hybridization analysis. However, despite extensive research on colchicine-induced polyploidy across various plant species, the specific colchicine concentration, exposure duration, and developmental stage required to reliably induce stable, non-mixoploid polyploidy in embryogenic calli of Napier grass and how these factors affect its regeneration efficiency and genetic stability remain largely unknown.

2.2.8 Impact of polyploidy on plant growth

Plant growth is the increase in size, mass or volume of a plant over time. It includes the accumulation of new tissues, such as leaves, stems, and roots, as well as expansion of existing tissues. Chromosome doubling in plant cells as a result of polyploidization has been shown to have a significant impact on plant growth. The effects of polyploidization can be both positive and negative, depending on the specific plant species, the degree of polyploidization, and environmental factors. One of the positive effects of polyploidization on plant growth was increased cell size. Polyploidization often led to an increase in cell size, which resulted in larger plant organs, such as leaves, flowers, and fruits (Chen et al., 2019). Moreover, polyploidization also led to changes in the expression of genes involved in growth and development, resulting in altered plant architecture which affected the growth pattern of plants, such as more robust root systems (Chen et al., 2018), the branching pattern and timing of flowering (Hegarty et al. 2006)

Additionally, polyploidization in plants was shown to have enhanced tolerance to environmental stresses, such as drought, salinity, and extreme temperatures (Leitch & Leitch., 2008). However, polyploidization can also have a negative effect on plant growth, such as reduced fertility, altered metabolism, and changes in gene expression. Such changes lead to altered growth patterns and developmental abnormalities (Gao et al., 2018). Similarly, polyploidization also led to altered responses to environmental stimuli, such as drought or nutrient availability, which impacted plant growth and survival (Liu et al., 2020). Even though there is extensive evidence on how polyploidy influences plant growth and stress tolerance in various species, there is limited understanding of how induced polyploidy specifically affects growth traits, physiology, and developmental responses in Napier grass under controlled tissue culture conditions.

2.2.9 Impact of Polyploidization on Plant Morphology

Plant morphology refers to the physical characteristics and structures of a plant, such as its shape, size, and arrangement of leaves, stems flowers, and roots. Polyploidization plays a significant role in the development of crops resulting in significant improvement on plant morphology, including changes in leaf size, shape, and texture, as well as changes in flower size, color and number. For example, polyploidization led to large leaves, thicker stems, and increased biomass in crops such as wheat and corns (Liu et al., 2015). In addition, polyploidization also had an effect on reproductive structures such as flowers and fruits.

A study by Zhang et al. (2020) showed that polyploidization in apples led to large flowers and higher fruit yield. Furthermore, hexaploid wheat (*Triticum aestivum),* which is a polyploidy species has undergone natural and artificial hybridization events that have contributed to the development of modern wheat varieties (Feldman and Levy., 2015) with large seed size (Ma et al., 2020).

Polyploidization process has also led to improved rice varieties, with several studies demonstrating that triploid rice hybrids have improved plant architecture, higher yield potential and larger grains compared to diploid rice (Liu et at., 2019; Zong et al., 2019). Although polyploidization is known to alter plant morphology in many crop species, there remains limited information on how induced polyploidy affects key morphological traits of Napier grass, particularly in relation to leaf architecture, biomass accumulation, and reproductive structure development under *in vitro* conditions.

2.2.10 Polyploidization Effect on Stomata Size and Number

Polyploidization can have a significant impact on plant morphology, including stomatal development. Stomata are specialized structures on the surface of plant leaves that control gas exchange and water loss. The number and size of stomata can be used as indicators of polyploidization. Several studies have investigated the effects of polyploidization on stomatal development. For example, in a study on the effects of polyploidization on *Populus euphratica*, it was found that the stomatal density and size increased with ploidy level, and that the polyploid plants had a higher stomatal conductance and photosynthetic rate than their diploid counterparts (Wang et al., 2019).

Similarly, a study on the effects of polyploidization on *Chrysanthemum lavandulifolium* showed that the stomatal size and density increased with increasing ploidy level, and that the polyploid plants had a higher photosynthetic rate and water use efficiency than their diploid counterparts (Li et al., 2018). Regardless of existing evidence from other species, the specific effects of colchicine-induced polyploidization on stomatal size, density, and associated physiological responses in Napier grass remain vaguely understood.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Introduction

This study was conducted between February 2023 and May 2024, at the Non-Ruminant Institute of Kenya Agricultural and Livestock Organization (KALRO) located in Kakamega county, Lurambi Sub-county Mahiakalo ward. The study was carried out in three phases; callus induction, colchicine treatment and regeneration, and field evaluation.

3.2 Collection of planting material

Two interspecific hybrid cultivars that are genotypically tetraploid (2n = 4x = 28) were used in this study; '*South africa'* (tolerant to Napier stunt disease), '*Bana grass'* (high yielding but susceptible to napier stunt disease) (Kannan et al., 2010) *South africa* was sourced from Kenya Agricultural and Livestock Organization (KALRO) while *Bana grass* was sourced from the Food Crops Research Institute of KALRO Kitale. All these genotypes are tetraploids (2n = 4x = 28).

3.3 Experimental design and treatment application

The experiment was conducted as a factorial with three factors: four levels of colchicine powder (Alphatma, Moscow, Russia) concentration (C0 (0%), C1 (0.05%), C3 (0.1%), and C3 (0.2%), three levels of exposure period (T1: 24 h, T2: 48 h, and T3: 72 h), and two Napier grass germplasms (V1 *South africa* Napier grass and V2 *Bana grass*), resulting in twenty-four treatment combinations. These were replicated thrice using a factorial completely randomized design (CRD), resulting in ninety-six experimental units. Initially, a pre-experiment was conducted on somatic embryogenesis to develop explants that were used in the subsequent steps of the study. The colchicine treatment and acclimatization in the greenhouse.

This pre-experiment was a factorial experiment in a completely randomized design (CRD) with 3 factors: 3 growth hormones for callus induction, 3 growth hormones for shoot regeneration and rooting, and 2 germplasms of Napier grass. This resulted in eighteen treatment combinations that were replicated six times, giving a total of 108 experimental units. From this, he best explants that formed embryogenic calli were selected for the next stage of the study. The following callus induction media were used: GM0 as a comparative control in hormone-free media, GM1 (MS media supplemented with 0.3 mg/L^{-1} BAP, 0.5 mg/L^{-1} 2,4-D, and 0.5 mg/L^{-1} IBA) and GM2 (MS media supplemented with 0.5 mg/L^{-1} BAP, 1.0 mg/L^{-1} 2,4-D, and 1.0 mg/L^{-1} IBA). For shoot regeneration, the following media were used: SRM0 as a comparative control in a hormone-free medium, SRM1 (MS media supplemented with 1 mg/L^{-1} BAP, 0.25 mg/L^{-1} 2,4-D, and 0.25 mg/L^{-1} IBA(Protist Lab Africa Ltd, Mfangano Street, Nairobi-Kenya), and SRM2 (MS media supplemented with 2 mg/L^{-1} BAP, 0.5 mg/L^{-1} 2,4-D, and 0.5 mg/L^{-1} IBA). For how long?

3.4 Callus Induction

Callus were induced from young leaf whorls explants (Shoot tillers) of two interspecific napier grass hybrids that were collected from the field (Gondo et al., 2017). The shoot tillers were washed with running tap water to remove sand and dust particles for 5 minutes. After washing 3 to four times, shoot-tips of length 1–7 cm were abscised from shoot tillers (explants) and the explants taken in the laminar flow cabinet (aseptic conditions) and soaked in distilled water for 25 to 30 minutes. Thereafter the explants were then transferred to 2.5% fungicide (with active ingredient dimethyldithiocarbamete) solution and soaked for 30 minutes. They were then washed 3 times with sterile distilled water to remove the fungicides for 6 minutes each washing taking 2 minutes. This was followed by surface sterilization done by immersing explants in 70 % (v/v) ethanol for 2 minutes, followed by, immersion in a 20% (v/v) NaOH (Protist Lab Africa Ltd, Mfangano Street, Nairobi-Kenya) and agitated for 15 minutes. Shoot tips of explants were rinsed three times with sterilized water, each wash lasting 2 minutes (Unami et al., 2016 and Gondo et al., 2017).

Tissue culture techniques were conducted in a laminar flow cabinet where shoot-tips of explants were prepared by trimming/further sectioned into 1–5 mm cross-sections using a sterile scalpel. The sectioned

Cross-sections were then pre-soaked in a solution containing 10 mg ascorbic acid, to prevent browning, then cultured on petri dish, in Murashige and Skoog (MS) medium supplemented with 0, 0.5, 1.0mgL-1 dichlorophenoxyacetic acid (2,4-D), BAP (0, 0.3, 0.5mgL-1), and 0, 0.5, 1.0 mgL-1 indole-3- butyric acid (IBA) for callus induction (Unami et al., 2022). The induction media was adjusted to PH 5.6 - 5.8 using 1mole/L HCL(Protist Lab Africa Ltd, Mfangano Street, Nairobi-Kenya) or NaOH (Protist Lab Africa Ltd, Mfangano Street, Nairobi-Kenya) prior to autoclaving at 121°C for 15 minutes (Unami et al. 2022) This culture was used to initiate the cultures, where each culture tube contained 20ml of medium. Cultures were incubated in the dark at 27 ± 2˚C (Unami et al., 2016) in a fabricated old model oven, fitted with a clock timer and a florescent bulb. Temperatures were calibrated to the required standards to function as a growth chamber. Explants were sub-cultured after 2 weeks to new medium of the same composition as above (Gondo et al., 2017). Sixty days after culture initiation, cultures were evaluated by recording the size, color, shape and texture of the embryogenic callus and selected for treatment with colchicine (Fabio et al., 2016; Ardabili et al., 2015).

3.5 Treatment with Colchicine and regeneration (Shoot and root)

Colchicine was applied at 134 days of culture; a factorial experiment was laid on a completely randomized design (CRD) with three replications. Embryogenic calli from the two interspecific hybrids were distributed at random on petri dishes containing different concentrations of colchicine (0%, 0.05%, 0.1%, and 0.2%), with each treatment lasting 24, 48 and 72hrs respectively. Embryogenic calli were then rinsed thoroughly with sterile distilled water for 2 minutes, to wash off colchicine and dried using a blotting paper (Fabio et al., 2016).

In order to proliferate shooting, a sub set of embryogenic calli were transferred to shoot regeneration media with MS supplemented with various concentrations of BAP (0, 1, 2mg L-1), 2, 4-D (0, 0.25, 0.25mg L-1), IBA (0, 0.25, 0.25mg/L-1). All media pH was adjusted to 5.6 - 5.8 prior to autoclaving (Ardabili et al., 2015) and cultured in a fabricated old model oven, fitted with a clock timer and a florescent bulb. Temperatures were calibrated to the required standards to act as a growth chamber providing 16 h (light):8

h (dark) photoperiod at 27 ± 2˚C. After 14 days of culture, elongated shoots were transferred to a rooting medium supplemented with NAA 1mg L-1, 2,4-D 1mg L-1 and 150mg Ascorbic acid. They were socked for 24hrs before transferring them to pots containing sterilized soil and taken in the screen house for acclimatization (Yu et al., 2009; Ardabili et al., 2015 ; Fabio et al., 2016).

3.6 Determination of ploidy level

Ploidy level of induced mutants was assessed by chromosome counts and genomic DNA, and confirmed by examining stomata number and size of stomata (Yu et al., 2009, Ardabili et al., 2015 and Fabio et al., 2016).

3.6.1 Chromosome counting by Karyotyping

Polyploid level of regenerated induced mutant were determined by karyotyping (Ardabili et al., 2015 and Fabio et al., 2016). This involved profiling and analyzing chromosomes based on their size, shape, and banding patterns. Young leaf whorls samples of about $2cm^2$ were collected from the greenhouse and fixed by immersing leaf tissues in 1% chromic acid and 10% formaldehyde solution for 24h at 4°C. Segments of the leaf were then softened by immersing them in sodium hydroxide (1N) for 10 min at 60°C and stained with aceto-carmine 4% for 10h, stained leaf tissues were placed on a glass slide and examined under a light microscope at X80 and X100 to visualize chromosomes.

3.6.2 Genomic DNA

3.6.2.1 Extraction

Genomic DNA was extracted from young emerging leaf tissue (0.3–0.7 g) of rooted plantlets early in the morning to ensure high-quality DNA, fixed with 70% ethanol and rinsed with molecular water (water free of ions, organic compounds and microorganisms) and placed in a cooler box with ice (Yu et al., 2009, Fabio et al., 2016). DNA was extracted using TES (Tris-EDTA-Sodium Chloride) procedure (Doyle, J.J and Doyle, J.L. 1990) where the frozen leaves were pulverized using a mortar and pestle until a fine paste was obtained. This was followed by lysis where the paste was transferred in a centrifuge tube and samples were vortexed for 30s. 500ul of TES DNA extraction buffer was then added to the tube to digest the cell wall and release cellular contents including the DNA into the solution. The solution was vortexed for another 30s to mix the solution thoroughly. Then samples in the centrifuge tubes were placed a water path at 65°C for 30mins while gently mixing by shaking within 5min interval. To precipitate proteins and other contaminants from the DNA, Ammonium acetate of volume 250ul was then added in the solution and vortexed for 30s then incubated for 10 mins. Centrifuging was done for 15 mins at 20,800 RPM and the supernatant transferred to new tubes. To this, 500ul of cold isopropanol was added to enhance DNA precipitation, then incubated at -20°C for 2 hrs. Samples were centrifuged again for 10mins at 20800RPM to pellet DNA and separate the DNA from the supernatant. Thereafter DNA pellets were washed with 800ul cold 70% ethanol to remove impurities, and then air dried lightly by carefully turning the tubes upside down to evaporate any remaining ethanol. The DNA was resuspended in the TE buffer and incubated at -20°C (Doyle and Doyle 1990) .

3.6.2.2 Gel electrophoresis

Agarose gel was prepared by dissolving 0.8% agarose in TAE buffer, then heating the solution for approximately five minutes. The melted agarose was poured into a casting tray with a comb to create wells and allowed to solidify. The prepared DNA samples and a 5KB DNA ladder were then carefully loaded into the wells while using the loading dye to visualize. (Rayburn et al., 2009)

The prepared gel was placed in the electrophoresis apparatus and covered with the TAE buffer and ran at 110 voltage and 150 current for a time duration of 9min. The bands were visualized on FUJI X-ray Film (Sambrook e2001).

3.6.2.4 Stomata size and Number

For stomata measurements, 35-d-old leaves that were about 1 cm^2 in size were collected from the greenhouse 2 months after regeneration. An area of approximately 0.2 cm^2 on the upper leaf epidermis was treated with a colorless nail polish. Once the polish dried, the layer was peeled off using adhesive tape. The tape was then affixed to clean and clear glass slide and the stomata density per mm^2 was counted with a light microscope at ×10, ×20, and ×40 (Chae et al., 2012 and Ardabili et al., 2015)

3.6.2 Performance determination of induced Mutants

Acclimatization was done in the screen house where seedlings of regenerated plants were grown in black polyethene seedling sleeves that contained sterile mix of organic manure and forest soil in the ratio 1:3 respectively. Each seedling sleeve had a dimension of 6cm by 9cm, that were arranged in a completely randomized design (CRD), replicated thrice (Figure 3). The sleeves were arranged at a spacing of 20cm by 20cm. At the center of each pot, a hole measuring 5cm by 5cm by 5cm was dug out to accommodate a single seedling. Prior to transplanting, NPK planting fertilizer was mixed with the sterile mix at a rate of 10 grams per plant, which was equivalent to 200:250 kg per hectare. Thereafter, standard routine agronomic practices were conducted. The collection of growth parameters data for regenerated mutant plants started on 1st May 2024 after transplanting to 30th June 2024.

3.7 Data collection.

3.7.1 Callus induction

Data on callus induction was collected as follows:

3.7.1.1 Embryogenic calli formation

i. Embryogenic calli induction

Calli induction frequency was evaluated each week for 4-8 weeks. The number of embryogenic calli induced from explants were counted under a stereomicroscope. Data for percentage embryogenic callus induction was calculated using the following formula**:**

Embryogenic calli induction frequency (%) = (Number of embryogenic calli induced/ explant cultured) × 100.

ii. Embryogenic calli growth

Calli growth were evaluated visually by recording observation by touching calli tissue using spatula or forceps to determine whether they are soft or firm. They were calculated as a percentage by taking embryogenic calli that had growth /the initial embryogenic calli induced multiplied by100.

iii Calli survival rate

a. Assessment of survival:

Survival of embryonic calli was assessed visually by observing calli and determining whether they are alive or dead. Callus that had lost its green color or became light brownish or cream was considered alive while the one that had blemish or necrosis were considered dead. The survival rate was determined by dividing the number of surviving calli by the total number of calli treated and multiplying by 100.

3.7.3 Effect of colchicine concentration on survival of explants and polyploid induction

Colchicine concentrations (0, 0.05, 0.1 and 0.2%) and treatment time (24, 48 and 72hrs) effect on survival and ploidy induction of explants were examined in a factorial experimental. Data was recorded based on visual observations made on the explants' morphology and survival

3.7.4 Ploidy evaluation

3.7.4.1 Chromosome determination by Karyotyping

Chromosomes were counted and recorded for each cell observed on the slides of a light microscope. The chromosome number was determined by counting the total number of chromosomes in a representative sample of cells. A minimum of 5-10 cells were counted to determine the average chromosome count, by adding up the chromosome counts from all the individual cells analyzed and dividing by the total number of cells.

3.7.4.2 Stomatal characteristics: Additionally, four stomata per leaf were measured for their length and diameter using a light microscope with a X20 and X40 objective and oculars respectively. The obtained values were then multiplied by a coefficient of 2.439 to obtain the true length of the stomata. Similarly, the density of stomata was determined as the number of stomata per square millimeter (Sari et al., 1999 and Yetisir and Sari, 2003)

3.7.4.3 Genomic DNA:

3.7.4.3.1 Band Pattern: This data was collected by comparing the collective arrangement of all bands on the gel, providing a visual representation of the DNA composition between induced mutants and controls indicating potential genomic changes

3.7.4.3.2 Relative Mobility: This is the measure of the distance of migrating bands when an electric current is passed through the chambers where by DNA fragments move towards the positively charged cathode. The relative mobility was calculated by distance covered by recording the distance covered of each migrating bands in (cm). Differences in distance covered by each band indicating genomic variation between putative induced plantlets and their progenitors.

3.7.4.3.3 Performance of Induced Mutants Plants

3.7.4.3.3.1 Plant architecture: The overall plant architecture was recorded and compared between induced mutants and controlled plants;

i. Plant height: The height of the plants was measured periodically using a measuring tape (cm) from the ground to highest point.

ii**. Number of tillers**: Tillers are shoots arising from the base of the plants and were counted on a weekly basis per plant by visually inspecting the plants, providing information on vegetative growth of the plant.

3.7.5 Data Analysis

Statistical analysis of variance and means comparison was done using R version 4.2.3 software. Tukey's test was used to determine the differences between culture media supplemented with different growth regulators, colchicine treatments and growth parameters viz; stomata number and size, plant height and tiller number significantly differ from each other at $p \leq 0.05$ significance level.

3.7.6 Ethical Considerations

All laboratory procedures complied with the ethical and biosafety regulations of the KALRO Kakamega Tissue Culture Laboratory, including proper handling of chemicals and disposal of contaminated cultures. Colchicine, being a hazardous mutagen, was handled with strict safety measures using appropriate personal protective equipment such as gloves, lab coats, and masks, and all colchicine-containing waste was disposed of according to institutional hazardous waste protocols. To ensure environmental safety, no genetically modified or induced mutant plants were released outside the laboratory, and all treated materials were maintained under controlled conditions to prevent unintended dissemination. The study further adhered to principles of responsible reporting, whereby all data were accurately recorded, analyzed, and presented without manipulation to maintain scientific integrity. Additionally, all external sources, methodologies, and published materials used in the study were properly cited to respect intellectual property and avoid plagiarism. Importantly, a formal research permit was obtained from the National Commission for Science, Technology and Innovation (NACOSTI), ensuring legal and ethical compliance with Kenya national research regulations.

3.7.7 Limitations of the Study

1. The efficiency of colchicine uptake by the explants may have varied across treatments, potentially influencing the polyploid induction rates.
2. The process of polyploid induction may have introduced somaclonal variations, which could have affected phenotypic traits independently of colchicine treatment.
3. Occasional tissue culture contamination reduced the number of viable samples in certain treatments, limiting replication and consistency.
4. The absence of flow cytometry constrained the accuracy of ploidy confirmation, making the study rely primarily on cytological techniques such as chromosome counting and stomatal analysis.
5. Differences in genotype responsiveness to both plant growth regulators and colchicine could not be fully controlled, possibly contributing to variation in experimental outcomes.
6. Time constraints restricted the study to early-stage evaluations, preventing long-term assessment of mature plant performance and stability.

3.7.8 Assumptions of the Study

1. All explants used in the study were assumed to be genetically uniform, healthy, and physiologically suitable for culture initiation.
2. The embryogenic calli generated from the explants were assumed to respond consistently to the applied PGR and colchicine treatments.
3. The morphological and cytological differences observed among regenerated plantlets were assumed to result primarily from colchicine-induced polyploidy rather than other external factors.

4. Laboratory environmental conditions—including temperature, humidity, and light intensity—were assumed to remain stable throughout the experimental period.
5. The effects of the applied colchicine concentrations on explant viability, callus development, and ploidy induction were assumed to be detectable within the two-month observation period.
6. All measurement instruments, including microscopes, imaging equipment, and growth chambers, were assumed to provide accurate and reliable data for analysis.

3.7.9 Validity and Reliability of the Study

The validity of this study was ensured by designing procedures, hormone treatments, colchicine concentrations, and cytological assessments based on well-established protocols in plant tissue culture and mutation breeding, thus guaranteeing strong content validity and ensuring that all essential components for evaluating polyploidy induction were addressed. Construct validity was achieved by using scientifically recognized indicators such as callus induction rate, explant survival percentage, stomatal size, and chromosome counts which accurately reflect polyploidy and plant developmental responses. Internal validity was maintained through the use of identical explant sizes, constant environmental conditions, uniform media formulations, and randomized treatments to minimize bias, while external validity was confined to *in vitro* Napier grass tissue culture systems and not field environments.

Reliability was strengthened through biological and technical replication of treatments, the use of standardized sterilization, culture, and treatment protocols, and the maintenance of consistent media preparation and incubation conditions. Additionally, instrument reliability was ensured by regular calibration and maintenance of microscopes, digital cameras, incubators, and laminar flow hoods, while repeatability was enhanced by taking measurements such as survival rate, callus growth, stomatal size, and chromosome counts multiple times and by the same trained researcher to ensure consistent and dependable results.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Response of genotype to tissue culture

Analysis of variance revealed significant genotype effects for the percentage of explants with callus at 4 weeks after culture initiation, necrosis intensity and amount of embryogenic callus at $p \leq 0.05$ (Figure 1). All genotypes produced most calli 97.5% 4 weeks after culture initiation (Figure 4). There were no significant differences ($p \leq 0.01$) among germplasm for 4 weeks. Consequently, no germplasm produced embryogenic callus 4 weeks after culture initiation. There were higher levels of necrosis in V1 germplasm while V2 recorded the lowest. Ideally, germplasm V2 recorded highest levels of contamination while V1 had the lowest.

After 7 to 8 weeks of culture, embryogenic callus on all germplasm was initiated in most media where it was evident that embryogenic callus was significantly affected by different plant growth hormonal combination (Figure 1). The percentage of embryogenic callus after 8 weeks was 80% for all genotypes on media with 1.0mgL-1 IBA, 1.0mgL-1 2,4-D and 0.5mgL-1 BAP while genotypes on media with 0.5mgL-1 IBA, 0.5mgL-1 2,4-D and 0.3mgL-1 BAP produced 30% callus. Explants cultured on hormonal free media did not produce any sign of growth of callus or embryogenic callus, they also had 100% necrosis (Figure 2). Table 4 showed that necrosis was there in all genotype where germplasm V1 had the highest after 4 weeks 6% and 8 weeks 10% while germplasm V2 had the lowest case of 4 weeks 5% and 8 weeks 9% necrosis.

In the present study, culturing using shoot tillers was preferred because they were less affected by contamination after various sterilization processes (Unami et at., 2016) MS medium supplemented with 1.0mgL-1 IBA, 1.0mgL-1 2,4-D and 0.5 BAPmg/L-1 was the most suitable in initiation of embryogenic callus after 8 weeks (Table 4, Figure 1). A Previous study by Unami et al. (2016) and Shan et al. (2000) highlighted the use of BAP or TDZ which played a significant role in cereal and grass species in shoot regeneration. This demonstrate that low levels of 2,4-D and IBA in combination with BAP are ideal and most suitable for shoot regeneration in *Cenchrus species* (Shan et al. 2000 and Unami et al, 2022). Napier grass regeneration from shoot tillers is influenced by its germplasm and initiation media, which have advance effects on initiation of callus and thereafter-embryogenic calli formation. In the present study, the interaction between germplasm and explants was significant possibly, as a result of gene expression and alteration, that solely depends on physiological stages (Vasil t al., 1986).

Table 4 Effects of different growth hormone combination on embryogenic calli induction.

Growth Media	Genotype	No.of Calli formed/% (4weeks)	No. of Necrosis formed/% (4weeks)	No. of embryogenic calli formed/% (4Weeks)	No. of embryogenic calli formed/% (8weeks)	No. of Necrosis formed/% (8weeks)
GM0	V1	0 (0)	96 (100) a	0 (0)	0 (0)	0 (0)
	V2	0 (0)	96 (100) a,b	0 (0)	0 (0)	0 (0)
GM1	V1	65 (87.83) c	11 (14.86) b	0 (0)	25(33.783) c	9(12.162)a
	V2	63 (85.13) d	9 (12.16) d	0 (0)	23 (31.081) d	4 (5.405)d
GM2	V1	68 (91.89) b	9 (12.162)d	0 (0)	51 (68.919) a	6 (8.108) c
	V2	69 (93.24) a	10 (13.514) c	0 (0)	49 (66.216) a,b	8 (6.757)b

Mean levels with $^{a–d}$ different numbers following each value within a column demonstrate significant differences according to Tukey's test ($p \leq 0.05$). GM0 indicates explants established in hormone-free media. GM1 indicates MS media supplemented with 0.3 mg/L^{-1} BAP, 0.5 mg/L-1 2,4-D, and 0.5 mg/L^{-1} IBA. GM2 indicates MS media supplemented with 0.5 mg/L^{-1} BAP, 1.0 MG/L-1 2,4-D, and 1.0 mg/L^{-1} IBA. Note: V1 and V2 is variety 1 and 2, GMO is home free media, GM1 is growth media one, while GM2 is growth media two. *Source: Wafula et al., 2025*

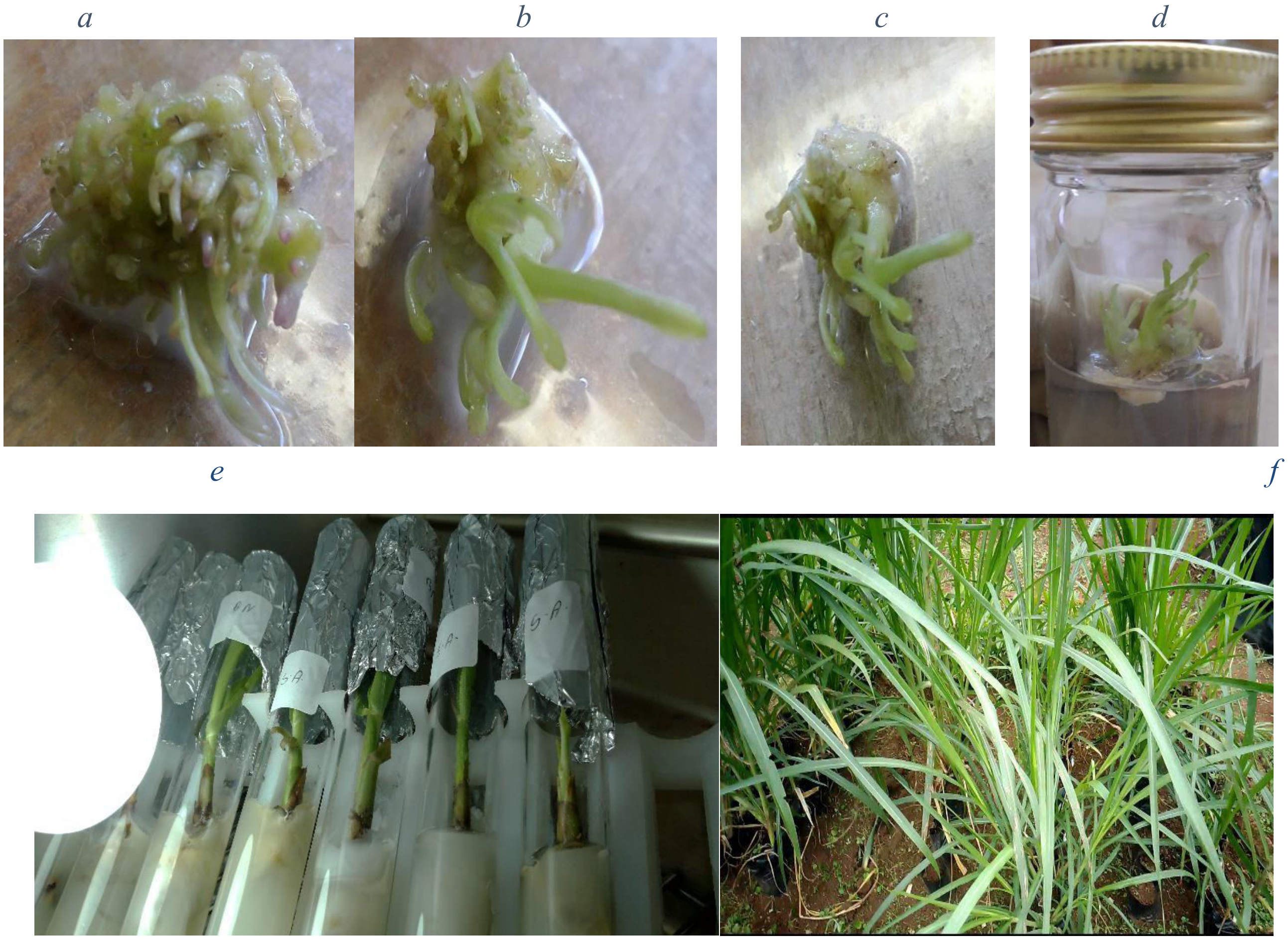


Figure 1 Regeneration of two genotypes of *Cenchrus purpureus* through somatic embryogenesis. **a** Embryogenic callus in MS media supplemented with 0.5mg/L-1 BAP, 1.0MG/L-1 2,4-D and 1.0mg/L-1 IBA. **b, c** and **d** Sprouting embryos in different growth stage in calli treated with colchicine. **e** Shoot regeneration and root induction with MS medium supplemented with 0.05mgL-1 IBA, 0.05mgL-1 2,4-D and 2mgL-1 BAP and transferred to media supplemented with NAA 1mgL-1 and 150mgL-1 Ascorbic acid for 14 day before being transferred to soil. **f** Regenerated of synthetic induced mutants with their progenitors after being potted and transferred in the greenhouse.Source: *Wafula et al., 2025*

4.2 Shoot and root Regeneration

Growth of shoots were initiated after 134 days of culture in most media (Table 4). Regeneration percentage had been significantly affected by different growth hormonal regulators combinations. Explants that were cultured on hormone free media did not show any sign of growth and were affected 100% by narcosis.

There was 40% shooting on MS medium supplemented with 0.05mgL-1 IBA, 0.05mgL-1 2,4-D and 2mgL-1 BAP which was the most suitable media for shoot regeneration while on medium with 0.25mgL-1 IBA, 0.25mgL-1 2,4-D and 1mgL-1 BAP had 20% (Table 4). After 14 days mutants plants were transferred in the greenhouse, rooting was induced after being cultured in media with MS supplemented with NAA 1mgL-1 and 150mgL-1 Ascorbic acid where they continued to grow on their own and produced roots which enabled them in absorption of water and minerals (Table 5, Figure 1).

A study conducted by Maeda et al., 2002 made the following observations of presence of green and white spots that were frequently seen on the surface of callus during callus induction with high regeneration ability. The use of auxin alone or in combination with cytokinins has been shown to play a vital role in organogenesis and somatic embryogenesis during critical periods of cell division in primordial cells which result in shoot regeneration in plants (Gaspasr et al. 1996; Pola et al. 2007). A study conducted by Fabio et al. (2016) documented the regeneration of interspecific hybrids of elephant grass and *Pearl millet* via somatic embryogenesis from shoot tips explants and reported highest regeneration rate in MS medium supplemented with 0.5 mg/L BAP and 2mgL-1 2,4-D using Leaf whorls containing immature inflorescences.

Consequently, Shan et al. (2000) and Pola et al. (2007) backed earlier reports on the combination of BAP or TDZ in shoot regeneration but in this report, they added IAA which was observed to increase the production of multiple shoots. Additionally, in the present study, rooting was induced by transferring plantlets to a liquid media containing 1mg/L-1 NAA in combination of 0.05g/L-1 of ascorbic Acid and 1mg/L-1 IBA for 24h before being potted and transferred to the greenhouse. Contrary to these findings, the use of NAA alone in solid or liquid media has been demonstrated to be the best in inducing rooting (Fabio et al, 2016; Pola and Sarada Mani 2006)

Table 5. Growth hormone combination effects on shoot induction derived from shoot tillers of two genotypes of Cenchrus purpureus.

Growth Media	Genotype	No. of regenerated clumps (%)	No. of regenerated shoots (%)
SRM0	VI	0(0)^{e}	0(0)^{e}
	V2	0(0)^{e}	0(0)^{e}
SRM1	V1	16(21.6)c	15(20.27)^{c}
	V2	14(19)^{cd}	11(14.86)^{d}
SRM2	V1	37(50)^{a}	32(43.24)^{a}
	V2	32(43)^{b}	30(40.54)^{ab}

Mean levels with $^{a–e}$ different letters following each value within a column demonstrate significant differences ($p \leq 0.05$). SRM0 indicates explants established in hormone-free media. SRM1 indicates MS media supplemented with 1 mg/L^{-1} BAP, 0.25 mg/L^{-1} 2,4-D, and 0.25 mg/L^{-1} IBA, SRM2 indicates MS media supplemented with 2 mg/L^{-1} BAP, 0.5 mg/L^{-1} 2,4-D, and 0.5 mg/L^{-1} IBA.Note: SRMO is hormone free media, SRM1 and SRM2 is shoot regeneration media one and two respectively *Source: Wafula et al., 2025*

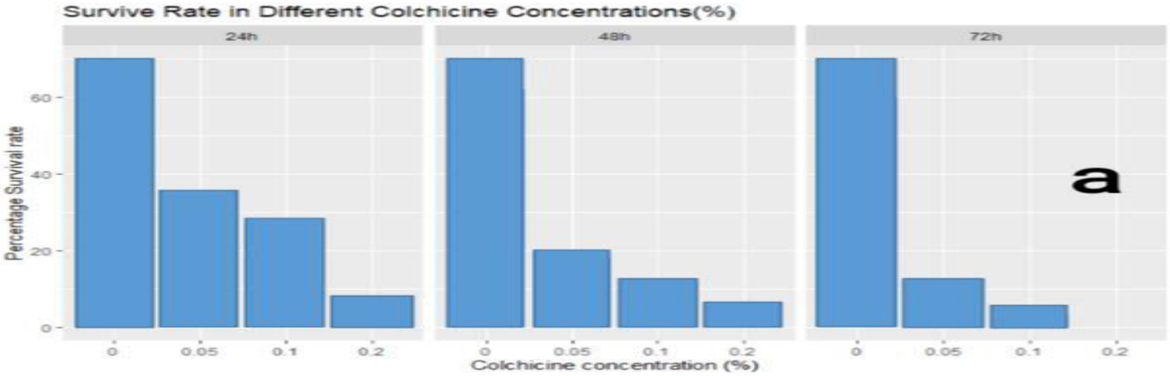
4.3 Effect of different colchicine concentrations on survival of explants and ploidy induction

Explants from the two germplasms were treated with different colchicine concentration as stated earlier on solid media. The percentage (%) of survival rate differed depending on colchicine concentrations levels, exposure duration and their interactions on callus survival ranging from 0-100% (Figure 2). Toxicity was observed on higher concentrations and longer time of exposer to treatments which severely affected survival rate of callus. Importantly, it was also observed through analysis of variance which demonstrated high levels of significance effects of colchicine concentration and time of exposure, and their interaction in octopoidy induction. The frequency of induction of octoploidy in synthetic polyploidy plantlets increased with increase in exposure time (Figure 2).

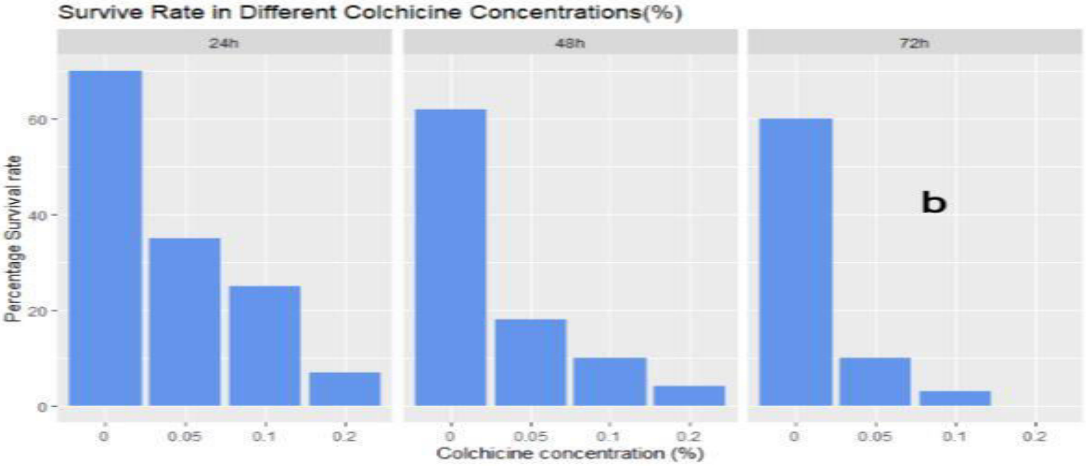
Colchicine concentration of 0.05% and exposure duration of 24h yielded the highest survival rate (35%) but led to low induced mutants 17 (48%), presumably due to lack of colchicine effect. On the other hand, treatment with 0.2% at 24h duration resulted in altered ploidy of 5 (7%) while 48h resulted in 3 (5%) callus survival. However, 72h duration resulted in 100% mortality of callus (Figure 3). The result also demonstrated that colchicine concentration of 0.1% with 48h duration of exposure was most suitable in inducing polyploidy 12 (48%) plant under *in vitro.* It was observed that the growth and induction of roots in mutant plants were severely affected by colchicine treatment viz the control. Furthermore, induced mutant plants were shorter than their progenitors, despite being the first to produce tillers they also had high number of tillers, and less stem diameter (Table 6, Figure 4

Leaf samples of synthetic mutants lines per germplasm were randomly selected for genomic DNA extraction and chromosome counting where it was evident that indeed chromosome doubling took place in some of regenerated plantlets that were distinct from there progenitors (Figure 2, Figure 6, Figure 7). The average genomic DNA size of regenerated plants was significantly different where single cell lines from the samples collected were characterized had same band pattern but some different from others. This resulted from different identical events which were depicted by gel electrophoresis using a 5kb ladder.

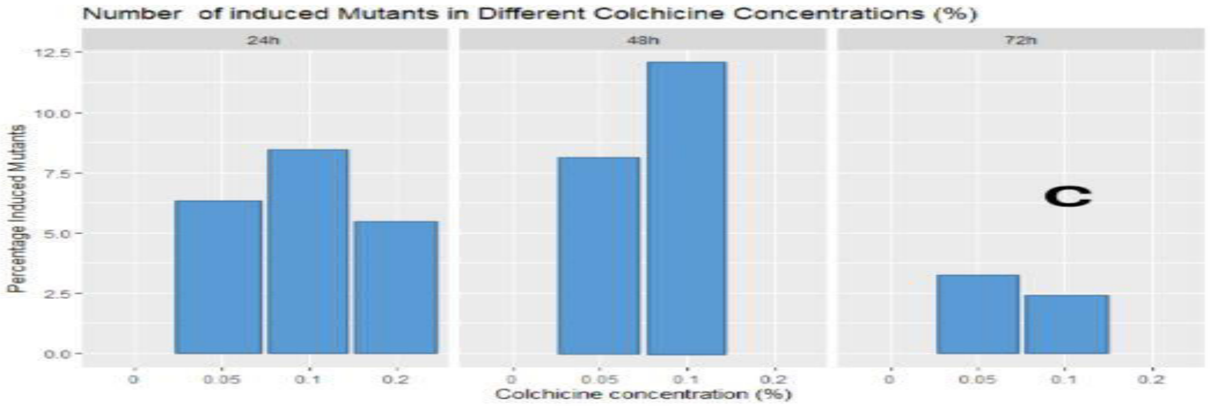
a)V1


Survive Rate in Different Colchicine Concentrations(%)
24h
48h
72h
Percentage Survival rate
0
20
40
60
0
0.05
0.1
0.2
Colchicine concentration (%)
a

b)V2


Survive Rate in Different Colchicine Concentrations(%)
24h
48h
72h
Percentage Survival rate
0
20
40
60
0
0.05
0.1
0.2
Colchicine concentration (%)
b

c) V1



d)V2

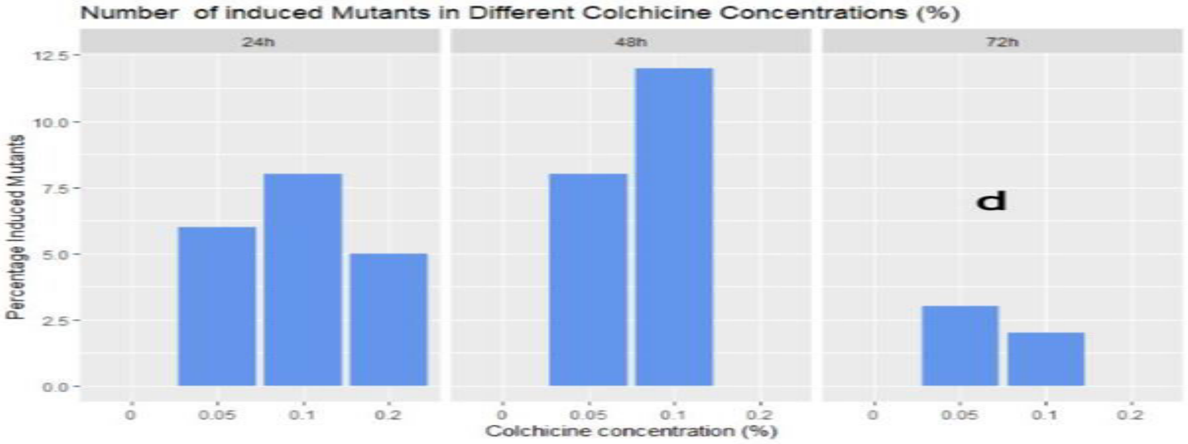


Figure 2. Percentage explant survival rate and synthetic induced mutants after exposure to different colchicine levels and time durations. (**a**) A and B show survival rate of explants after exposure to colchicine: C0 = 0, C1 = 0.05, C2 = 0.1, and C2 = 0.2%, and exposure duration: 24hrs, 48hrs, and 72 hrs. (**b**) C and D shows number of mutants induced in different colchicine concentrations. *Source: Wafula et al., 2025*

The present study on *in vitro* induction of polyploidy demonstrates the important role of induced mutation as an efficient and cost-effective method of breeding *Cenchrus purpureus*. However, *in vitro* induction of mutation using chemical mutagenesis like colchicine has limitations vis, high doses with longer exposure duration are lethal to plants as they significantly reduced calli survival rate and resulted in deformed plants

(Mba et al., 2009). In this study, it was evident that there was high toxicity when colchicine concentrations were increased to 2% with longer duration of exposure in all genotypes, while 0.1% colchicine concentration with a treatment duration of 2 days led to maximum number of induced mutants. Other authors have reported a similar relationship between colchicine doses and survival rate of explant (Chen et al., 2007; Mba et al., 2009).

Nevertheless, since the main objective of exposing plant cells to colchicine is to induce mutation, treatment that reduce survival rate can be beneficial due to the reduction in progenitor's number and selection of induced mutants (Vainola t al., 2001). The plants leaf whorl consists of outer and inner compartment with high numbers of primordial cells. The outer compartment results in production of new plant cells while the inner compartment which houses the initial high number of primordial cells, is responsible for the production of other plant cells. Exposure of plant cells to different mutagenic doses affects plants positively and negatively by manifesting in primordial cells when they are actively dividing (Kiong et al., 2008; Dhamayanthi et al., 2010)

4.4 Phenotypic effect on leaf characteristics

Induced mutants' stomata size and number was significantly different compared to their progenitors where they exhibited larger stomata with stomatal length of 20 or more (>120um) than their progenitors which were very dissimilar. However, they had lower stomata density (Table 3, Figure 1). Similarly, analysis of variance detected high correlation in tillering ability after 6 weeks of establishment in the greenhouse where induced mutants were the first to produce tillers (Table 6, Figure 4). However after 8 weeks, despite all plants being able to produce tillers there was a positive correlation in number of tillers between induced mutants with their progenitors (Table 6, Figure 4).The mean of plant height between induced mutants and their progenitors were greater as most induced plants had slow growth rate as a result of chromosome aberration (deletion, duplication, inversion and translocation), physiological and toxic effect which presumably reduced cell survival (Table 6, Figure 1). As so, the mean stem diameter of synthetic induced mutants were slightly smaller as compared with their progenitors (Table 6, Figure 4)

The current study shows the presence of mutants that are genetically dissimilar due to phenotypic segregation. Phenotypic diversity evaluated in induced mutants included plant height, early and late tillering, and stomata size and number. The results of synthetic induced mutants revealed significantly bigger stomata with a lower stomatal density, findings that were also reported by Queensenberry et al., (2010) and Liu et al., (2007). Similarly, Tale et al. (2005) reported that estimation of ploidy level in closely related species can be achieved using stomatal guard cell size as it is often significantly bigger in induced mutants than there progenitors. The use of stomata length was equally reported in other crop grass pasture such as rye grass and barley (Speckmann et al., 1965 ; et al., 1988),*Miscanthus x giganteus* (Yu et al., 2009).

In the present study, preliminary screening of the synthetic induced mutant by comparison of stomata size and number with their corresponding counterparts was successful. Some of the selected plantlets were confirmed as synthetic induced mutants by chromosome counting and genomic DNA. Observations made in the chlorophyll of some induced mutants after exposure to different colchicine doses revealed plantlets with yellow and striped leaves, i.e albino leaf. The research findings of Maluszynski et al. (2009) showed that the presence of chlorophyll deficiencies is a good indicator of genetic action of mutagens, where increasing mutagens doses lead to a greater frequency of chlorophyll plantlets. The negative effects of chlorophyll in the early stage of growth are of essence in breeding and evaluation for genomic effects and sensitivity of mutagens in crops (Tulmann Neto et al., 2011).

The other main morphological changes observed in the present study were early/ late tillering, reduced plant height and stem diameter in synthetic mutant of the two *Cenchrus* germplasms compared with their counterparts. Similar results were previously observed in *Miscanthus sinensis* (Glowacka et al., 2010) who reported that physiological delays are apparently due to delayed development in synthetic induced mutants as a result of chromosome aberration.

A positive correlation in terms of DNA genome size has also been observed, except that some *South africa* Napier grass plantlets and their progenitors could produce tillers after 6 weeks (but *Bana grass* had more tillers compared to *South africa napier grass*. However, the present study also reports that all induced mutants and some progenitors produced tillers but with diverse number of tillers depending on genotype, colchicine concentration, and exposure time. A previous study by Dhanavel et al. (2008) also reported variation in plant development including stem diameter, tillering ability and days taken by plants to mature as a result of mutation. It is very evident from this study that through mutagenesis, morphological traits of importance could be used to improve the overall performance in terms of yield and herbage of pasture and forage for enhanced livestock productivity. The study also suggest that in selection for mutants with traits of importance, the effectiveness and efficiency of the mutagen is paramount. In this case, phenotype selection of induced mutants should not undeliberately result in physiological and chromosome abnormality as a result of deletion, inversion, duplication and translocation, and lethal dose. The effect on plant cell in the long run affect the cell by decreasing its survival rate and eventually eliminate the mutant.

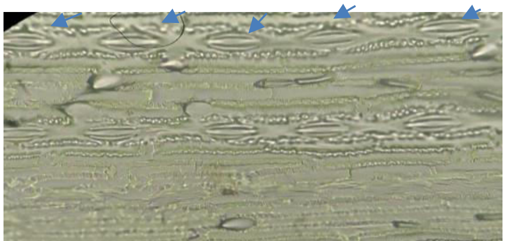
Table 6 Quantitative and qualitative characteristics in induced mutant's plantlets as compared with progenitors of Cenchrus purpureus plantlets.

Germplasm	stomata number (per mm^2)	Stomata size (mm^2)	Plant height (cm)	No. of Tillers after 6 weeks	No.of Tillers after 8 weeks	Stem diameter after 8 weeks (cm)
C0T0V1	21^{a}	122.54^{c}	190.23^{a}	0^{c}	2^{cd}	5.2^{a}
C0T0V2	19^{ab}	120.65^{cd}	145.31^{c}	0^{c}	5^{b}	4.7^{ab}
C2T2V1	11^{c}	162.44^{a}	123.86^{d}	2^{ab}	3^{c}	4.6^{bc}
C3T1V2	12^{c}	158.45^{b}	170.18^{b}	3^{a}	6^{a}	4.5^{bc}

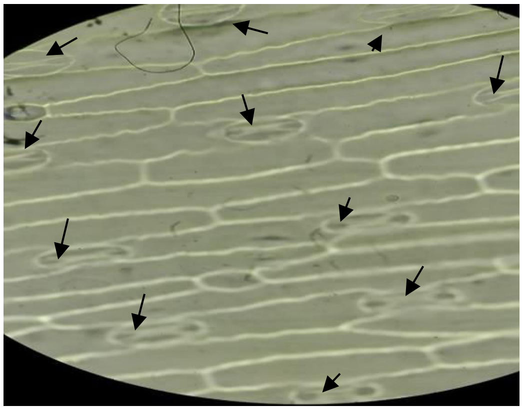
t-Tests were performed between compiled means from two control lines and those from synthetic induced mutants of each germplasm. Mean levels with $^{a–d}$ *different letters following each value within a column show significant differences (*$p \leq 0.05$*). Source: Wafula et al., 2025*

X20

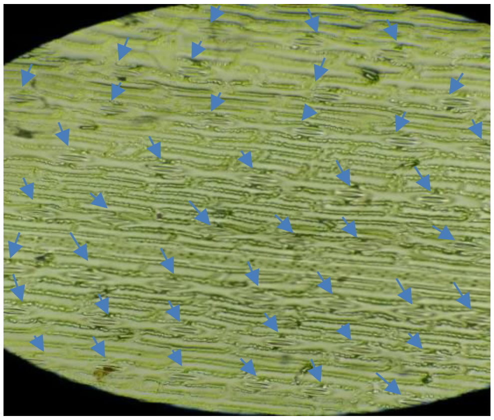
X40



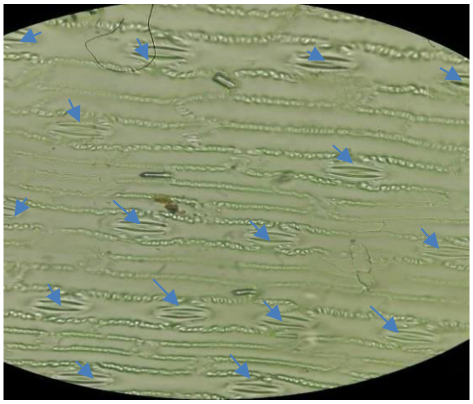
A X20



B X40



C X20



D X40

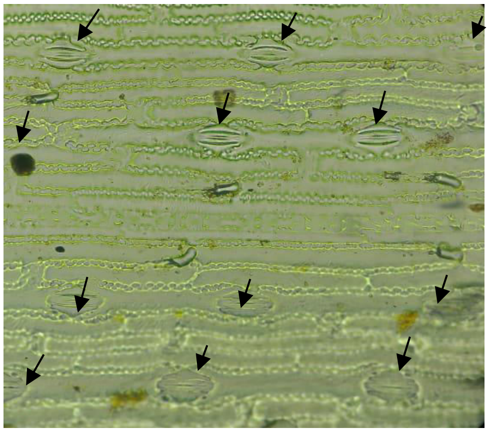


Figure 3 Difference in stomata size and number between synthetic induced mutant and their progenitors at x20 and x40 magnification. Blue arrow points at progenitors stomata while Black arrow points at synthetic induced mutants. a) C0T0V1 control viewed under x20, b) C0T0V1 control viewed under x40. c) C2T2V1 synthetic mutant viewed under x20. d) C2T2V1 synthetic mutant viewed under x40. A) C0T0V2 control viewed under x20, B) C0T0V2 control viewed under x40. C) C2T2V2 synthetic mutant viewed under x20. D) C2T2V1 synthetic mutant viewed under x40. Source: *Wafula et al., 2025*

Figure 4 . Tillering ability of two *Cenchrus purpureus* germplasms and their synthetic mutants after 6 weeks;A) C2T2V2 synthetic mutant. B) C0T0V2 progenitor. C) C2T2V1 synthetic mutant, and D) C0T0V1 progenitor, and 8 week; E) C2T2V2 synthetic mutant. F) C0T0V2 progenitor. G) C2T2V1 synthetic mutant, and H) C0T0V1 progenitor. *Source: Wafula et al., 2025*

Figure 5 Abnormalities shown by synthetic polyploidy after treatment with colchicine. A,B and C shows albino plants and leaf chlorosis abnormalities two weeks after treatment with 0.1% colchicine concentration and 48h duration, and 0.2% with 24h and 48h duration of exposure. E, F G and H shows abnormalities several weeks after establishment. E shows chromosome incompatibility that result to death of the plant. F and D shows leaf chlorosis of new leaf whole that is dying off. G and H show new leaf whole emerging with vigor immediately former dying off. *Source: Wafula et al., 2025*

Figure 6 Chromosome number in Cenchrus purpureus visualized under a light maicroscope at ×100. (a) COTOV1 progenitor (2n=4×=28). (b) C2T2V1 synthetic induced mutant (2n=8×=56. (c) COTOV2 progenitor (2n=4×=28) and (d) C2T2V2 synthetic induced mutant (2n=8×=56). * Measurements of Some chromosomes number were diverse among counts, possibly due to overlaying chromosomes. *Source: Wafula et al., 2025*

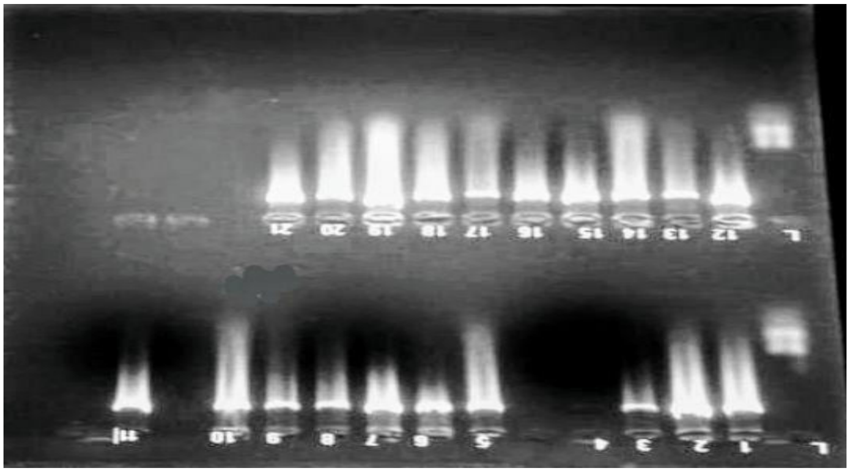


Figure 7 Genomic DNA of induced mutants and their progenitors during gel electrophoresis. Shift in band intensity/mobility shows genomic change due to polyploidy, indicating new bands/new alleles. Well L is the ladder, wells 1, 2, 6, 7, 10, and 11 represent V1 putative mutants, while wells 3, 5, 8, and 9 represent V1 progenitors. On the other hand, wells 12, 15, 18, 19, and 21 represent V2 putative mutants, while 13, 14, 16, 17, 1, and 20 represent V2 progenitors. Progenitors have lower-molecular-weight bands (28 chromosomes) viz the mutants, making them travel farther from the well and appear slightly lower on the

gel. On the other hand, putative mutants have larger-molecular-weight bands (56 chromosomes), making them travel much slower and travel a shorter distance from the well. *Source: Wafula et al., 2025*

CHAPTER 5

5.1 SUMMERY OF FINDINGS

This study investigated the effects of plant growth regulators (PGRs) and colchicine concentrations on embryogenic callus induction, shoot regeneration, and polyploidy induction in Napier grass (*Cenchrus purpureus*). The study revealed that the combination of 1.0mgL-1 IBA, 1.0mgL-1 2,4-D and 0.5 mg/L-1BAP was most effective for inducing embryogenic calli. Shoot and root regeneration was optimized using MS medium supplemented with 2 mg/L^{-1} BAP, 0.5 mg/L^{-1} 2,4-D, and 0.5 mg/L^{-1} IBA and root formation was achieved by MS supplementation with NAA 1mgL-1 and 150mgL-1 Ascorbic acid. This underscores the importance of optimizing PGR combinations in Napier grass tissue culture. Colchicine concentrations significantly affected callus survival and polyploid induction, with medium concentrations (0.1%) enhancing survival and ploidy induction, while higher concentrations (≥0.2%) reduced viability and regeneration, indicating the need for precise treatment optimization. Cytological analysis through chromosome counting and stomatal measurements confirmed successful induction of polyploidy, with low to moderate colchicine levels inducing polyploidy. Overall, the findings demonstrate that colchicine-induced polyploidy *in vitro* can enhance biomass and morphological traits in Napier grass, offering a strong foundation for developing improved polyploid lines for enhanced livestock feed production.

5.2 CONCLUSION

1. 1.0 mg IBA, 1,0mg 2,4D and 0.5mg BAP are the best growth hormone combination for embryogenic callus formation

2. 0.1% colchicine treatment at 48 hours yields the best performance in both South Africa napier and Bana grass

3. Polyploidy induced napier has high tillering ability and early maturity

5.3 RECOMMENDATION

In conclusion, this study successfully report advancement made in napier grass following chromosome doubling and somatic embryogenesis where induced mutant plantlets were regenerated. Polyploidy was confirmed by chromosome counting, stomata size and number, genomic DNA and other morphological characteristic. However, flow cytometry need to be used to confirm ploidy level. The superior mutant plantlets can be selected and recommended for characterization across representative agro-ecologies for large-scale production and used in *Cenchrus purpureus* breeding program in Kenya and its environs. Future studies should focus on protoplast fusion to compact sterility in napier grass and allow fussion of such plants at the cell level, where desirable traits can be passed among sterile *Cenchrus purpureus*

5.4 SUGGESTIONS FOR FURTHER RESEARCH

Based on the findings and limitations of this study, the following areas are recommended for further research:

1. Future studies should evaluate the agronomic performance of colchicine-induced polyploid Napier grass under field conditions, including biomass yield, nutritional quality, and persistence across multiple growing seasons.

2. Further research could assess the response of polyploid Napier grass to abiotic stresses such as drought, salinity, and low soil fertility to determine whether polyploidy confers enhanced environmental adaptability.

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APPENDICES

APPENDIX 1: Table 1. Treatment Combinations for Colchicine, Time and Variety

C0T0V1	C1T0V1	C2TOV1	C3T0V1
C0 T0V2	C1T0V2	C2T0V2	C3T0V2
C0T1V1	C1T1VI	C2T1V1	C3T1V1
C0T1V2	C1T1V2	C2T1V2	C3T1V2
C0T2V1	C1T2V1	C2T2V1	C3T2V1
C0T2V2	C1T2V2	C2T2V2	C3T2V2
C0T3V1	C1T3V1	C2T3V1	C3T3 VI
C0T3V2	C1T3V2	C2T3V2	C3T3V2

Colchicine concentration	Napier grass germplasms	Duration in time
C0 (0%):Control	South Africa (V1)	Time zero (T0)
C1 (0.05%):Low colchicine concentration	Bana grass (V2)	Time one, 24h (T1)
C2 (0.1%):Medium colchicine concentration		Time two, 48h (T2)
C3 (0.2%): High colchicine concentration		Time three, 72h (T3)

APPENDIX 2: Table 2 Experimental layout for colchicine treatment in the laboratory

R 1.

C3T1VI	C0T0V1	C1T3V2	C3T2V1
C2T2V1	C2T3V2	C0T0V2	C1T1V2
C3T2V2	C2T2V2	C3T3V2	C2T1V2
C3T1V2	C1T1V1	C2T1V1	C1T2V2
C3T3V1	C1T2V1	C2T3V1	C1T3V1
C3TOV1	C1TOV2	COT3V1	COT3V2
COT1V1	C2TOV2	COT1V2	C1TOV1
C3TOV2	COT2V2	COT2V1	C2TOV1

R2

C1T3V1	C2T2V2	C0T0V1	C1T1V2
C3T3V2	C3T2V1	C1T1V1	C0T0V2
C1T3V2	C2T1V2	C2T3V2	C1T2V1
C2T1V1	C3T3V1	C3T1V2	C3T2V2
C2T2V1	C3T1V1	C2T3V1	C0T0V1
COT1V2	C1TOV1	COT2V1	C3TOV2
COT2V2	COT3V2	C0T3V1	C2TOV1
COT1V1	C2TOV2	C3TOV1	C1TOV2

R3

C1T3V2	C3T2V1	C1T1V1	C0T0V2
C3T3V2	C2T1V2	C2T3V2	C1T2V1
C2T2V2	C1T1V2	C0T0V1	C1T3V1
C2T2V1	COT0V1	C2T3V1	C3T1V1
C2T1V1	C3T3V1	C3T2V2	C3T1V2
C1TOV2	COT1V1	COT3V1	COT2V1
C0T1V2	COT2V2	C2TOV2	C1TOV1
C2TOV1	C3TOV2	COT3V2	C3TOV1

APPENDIX 3: Table 3 Field experimental lay out for performance assessment of induced mutants

R1	C3T1VI	C0T0V1	C1T3V2	C3T2V1
	C2T2V1	C2T3V2	C0T0V2	C1T1V2
	C3T2V2	C2T2V2	C3T3V2	C2T1V2
	C3T1V2	C1T1V1	C2T1V1	C1T2V2
	C3T3V1	C1T2V1	C2T3V1	C1T3V1
	C3TOV1	C1TOV2	COT3V1	COT3V2
	COT1V1	C2TOV2	COT1V2	C1TOV1
	C3TOV2	COT2V2	COT2V1	C2TOV1

R2	C1T3V1	C2T2V2	C0T0V1	C1T1V2
	C3T3V2	C3T2V1	C1T1V1	C0T0V2
	C1T3V2	C2T1V2	C2T3V2	C1T2V1
	C2T1V1	C3T3V1	C3T1V2	C3T2V2
	C2T2V1	C3T1V1	C2T3V1	C0T0V1
	COT1V2	C1TOV1	COT2V1	C3TOV2

	COT2V2	COT3V2	C0T3V1	C2TOV1
	COT1V1	C2TOV2	C3TOV1	C1TOV2

R3	C1T3V2	C3T2V1	C1T1V1	C0T0V2
	C3T3V2	C2T1V2	C2T3V2	C1T2V1
	C2T2V2	C1T1V2	C0T0V1	C1T3V1
	C2T2V1	COT0V1	C2T3V1	C3T1V1
	C2T1V1	C3T3V1	C3T2V2	C3T1V2
	C1TOV2	COT1V1	COT3V1	COT2V1
	C0T1V2	COT2V2	C2TOV2	C1TOV1
	C2TOV1	C3TOV2	COT3V2	C3TOV1

APPENDIX 4: BUDGET

S/NO	ITEM	QUATITY REQUIRED	UNIT COST(Ksh)	TOTAL COST (Ksh)
01	**GROUP 1**			
02	Research assistant	2	20,000	20,000
03	Lab technician	1	20,000	20,000
04	**GROUP 2**			
05	Colchicine	50g	18,000	900,000
06	Murashige and Skoog (MS) medium	100g	8,000	8,000
07	Buffer solution PH 4.00	500 ml	2,200	2,200
08	Buffer solution PH 7.00	500ml	2,200	2,200
09	Tween 20	500ml	3,000	3,000
10	Phytagel	500gms	68,440	68,440
11	Naphthaleneactic acid (NAA)	50gms	1740	3480
12	Cupric sulphate	500g	2,088	2,088
13	sucrose	500g	986	986
14	L-Cystein	25g	3,000	3,000
15	6-Benzylaminopurine	50g	3,000	30,000

16	Hexadecyltrimethylammonium bromide	1lt	8,500	10,500
17	Nacl	1lt	500	500
18	Tris-hydrochloric acid	200g	5,000	8,500
19	Ethernol	4lt	5,800	5,800
20	Ammonium acetate	500g	1,500	1,500
21	Dthiothreitol	5g	8,000	8,000
22	Rnase A	100mg	24,000	24,000
23	Proteinase K	100mg	25,000	25,000
24	Isopropanol	2.5lt	3,000	3,000
25	Chloroform	2.5lt	3,480	3,480
26	Sterile distilled water	1lt	7,000	7,000
27	Penicillin and-Streptomycin	40ml	4,000	8,000
	GROUP 3			
28	Printing and binding cost for the proposal (5 copies). Each proposal is 46 pages	5 copies	2,500	12,500
29	30cm Ruler	1	125	200
30	A color chat/ Standard color scale	1		2,000
32	Nail polish	100ml		1,882
11	Serviettes (Cosy)	24	120	2,880
12	Tissue paper (Cosy)	Dozen	1,200	1,200
13	Labels	Dozen	3,000	3,000
14	Liquid Detto	1lt	2,500	12,500
15	Teepol	5lt	600	3,000
16	Kabendezin	1lt	2,500	12,500
17	Spray gun	2	250	500
18	Match box	2 box	100	200
19	Note book	4	120	480
20	Office pen	1 Box	500	500
21	Surgical gloves	4 Boxes	2,500	10,000
22	Face masks	3 Box	3,000	9,000
	GROUP 3			
23	Flexible measuring tape/Measuring cord	1m	1,500	1,500
24	DAP fertilizer	25kg	3,500	3,500
25	Planting pots	50	350	17,500
26	CAN fertilizer	25kg	3,400	3,400

27	Plant material			5,000
28	Blotting paper		5,000	5,000
29	Inflorescent bulb	2	6,000	6,000
30	Knapsack sprayer	20 l	6,700	6,700
30	Aluminum Foli	30m	3,500	3,500
31	Ridomil Gold	1kg	3,000	3,000
43	**Other Expenses**			
44	Conference attendance	2	15,000	30,000
45	Publication fees	2	20,000	40,000
46	Bench fee	6 months	20,000	120,000
47	Greenhouse fee	6 months	30,000	180,000
46	Miscellaneous Cost			174,909
	GRAND TOTAL			**1,923,999**

APPENDIX 5: RESEARCH TIME SCHEDULE

ACTIVITY	**May 2022 - Aprill 2023**	**June - August 2023**	**September-October-2023**	**November-December-2023**	**November-Feb 2024**
Proposal writing	🟦				
Literature review	🟦				
Proposal defending		🟦			
Callus induction			🟦		
Polyploidy induction				🟦	
Karyotyping Analysis					🟦
Acclimatization and Field Evaluation					🟦
Thesis writing					🟦
Thesis submission					🟦

Submission and defense					🟦