OPTIMIZING *In-vitro* PROTOCOL AND DIVERSITY STUDIES OF VANILLA (*Vanilla spp.*) FROM FIVE COUNTIES IN KENYA

LEAH NASIMIYU SIMIYU

A Thesis Submitted to the Graduate School in Partial Fulfilment of the Requirements for the Master of Science Degree in Plant Biotechnology of Egerton University

EGERTON UNIVERSITY

FEBRUARY, 2023

DECLARATION AND RECOMMENDATION

Declaration

I declare that this thesis is my original work and has not been submitted for examination in any institution.

Signature……………………………………...   Date…13TH FEBRUARY 2023…….

Leah Nasimiyu Simiyu

KM22/11733/16

Recommendation

This thesis has been submitted with our approval as university supervisors for examination according to University regulations.

Signature……………………………………   Date…13TH FEBRUARY 2023…….

Dr. Joseph Ngwela Wolukau, Ph.D

Department of Crops, Horticulture and Soils

Egerton University.

Signature……………………………..   Date…13TH FEBRUARY 2023…….

Dr. Maurice Oyoo, PhD

Department of Crops, Horticulture and Soils

Egerton University.

COPYRIGHT

©2023 Leah Nasimiyu Simiyu

All rights reserved. No part of this thesis should be reproduced, stored in retrieval and a database system, or transmitted in any form or by any means, scanning, photocopying, recording, or otherwise, without prior permission from the author or Egerton University.

DEDICATION

I dedicate this thesis work to our Almighty God for guidance and to my entire family for their moral and financial support.

ACKNOWLEDGEMENTS

I'm grateful to Egerton University for giving me a chance and creating a conducive environment to pursue my Master's degree in Plant Biotechnology. I extend my gratitude to the Centre of Excellence in Sustainable Agriculture and Agribusiness Management (CESAAM) at Egerton University for funding my project. I am thankful to Kenya Agricultural and Livestock Research Organization (KALRO) for granting me permission to use their research facilities for conducting laboratory experiments. I thank my university supervisors Dr. Joseph N. Wolukau and Dr. Maurice E. Oyoo for their guidance and support through the period of research studies. I would like to thank Dr. Pascal O. Ojwang, Egerton University and Dr. Godwin Macharia, Centre Director, KALRO, Njoro for their support during the conduct of this work. I am grateful to staff at KALRO, Njoro, Mr. Manfred Miheso and Mr. Nicodemas Kimani for their technical assistance during field and laboratory work.

ABSTRACT

Vanilla production can be a source of income to the farmers in Kenya. *In-vitro* regeneration is a rapid mass multiplication of quality plantlets; however, this technology has not been exploited at large scale for vanilla production in Kenya. Morphological and genetic diversity is an important tool for crop improvement. Production of vanilla in Kenya is partly limited due to inadequate knowledge on vanilla diversity. The objectives of the study were to (i) determine the effect of kinetin (KN) and indole-3-acetic acid (IAA) concentration levels on stem nodal segments of vanilla in Kenya, (ii) characterize vanilla accessions in Kenya using phenotypic traits (iii) characterize vanilla accessions in Kenya using microsatellite DNA markers. Shooting and rooting were established using 0, 0.4, 0.8, 1.2, 1.6, 2.0 mg L^{-1} concentration levels of KN and IAA on stem nodal segments of vanilla. *In-vitro* treatments were laid in Completely Randomized Design with five replications. Data were collected on plants with shoots, shoots per plant, number of leaves, number of nodes, shoot length, number of plants with roots, roots per plant, root length, dead plants, dormant plants and contaminated media. Data was subjected to analysis of variance at $p \leq 0.05$ level of significance using general linear model procedure of SAS version 9.1. Means were separated by Tukeys' Honestly Significant Difference Test at 5%. Phenotypic traits were used to estimate the level of accessions variation. POPGENE version 1.32 was used to compute the genetic parameter, while Powermarker version 3.25 was used to determine molecular variance. Treatment with kinetin at 1.2 mg L^{-1} resulted in superior number of plants with shoots (4.2±0.2), shoots per plant (2.6±0.4), number of nodes (2.4±0.4) and shoot length (11.0±0.6cm). IAA 2.0mgL^{-1} resulted in the longest root length (4.6±0.6 cm). Cluster analysis for qualitative traits grouped accessions into two major clusters and five sub-clusters based on the county of origin. Cluster analysis for quantitative traits grouped accessions into three groups. Amplicons ranged from 1 and 4 with 27 (96.43%) alleles being observed. Effective alleles mean was 1.63. Gene diversity mean was 0.35, while the mean shannon information index and polymorphic information (PIC) content values were 0.5 and 0.35, respectively. The highest PIC recorded was 0.375. Kinetin at 1.2mgL^{-1} and IAA at 2.0mgL^{-1} were considered the best treatments for rapid mass multiplication of vanilla and can be used at large scale production. Phenotypic traits failed to group vanilla accessions into respective counties. Genetic markers failed to reveal high polymorphism among the studied vanilla accessions.

TABLE OF CONTENTS

DECLARATION AND RECOMMENDATION ii
COPYRIGHT iii
DEDICATION iv
ACKNOWLEDGEMENTS v
ABSTRACT vi
LIST OF FIGURES xii
LIST OF TABLES xiii
LIST OF ABBREVIATIONS xiv
CHAPTER ONE 1
INTRODUCTION 1
1.1 Background information 1
1.2 Statement of the Problem 4
1.3 Objectives 4
1.3.1 General objective 4
1.3.2 Specific objectives 4
1.4 Hypotheses 5
1.5 Justification of the study 5
1.6 A Scope and limitations 5
CHAPTER TWO 7
LITERATURE REVIEW 7
2.1 Origin and distribution of vanilla species 7
2.2 Botany of vanilla species 7
2.3 Genetics of vanilla species 8
2.4 Natural vanillin content and uses 8
2.5 Vanilla species propagation 9
2.5.1 Diseases affecting vanilla species 9
2.5.2 Pests affecting vanilla species 10
2.6 Vanilla production and market trend in Kenya and worldwide 10
2.6.1 Export of vanilla from Kenya 12
2.6.2 The import trend of vanillin in Kenya 12
2.7 Conventional propagation technique and its limitations 13

2.7.1 Micropropagation of vanilla species 13
2.7.2 *In-vitro* regeneration protocol of vanilla species 14
2.8 Morphological characterization of vanilla species 15
2.9 Genetic characterization of vanilla species 15
2.9.1 Use of Simple Sequence Repeats (SSRs) marker in plant improvement.......................16
2.9.2 Use of Inter Simple Sequence Repeats (ISSR) in plant improvement...........................17
2.9.3 Application of Random Amplified Polymorphic DNA (RAPDs) in plant biodiversity studies 18
2.9.4 Using Restriction Fragment Length Polymorphism (RFLPs) in plant improvement 18
2.9.5 Amplified Fragment Length Polymorphism (AFLPs) in plant improvement 19
CHAPTER THREE 21
OPTIMIZING *In-vitro* REGENERATION PROTOCOL FOR VANILLA *(Vanilla spp.)* IN KENYA...21
Abstract 21
3.1 Introduction..22
3.2 Materials and methods 24
3.2.1 Plant materials sampling and Experimental site 24
3.2.2 Surface and equipment sterilization...24
3.2.3 Ex-plants collection 25
3.2.4 Ex-plants surface sterilization...25
3.2.5 Nutrient media composition..26
3.2.6 Preparation of nutrient media and sterilization..26
3.2.7 *In-vitro* conditions and inoculation...26
3.2.8 Multiplications of shoots..27
3.2.9 Experimental design, layout and data collection 27
3.3 Data analysis 28
3.4 Results and discussion 29
3.4.1 Number of plants with shoots 30
3.4.2 Number of shoots per plant..31
3.4.3 Number of leaves 32
3.4.4 Number of nodes...33
3.4.5 Shoot length 34
3.4.6 Rooting of Shoots 35

3.4.7 Number of plants with roots 36
3.4.8 Number of roots per plant 37
3.4.9 Root length 38
3.4.10 Micropropagation constraints 39
3.5 Conclusion and recommendation 41
CHAPTER FOUR 42
MORPHOLOGICAL DIVERSITY OF KENYAN VANILLA (*Vanilla spp.*) ACCESSIONS 42
Abstract 42
4.1 Introduction 43
4.2 Materials and methods 44
4.2.1 Experimental sites 44
4.2.2 Morphological traits 46
4.3 Statistical analyses 47
4.3.1 Qualitative traits 47
4.3.2 Quantitative traits 48
4.4 Results and discussion 49
4.4.1 Results of qualitative traits analysis 49
4.4.2 Factorial analysis 51
4.4.3 Cluster Analysis 53
4.4.4 Results of quantitative traits analysis 55
4.4.5 Correlations analysis of quantitative traits of vanilla accession from different counties in Kenya 56
4.4.6 Factor Analysis of quantitative traits of vanilla accessions in Kenya 57
4.4.7 Principal Component Analysis 58
4.5 Conclusion and recommendation 62
CHAPTER FIVE 63
DIVERSITY ASSESSMENT OF VANILLA *(Vanilla spp.)* ACCESSIONS IN FIVE COUNTIES OF KENYA USING SIMPLE SEQUENCE REPEATS (SSRs) MARKERS 63
Abstract 63
5.1 Background of the study 64
5.2 Materials and methods 66

5.2.1 Experimental site 66
5.2.2 Collection of plant accessions....68
5.2.3 Total Nucleic Acid Extraction 68
5.2.4 DNA Quantification....68
5.2.5 Selection of SSR markers and PCR genotyping 68
5.2.6 Scoring of the markers....71
5.2.7 Phylogenetic analysis....71
5.3 Results and discussion 72
5.3.1 Quality and Quantity of DNA....72
5.3.2 Polymorphism of the markers....72
5.3.3 Gene diversity 74
5.3.4 Genetic of vanilla among distance counties....76
5.3.5 Factorial analysis 77
5.3.6 Phylogenetic Trees....78
5.4 Conclusion and recommendation....80
CHAPTER SIX 81
GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS 81
6.1 General discussion 81
6.2 Conclusion 82
6.3 Recommendations....83
6.4 Areas for further research 83
REFERENCES....84
APPENDICES 104
Appendix A: ANOVA showing the effect of varied kinetin concentrations levels on number of plants with shoots of vanilla accessions from Busia county....104
Appendix B: ANOVA showing the effect of varied kinetin concentrations levels on number of shoots per plant of vanilla accessions from Busia county 104
Appendix C: ANOVA showing the effect of varied kinetin concentrations levels on number of leaves of vanilla accessions from Busia county....104
Appendix D: ANOVA showing the effect of varied kinetin concentrations levels on number of nodes of vanilla accessions from Busia county 104
Appendix E: ANOVA showing the effect of varied kinetin concentrations levels on shoot length of vanilla accessions from Busia county....105

Appendix F: ANOVA showing the effect of varied IAA concentrations levels on the number of plants with roots of vanilla accessions from Busia county 105
Appendix G: ANOVA showing the effect of varied IAA concentrations levels on the number of roots per plant of vanilla accessions from Busia county 105
Appendix H: ANOVA showing the effect of varied IAA concentrations levels on root length of vanilla accessions from Busia county 105
Appendix I: ANOVA showing constraints of kinetin and IAA (Dead plants) 106
Appendix J: ANOVA showing constraints of kinetin and IAA (Dormant plants) 106
Appendix K: ANOVA showing constraints of kinetin and IAA (contaminated media) 106
Appendix L: Data collection table for the effect of varied levels of kinetin on shoots of vanilla accessions from Busia county 106
Appendix M: Data collection table for the effect of varied level of IAA on roots of vanilla accessions from Busia county 108
Appendix N: Data collection table for micropropagation constraints, dead plants 109
Appendix 0: Data collection table for micropropagation constraints, dormant plants 110
Appendix P: Data collection table for micropropagation constraints, contaminated media.111
Appendix Q: Data collection table for qualitative traits of vanilla accessions from five counties of Kenya 112
Appendix R: Data collection table for quantitative traits of vanilla accessions from five counties of Kenya 114
Appendix S: DNA extraction protocol 116
Appendix T: Preparation of 1% agarose gel electrophoresis for separating DNA by size 118
Appendix U: Table of Nanodrop 119
Appendix V: Table for DNA bands scores 121
Appendix W: Research permit..124
Appendix X: Research publication 126

LIST OF FIGURES

Figure 1: Production trends of vanilla in Kenya from 2006 to 2020 in volume 12

Figure 2: Export quantity trend of vanilla in from Kenya, 1993 – 2020 12

Figure 3: The export trend of vanilla in Kenya, 1993 – 2019 13

Figure 4: Map of the experimental site, Busia County, Kenya 24

Figure 5: Plants with the shoot at varied kinetin concentration levels after two weeks of shooting of the vanilla plantlets 31

Figure 6: Number of leaves of vanilla crops in Kenya at varied kinetin concentration levels after 8 weeks 33

Figure 7: Shoots length representative at varied Kinetin concentration levels after 8 weeks . 35

Figure 8: Images of the effects of different concentrations of IAA on rooting of vanilla shoots, 3 weeks after inoculation 37

Figure 9: Forms of constraints during Micropropagation 40

Figure 10: Map of Kenya showing the physical location of the experimental sites 45

Figure 11: A factorial analysis based on qualitative traits of vanilla accessions from five counties of Kenya 52

Figure 12: A dendrogram of vanilla accessions based on qualitative traits characterized from five counties in Kenya 54

Figure 13: Two-panel Bi-plots of the first two Principle components of the vanilla accessions 60

Figure 14: Dendrogram based on quantitative traits 61

Figure 15: Map of the experimental sites 67

Figure 16: Gel images of L - Ladder (100bp), single and multiple bands of primers 72

Figure 17: Factorial plot showing variability among the vanilla accessions 78

Figure 18: A dendrogram showing the relationship among the vanilla accessions 79

LIST OF TABLES

Table 1: World vanilla crop production in 2016 11

Table 2: Effect of kinetin concentration levels on the shooting of vanilla plantlets 29

Table 3: Effect of IAA concentration levels on rooting of vanilla shoots 36

Table 4: Constraints of a combined concentration levels of kinetin and IAA on vanilla plants throughout the *in-vitro* experiments 39

Table 5: The qualitative and quantitative traits according to IPGRI (2001) used to characterize vanilla accessions from five counties of Kenya 46

Table 6: Frequency distribution table of qualitative morphological traits of vanilla accessions from five counties in Kenya 50

Table 7: Quantitative traits of vanilla accessions from five counties in Kenya 56

Table 8: Linear correlation table of the quantitative traits of vanilla accessions characterized from five counties in Kenya 57

Table 9: Un-rotated factor loadings for the variables of vanilla accessions based on factor analysis 58

Table 10: Eigen Values, Standard deviation, Proportion of variance and Cumulative proportions of morphological traits that contributed to the five PCs 59

Table 11: List of primer sequences of 14 microsatellite loci in the vanilla orchid 70

Table 12: Summary statistics of genetic diversity indices of vanilla accessions studied in five counties in Kenya 74

Table 13: Nei's gene diversity indices for subdividing the populations 76

Table 14: Nei's genetic identity (above diagonal) and genetic distance (below diagonal) 77

Table 15: Analysis of molecular variance (AMOVA) for the 76 vanilla accessions from 5 select counties of Kenya 77

LIST OF ABBREVIATIONS

BAP	6-Benzylaminopurine
CTAB	Cetyltrimethylammonium Bromide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
ELISA	Enzyme Linked Immuno-Sorbent Essay
IBA	Indole-3-Butyric Acid
IPGRI	International Plant Genetic Resource Institute
NAA	Naphthalene Acidic Acid
SNO.	Serial Number
USD	United State Dollar

CHAPTER ONE

INTRODUCTION

1.1 Background information

Vanilla (*Vanilla spp.)* belongs to the Orchidaceae family and originated from Mexico (Briddon *et al.,* 2019). Orchidaceae family contains more than 25,000 species distributed in 800 genera (Khasim *et al.,* 2020). Vanilla species were introduced into tropical countries like Asia and Africa from the original Mexican cultivated stock (Havkin-Frenkel & Belanger, 2018). *Vanilla planifolia* Andrews (syn. *V. fragrans*), *Vanilla tahitensis* and *Vanilla pompona* Scheide are commercially cultivated species for the production of natural vanillin flavour (Briddon *et al.,* 2019). According to Limi (2019), Madagascar was the leading producer and exporter of vanilla crop from the year 2012 to 2017. According to FAO (2018), in 2016 Madagascar, Reunion and Kenya produced 2,926, 2,304 and 15 tonnes of vanilla respectively.

Vanilla flavour, vanillin is the second most expensive spice traded in the world market after saffron (Kafi *et al.,* 2018). Globe Newswire (2022) reported that in 2021 worldwide vanilla market was valued at USD 1,434.51 million and is projected to reach USD 1,956.09 million by 2028. Selina (2022) reported that the prices of vanilla in Kenya per tonne for the years 2017, 2018 and 2019 were USD 625.00, USD 4,000.00 and USD 3,000.00 respectively. The trending price from the year 2021 to 2022 in Kenya was KES 326.8 per kg, an average price for a tonne of vanilla in Mombasa and Nairobi was USD 3000 (Selina, 2022).

According to Oyugi (2018), the crop can fetch over KES. 16,000 per vine after harvest when under recommended husbandry practices. Kenya exports vanilla to Germany, Uganda and Democratic Republic of Congo (De-Vos, 2020). According to FAO (2020), in 2019, Kenya produced 45 tonnes of vanilla and this is not projected to drop. The portion of vanilla produced that was exported in 2019 was less than 1% and the country ranked number 82 in the world export markets (FAO, 2020). Vanilla exported from Kenya in 2016, 2017, 2018, 2019 and 2020 was 2, 8 2, 1, and 0 tonnes respectively (FAO, 2022).

Vanilla crop has huge monetary value attached to it (Nany & Grisoni, 2021). Vanilla flavour has a wide area of applications that include flavouring food, beverages, soaps, ointments, perfumes, cosmetics and in pharmaceuticals industries (Upadhyay & Singh, 2021). Natural vanillin comprises of a large number of aromatic compounds with sweet fragrances (Hakeem & Aftab, 2021). Natural vanillin flavour is obtained as an extract from cured vanilla beans (Schmidt & Cheng, 2017). Vanillin is found to have antimutagenic, anticarcinogenic and antimicrobial properties. The antimutagenic property has ability to reduce chromosomal

damage caused by X-ray and ultraviolet (UV) light (Uysal *et al.,* 2019). Anticarcinogenic property have effects in a family of DNA-PK inhibitors (Pollard & Curtin, 2018) while antimicrobial property acts against the yeasts (Molina *et al.,* 2020).

According to FAO (2022), from the year 2016 to 2020 Kenya imported 1, 1, 2, 0 and 0 tonnes respectively. The demand for vanilla flavour in Kenya went down in 2019 as compared to 2018 (Fathima-Rafieah & Divakaran, 2021). The demand reduced because the country imports more synthetic vanilla essence rather than natural vanilla (Volza, 2020). Kenya imports synthetic vanilla essence from India, South Africa and Italy (Volza, 2020). Kenya is rated as the world's largest importer of synthetic vanilla essence (Volza, 2020).

Most parts of Kenya have favourable climatic conditions for cultivating vanilla crop (Yahyah *et al.,* 2020) with ready local and export markets upon its maturity (Sowbhagya, 2019). This presents a great opportunity for the developing countries like Kenya to take advantage of exporting vanilla spice. Despite vanilla crop being of great economic value and marketable, many vanilla farmers have abandoned its production because of value chain constraints in production and processing (Chauvin *et al.,* 2017). According to Chauvin *et al.* (2017), vanilla production system requires quality planting materials, highly skilled production and processing personnel.

Traditional propagation of vanilla is labour intensive because all work is done manually (Nany & Grisoni, 2021). Intensive labour is experienced during planting tutor trees, training vanilla vines, weeding, hand pollination on each vanilla flower and harvesting the mature pods (Nany & Grisoni, 2021). According to Nany (2021), establishing plantations and intensifying production where plant suppliers do not have adequate and quality planting materials reduces the rate of improving productivity.

Vanilla is commonly propagated by stem cuttings which arrests subsequent plant growth and development, and serves as a means for spreading varied diseases (Soni *et al.,* 2022). Tissue culture techniques have emerged as an alternative method for large scale production of quality planting materials within a relatively short period of time (Robinson, 2020). Vanilla crops have been propagated both through direct organogenesis and indirect organogenesis (Mariska & Fronika-Sianipar, 2020). A number of studies on multiplication of vanilla species have been reported in other countries in Africa and worldwide through the culture of stem nodes (Havkin-Frenkel & Belanger, 2018) root tips (Dolce *et al.,* 2019), shoot tips (Havkin-Frenkel & Belanger, 2018) and callus culture (Abdin *et al.,* 2017) but limited *in-vitro* information is documented in Kenya on vanilla crop.

The present study was undertaken to determine the suitable level of Kinetin and IAA concentration on *in-vitro* regeneration of stem nodal segments of vanilla species grown in Busia County, Kenya.

Since vanilla crop was introduced in Kenya in 1954, limited research information has been documented on available vanilla species found in the country. Determining variation in crop species is an important tool for germplasm conservation, management and improvement (El-Esawi, 2019). Therefore, it was necessary to assess the extent of diversity in vanilla species found in Kenya.

The studies were conducted to determine the effect of kinetin and IAA concentration levels on stem nodal segments of vanilla crops for *in-vitro* regeneration of the planting materials and assess diversity of vanilla species found in Kenya.

1.2 Statement of the problem

Vanilla is the second most costly spice in the world after saffron. Many studies have been done on *in-vitro* regeneration of vanilla crop through indirect (callus culture) and direct organogenesis (stem nodes, root tips, shoot tips) in other countries at large scale production. Unfortunately, *in-vitro* regeneration of vanilla crop in Kenya is neglected and limited research on *in-vitro* production is documented. This partly contributes to scarcity of the planting materials in Kenya. Success of any plant biotechnology and breeding programmes depends on availability of extensive level of genetic variability in plants. Vanilla species found in Kenya have not been characterized both at morphological and molecular levels, this contributes to difficulties to improve important traits in vanilla crop. Vanilla crop is grown by few farmers in Kenya partly because of scarcity of planting materials, aspect of plant environmental adaptability and unimproved species leading to low rate of production in the country. One metre of vanilla vine is sold at KES.600 which is unaffordable to most farmers. Vanilla flavour essence used in Kenya are produced artificially from coal tar derivatives, lignin, methyl and ethyl chemicals that taste the same as natural vanillin and other vanilla essence imported from other countries. These chemicals are harmful to human body. Through this research, micropropagation and characterization will help to optimize *in-vitro* protocol of vanilla crop and identified important vanilla species available in Kenya. These will promote natural production of vanillin content that have a lot of economic value. It will also enhance commercialization of vanilla crop in Kenya which will earn the country foreign exchange and act as a source of income to the farmers.

1.3 Objectives

1.3.1 General objective

To contribute towards increased natural vanillin production through optimizing *in-vitro* protocol and diversity assessment of vanilla accessions from five counties of Kenya.

1.3.2 Specific objectives

i. To determine the effect of phytohormone (kinetin and IAA) concentration levels on stem nodal segments of vanilla accessions in Kenya.

ii. To characterize vanilla accessions from different counties of Kenya using morphological markers.

iii. To determine the genetic diversity among vanilla accessions from different counties of Kenya using simple sequence repeat DNA markers.

1.4 Hypotheses

i. There is no significant effect of phytohormone (kinetin and IAA) concentration levels on stem nodal segments of vanilla accessions in Kenya.
ii. There is no significant morphological variation among vanilla accessions from different counties of Kenya.
iii. There is no genetic variation among vanilla accessions from different counties of Kenya as revealed by simple sequence repeat DNA markers.

1.5 Justification of the study

Vanilla is a crop of great economic potential to most food, cosmetic and pharmaceutical industries. Vanilla crop is highly valued due to its vanillin content. Vanillin has been found to have antimutagens properties (El-Shemy, 2017). A study carried out by El-Shemy (2017), showed that antimutagens properties have the ability to reduce chromosomal damage caused by X-ray and ultraviolet (UV) light. Vanillin has antimicrobial properties that act against yeasts (Sabel *et al.,* 2017). Despite its value, limited research has been done on *in-vitro* regeneration and improvement of genetic value on vanilla species in Kenya. Conventional propagation can still be used but has limitations as compared to tissue culture that offers a means for rapid and mass multiplication of quality, pest and disease free planting materials. Assessing morphological and genetic relationships among and within the crop species is an important tool for crop improvement to any successful plant biotechnology (Al-Khayri *et al.,* 2018). Based on traditional method, plant traits characterizations are based on morphological descriptor, however, most of these traits are influenced by environment (Bai, 2017). Phenotypic characterization is highly influenced by environment, hence it is important to complement morphological and genetic markers.

1.6 A Scope and limitations

Vanilla accessions from 4 counties (Bungoma, Busia, Kilifi and Kwale) were collected, planted under shade net in hot tanks for shooting and rooting at Egerton University. Only accessions from Busia County adapted to the new environment and were used as mother plants for *in-vitro* regeneration. MS media supplemented with kinetin (KN) and indole acetic

acids (IAA) at different concentration levels were used to determine their effects on stem nodal segments. Only KN and IAA were evaluated due to limited research funds.

Morphological characterization was done *in-situ* in 5 counties in Kenya (Bungoma, Busia, Mombasa, Kwale and Kilifi). Total number of vanilla accessions characterized both at morphological and molecular levels were 76 because of the few number of farmers and institutions that grow vanilla crops in Kenya.

CHAPTER TWO
LITERATURE REVIEW

2.1 Origin and distribution of vanilla species

Vanilla genus belongs to the Orchidaceae family which contains more than 25,000 species (Parizaca, 2019). Vanilla species are native to tropical forests of Mesoamerica (Havkin-Frenkel & Belanger, 2018). In the nineteenth century, vanilla was introduced into tropical countries like Asia and Africa (Nair *et al.,* 2022). Vanilla is a terrestrial epiphyte used to produce important agricultural products (Nargar & Chen, 2020). *Vanilla tahitensis* J.W. Moore, *Vanilla pompona* Scheide *and Vanilla planifolia* Andrews are the common species of economic value (Briddon *et al.,* 2019). It was reported by Limi (2019) that in the period 2012 to 2017 Madagascar was the world's leading producer of vanilla crop, followed by Indonesia, Papua New Guinea, India and Uganda.

2.2 Botany of vanilla species

Plant botany is the biological study that deals with plant structures, properties, and biochemical processes (Stevens, 2019). Kowsalya *et al.* (2017) studied the vegetative anatomy and systematic of the vanilla species. Vanilla plants have green vines with leaves (*Vanilla planifolia* Andrews, *Vanilla tahitensis* J.W. Moore *and Vanilla pompona* Scheide) (Briddon *et al.,* 2019). Leafy vanilla plant vines produce long, branched, terrestrial or absorbing roots which penetrate the substratum and are unlimited to extension growth (Li *et al.,* 2022). Vegetative anatomical characters of vanilla species have some phylogenetic values that differentiate vanilla species (Kowsalya *et al.,* 2017).

Vanilla planifolia Andrews is a perennial climbing orchid with a thick, cylindrical and succulent green vine with longer pods (Sanchez-Galindo *et al*., 2018). According to Sanchez-Galindo *et al*. (2018), *V. planifolia* have adventitious, long, aerial roots of 2 mm in diameter, produced singly opposite the leaves and adhere firmly to the support. The description of Sanchez-Galindo *et al*. (2018), the stems are long, cylindrical, 1-2 cm in diameter, branched, succulent, brittle and dark green. The stem internodes are at 5-15 cm intervals (Sanchez-Galindo *et al*., 2018). The leaves are long (8- 20 cm), broad (3-6 cm), flat, fleshy, alternate and oblong elliptic, and at the joint of each stem a bud or root is formed alternatively (Sanchez-Galindo *et al*., 2018).

According to Sanchez-Galindo *et al*. (2018), inflorescences are stout, simple, long (5-8 cm) and bears 15-30 flowers which open from the base upwards. Flowers open and last only

for a day. The large, waxy, pale green flowers are about 7-10 cm in diameter (Sanchez-Galindo *et al*., 2018). *Vanilla planifolia* pods turn dark chocolate brown after curing (Briddon *et al.,* 2019).

Vanilla tahitensis is a man-made hybrid and it is indigenous to Tahiti (Brunschwig *et al.,* 2017). According to Briddon *et al.* (2019), *Vanilla tahitensis* has zigzag slender stems, narrow elliptic leaves, yellow-green flowers which are followed by bunches of beans like pods. *Vanilla tahitensis* has terrestrial roots that anchor within the soil and epiphytic roots anchoring to the tree trunks (Briddon *et al.,* 2019). The indehiscent pods are 12-14 cm long and about 1.5-1.8 cm wide which turn red-brown upon curing (Briddon *et al.,* 2019).

Vanilla pompona produces inferior quality vanilla beans, but is the hardiest species (Khoyratty *et al.,* 2018). *Vanilla pompona* is a perennial climbing plant with a succulent stem of 5 metres or more in length (Brunschwig *et al.,* 2017). The plant support itself by means of aerial roots produced from the stem nodes (Briddon *et al.,* 2019). The pods of *Vanilla pompona* are almost cylindrical, short (10-12.5 cm long) and large (2-2.5 cm in diameter) (Khoyratty *et al.,* 2018).

2.3 Genetics of vanilla species

The basic chromosome number of the vanilla genus is 16 (x=16) (Briddon *et al.,* 2019). *Vanilla planifolia, Vanilla pompona* and *Vanilla tahitensis* are diploid with 2n=32 (Briddon *et al.,* 2019). Brunschwig *et al.* (2017) detected that somatic chromosome number of vanilla species ranged from 2n=20 to 32, 28 being the most frequent number. Additionally, abnormalities in pollen grain mitosis combined with high pollen sterility and chromosome associations during pollen mitosis were reported by Brunschwig *et al.* (2017) in vanilla species. The studies of Yang *et al.* (2019) indicated that there was possible occurrence of cytotypes in the seedling progenies of vanilla species. Yang *et al.* (2019) also reported Polyploidization in the study that described the source of morphological variation in cultivated vanilla species.

2.4 Natural vanillin content and uses

The primary quality determinant for cured vanilla beans is the aroma/flavour character (Adawiyah *et al.,* 2019). The unique flavour and fragrance of vanilla beans are influenced by vanillin ($C_8H_80_3$) content, the most abundant aromatic constituent of vanilla beans (Gonzalez *et al.,* 2017). Vanillin is not present at the time of harvest but is produced during curing by enzyme action on glucosides (Tyupin, 2021). The cured bean contains 1.5-3.5% vanillin content (Yulianto & Paramita, 2019). Natural vanillin content is the most popular

and widely used flavour on both a monitory and tonnage basis (Moller & Gallage, 2017). Natural vanilla flavour extracted from cured vanilla beans is universally used as an aromatic flavouring in food, pharmaceutical, beverages and cosmetic industries (Erady *et al.,* 2020). Vanillin content was found to have antimutagenic properties, which was further evident when significantly reduced the number of micronuclei was at all dose levels (Ravindran, 2017). Vanillin also had anticarcinogenic effects in a family of DNA-PK inhibitors (Ahmed *et al.,* 2019). According to Sen (2022), vanillin has antimicrobial properties that act against yeasts.

2.5 Vanilla species propagation

Vanilla grow well in warm, humid climates at temperatures between 21 and 32 °C (33.8–89.6 °F) (Havkin-Frenkel & Belanger, 2018). Vanilla requires a light and well-drained soil rich in potassium and calcium, with a pH between 6.0 and 7.0 (Ortigao, 2020). Vanilla is a climbing plant that requires a support (Briddon *et al.,* 2019). It is usually planted alongside a companion tree like Jatropha plant referred to as a tutor tree (Hill-Saya, 2020). Vanilla is traditionally propagated by stem cuttings from a mother plant (Briddon *et al.,* 2019). According to Briddon *et al.* (2019), 1.5 metres (5 ft) are cut, lower leaves removed and planted at the base of the supportive tree. Cuttings should be planted at least 2 metres (6.6 ft) apart (Briddon *et al.,* 2019).

Brunschwig *et al.* (2017), vanilla plants vines should be kept at a manageable height. When the plants reach a height of 1.6–1.8 metres, they should be bent back over the nearest suitable branch and the end of the shoot planted back into the ground and covered with soil (Brunschwig *et al.,* 2017). Vanilla shoots are also cut at the desired height and planted next to the same tutor tree after the wound has dried to create a new rooted plant (Erawati *et al.,* 2021). Mulching is done using organic mulch like grass to suppress weeds and conserve soil moisture (Cizkova *et al.,* 2021). Vanilla is naturally pollinated by hand and sometimes pollination is done by using small Mexican bees if present (Ravindran, 2017). Vanilla is ready for harvest between 6 and 9 months after flowering when the pods are still dark green and the tip is beginning to turn yellow (Chambers, 2019).

2.5.1 Diseases affecting vanilla species

Vanilla species are susceptible to many bacterial, viral and fungal diseases (Havkin-Frenkel & Belanger, 2018). Bacterial soft-rot disease is caused by *Erwinia carotovora* bacteria (Khedr, 2019). The disease infects vanilla leaves and shoots (Havkin-Frenkel & Belanger, 2018). Viral diseases are caused by cymbidium mosaic virus, odontoglossum ring-spot virus

and vanilla necrosis virus (Havkin-Frenkel & Belanger, 2018). According to Havkin-Frenkel (2018), virus diseases caused by cymbidium mosaic and odontoglossum ring-spot viruses are usually symptomless. The diseases are mild chlorotic streaks or mild mottles observed on the leaves (Havkin-Frenkel & Belanger, 2018). Most vanilla species affected by viral diseases are *Vanilla planifolia* and *Vanilla tahitensis* (Brunschwig *et al.,* 2017).

Fungi stem rot disease is caused by *Fusarium oxysporum* and *Fusarium solani* (Askun, 2018). Stem rot starts at the leaf axil and spreads to the nodal areas resulting in rotting and drying of the stem above the point of infection (Askun, 2018). Fungi Anthracnose disease is caused by *Colletotrichum gloeosporiodes* (Perdani *et al.,* 2021). It attacks the stem apex, leaves and roots resulting in wilting, falling of the fruits and brown spots appearing on leaves (Ingram *et al.,* 2020). Fungi phytophthora blight is caused by phytophthora fungi that cause rotting of the beans, leaves and stems (Singh *et al.,* 2020). Fungi sclerotium rot disease is caused by *Sclerotium rolfsii* (Motagi *et al.,* 2020). The disease affects the leaves of vanilla plants (Brunschwig *et al.,* 2017). The beans develop reddish brown sunken lesions initially and later the entire bean rots (Brunschwig *et al.,* 2017). Fungi dry rot disease is caused by *Fusarium spp.* and *Rhizoctonia spp.* (Fernandes *et al.,* 2021). Fernandes *et al.* (2021), this disease affects plant stems, roots and leaves shrinks and become yellow.

2.5.2 Pests affecting vanilla species

The common pests that affect vanilla plants are vanilla vine weevil (*Sipalus spp.*) and white grubs (*Scarabaeidae spp.*) that damages vines and shoots of vanilla (*Vanilla planifolia*) (Briddon *et al.,* 2019). Both weevils and grubs feed on vanilla plants leading to necrosis and rotting of the affected portions (Carbajal-Valenzuela *et al.,* 2022). Most of the time weevils feed on vines and leaves while the grubs feed on the inner tissues of the vine creating tunnels (Ayemele *et al.,* 2017).

2.6 Vanilla production and market trend in Kenya and worldwide

Vanilla flavour is the second most expensive essence in the world markets after saffron (Boone *et al.,* 2022). According to Oyugi (2018), vanilla crop has substantial monetary value. The crop can fetch a farmer over KES. 16,000 per vine, if the recommended husbandry practices and post-harvest handling procedures are followed. A vine of vanilla can bear up to 80 beans. Vanilla has a wide area of application that include flavouring food, drinks, soaps, ointments, perfumes, cosmetic and medicines. One vanilla bean measuring 10-12cm long is sold at KES. 200 and a hectare can accommodate over 200 vines.

Table 1: World vanilla crop production in 2016

Country	Rank	Production (tonnes/year)
Madagascar	1	2,926
Indonesia	2	2,304
China	3	885
Mexico	4	513
Papua New Guinea	5	502
Turkey	6	303
Uganda	7	211
French Polynesia	8	203
Portugal	9	180
Reunion	10	24
Malawi	11	20
Kenya	12	15
Comoros	12	15
Zimbabwe	14	11
Cook Islands	15	2
Seychelles	16	1

Source: FAO (2018)

According to FAO *(*2018), Kenya produced 15 tonnes of vanilla in 2016 and it was ranked 12th in the world production. Shabbir (2018) reported that vanilla crop is also well grown in Uganda, Comoros and Madagascar both on small and large scale. Most parts of Kenya have favourable climatic conditions for cultivating vanilla crop and there is both local and export markets ready, though vanilla farmers have abandoned its production (Shabir, 2018). The major markets for vanilla beans include European countries and United States (Shabbir, 2018). According to FAO (2022), production of vanilla crop in Kenya ranged from 12-15 tonnes per year from 2006 to 2020 (Figure 1).

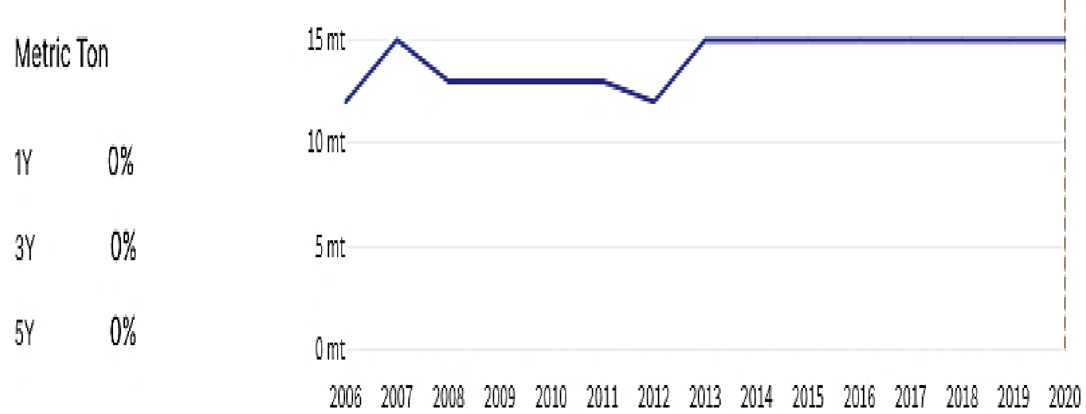


Figure 1: Production trends of vanilla in Kenya from 2006 to 2020 in volume
Source: FAO (2022)

2.6.1 Export of vanilla from Kenya

The quantity of vanilla exported to the world trade markets from Kenya in 2016-2020 are shown in Figure 2. Vanilla quantity exported from Kenya in 2016 to 2020 in tonnes were 2, 8, 2, 1and 0, respectively (FAO, 2022).

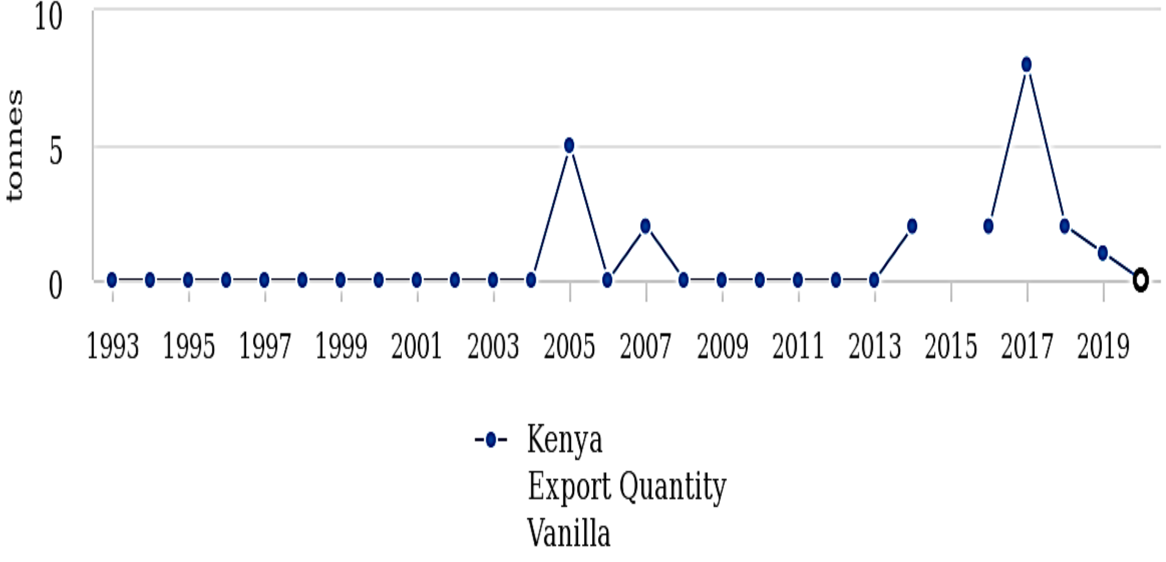


Figure 2: Export quantity trend of vanilla in from Kenya, 1993 – 2020

2.6.2 The import trend of vanillin in Kenya

Report made by FAO (2022), from the year 2016 - 2019, Kenya importation quantity of natural vanilla flavour ranged from 1 to 2 tonnes per year (Figure 3). The demand for vanillin

in Kenya dropped by 50 percent in 2019 as compared to 2018 (Fathima-Rafieah & Divakaran, 2021).

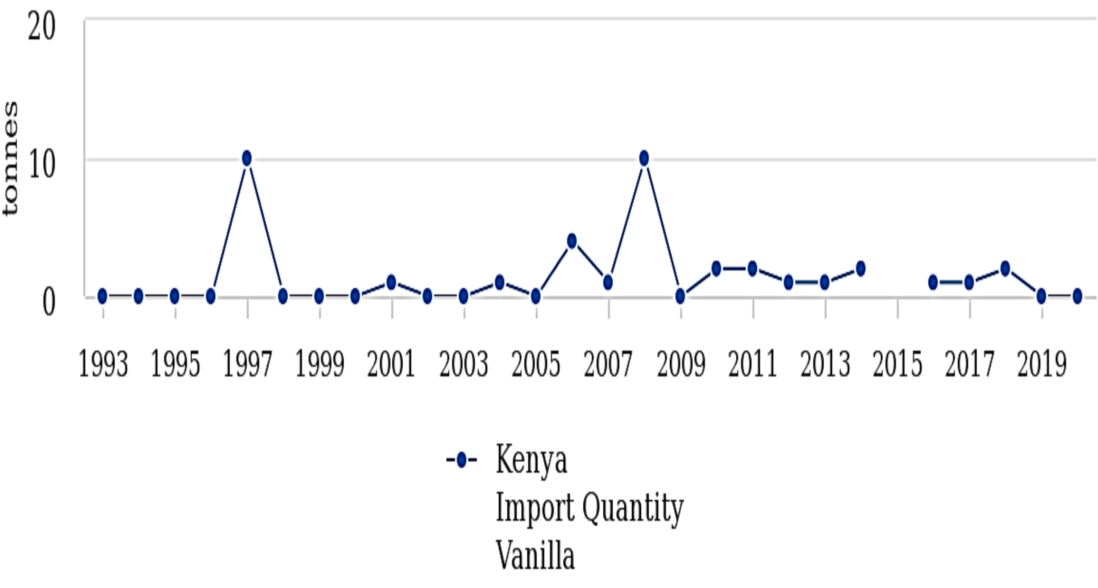


Figure 3: The export trend of vanilla in Kenya, 1993 – 2019

2.7 Conventional propagation technique and its limitations

Vanilla is propagated by use of stem cuttings which is often uneconomical, slow, labour intensive, time consuming, arrests subsequent plant growth and development, and serves as a means for the spread of varied diseases (Verdi *et al.,* 2020). Market demand for propagules is hardly met through cuttings (Masih & Jolly, 2017). According to Robinson (2020), tissue culture technique is an alternative method for large scale production of planting materials within a relatively short period of time. Micropropagation techniques are described as more reliable in the vanilla cultivating areas (Erawati *et al.,* 2020).

2.7.1 Micropropagation of vanilla species

Micropropagation techniques are used in mass multiplication of plants which are of high quality, pest and disease free and identical to the selected genotype within a very short period of time (Starrantino & Caruso, 2021). Application of tissue culture techniques is an alternative means of asexual propagation of plant species (Nhut, 2022). *In-vitro* propagation of vanilla by culturing axillary buds (Faisal & Alatar, 2019), aerial root tips through callus protocorms (Watanabe & Sakaguchi, 2017), apical meristems, shoot tips and nodal segments

(Radotic & Pavlovic, 2017) has been observed earlier. Micropropagation using shoot tips or nodal segments has been found to be an appropriate technique (Faisal & Ahmad, 2018).

True-to-type, clonal fidelity is important for utilizing the advantages of Micropropagation (Dharman & Anilkumar, 2021). Based on the choice of ex-plants, a major problem encountered with *in-vitro* culture is the occurrence of somaclonal variations amongst sub-clones of one parental line arising as a direct consequence of *in-vitro* culture conditions (Gulyaeva *et al.,* 2020). Micropropagated plants obtained from organ segments, especially from nodal, axillary buds and shoot tips have been observed to maintain clonal fidelity as organized meristems are more resistant to genetic changes compared to unorganized callus under *in-vitro* culture (Arifuzzaman *et al.,* 2016).

Mass multiplication of plants by tissue culture is a costly and labour-intensive technology (Cyriac *et al.,* 2020). The gelling agents being used are not inert medium components and do not allow easy automation of commercial mass propagation (Hegele *et al.,* 2021). Agar and gelrite are the most commonly used non-nutrient gelling agents which are the cheapest ingredients of the culture medium (Hegele *et al.,* 2021). Liquid media is one of the automation and improved technology (Hegele *et al.,* 2021). Liquid culturing has been considered as an ideal technique for mass propagation because it reduces manual labour and renders automation opportunities of the entire process (Hegele *et al.,* 2021).

According to Hegele *et al.* (2021), plant tissues from several species are known to perform better when cultured in liquid medium rather than on solid medium (agar). Liquid medium is considered for reducing plantlet production costs and the system provides more uniform culturing conditions (Marazzi *et al.,* 2017). Use of liquid medium allows scale - up in bioreactors minimizing the number of operations with the advantages of medium manipulation, hence reducing the cost of production of micropropagules (Lee *et al.,* 2019). Bioreactors are modified microbial liquid media that are unsuitable for higher plants due to their high sensitivities to shear force leading to mechanical damages (Tekere, 2019). Aerated types exhibit foam formation, particularly in bubble aerated bioreactors (Bernauer *et al.,* 2021). Vanilla shoots when grown in liquid medium undergo hyperhydricity or vitrification problem. Therefore, it is better to grow vanilla shoots on solid medium (agar) (Erawati *et al.,* 2021).

2.7.2 *In-vitro* regeneration protocol of vanilla species

In-vitro regeneration attempts and standardization of the basal medium and phytohormones for micropropagation of plant species with respect to economically important

species have been practiced worldwide (Aarti-Patel *et al.,* 2018). Most *in-vitro* regeneration protocols have been developed and standardized on medicinal plants like *Cantranthus roseus* (Kumar, 2018), biodiesel plants like *Jatropha curcus* (Raj & Mohan, 2016) and herbal pesticidal plants (Alamgir, 2017). Nodal explants of these species were micropropagated successfully when cultured onto MS medium containing kinetin and IAA at 1.5, 0.5, 0.1 mg L^{-1}respectively. Limited *in-vitro s*tudies have been done on vanilla crop in developing countries including Kenya. Therefore, testing various cytokinin (Kinetin) and auxin (IAA) concentration levels was necessary.

2.8 Morphological characterization of vanilla species

Morphological characterization refers to a broad subject that involves phenotypic descriptors which distinguish one plant from another or one accession against the other (Doussoh *et al.,* 2018). Morphological descriptors which are usually included in morphological characterization are mature plant height, leaf width, leaf length, flower colour and root type (Bahar *et al.,* 2018). Morphological and physiological features of plants are used to understand the genetic variation (Gamrat *et al.,* 2021). Morphological features are indicative of the phenotype and they are affected by environmental factors and growth practices (Gamrat *et al.,* 2021).

Many studies have been done on morphological diversity studies of vanilla species particularly on *Vanilla aphyla, Vanilla planifolia, Vanilla tahitensis* and *Vanilla pompona* which showed low levels of similarity (Briddon *et al.,* 2019). There are several sources of germplasm in developing countries (Rodrik, 2018). Despite most developing countries having sources of vanilla germplasm, limited information is documented for vanilla species grown in Kenya. According to Lu (2019), identification of variation in plants at the DNA level is described as more reliable.

2.9 Genetic characterization of vanilla species

Genetic characterization refers to the detection of variation as a result of differences in either DNA sequences, specific genes or modifying factors (Elisha *et al.,* 2022). The genetic diversity has an impact on levels of biodiversity (Hodgkin & Brown, 2015). Understanding the patterns of genetic diversity of species is important because it acts as a base for crop improvement in breeding program (Singh *et al.,* 2021). New approaches to the study of genetic variation from wild species to cultivated varieties mediated by information on molecular markers are promising to exploit wild genetic resources (Yadav *et al.,* 2022).

Biodiversity involves applications of morphological, biochemical and molecular markers to determine the amount of genetic variability (Hanci, 2019). Characterization of diversity has long been based on morphological traits only (Tanwar & Bisen, 2018). However, morphological variability is often restricted, characters may not be obvious at all stages of the plant development and appearance may be affected by environment (Latheef & Kavino, 2018). Variety of different genetic markers has been proposed to assess genetic variability as a complementary strategy to more traditional approaches in genetic resources management (Rao & Rajasekharan, 2019). Molecular tools provide valuable data on diversity through their ability to detect variation at the DNA level (Takami, 2019). Molecular techniques differ in a way the sampling within the genome is done and the type of data generated (Takami, 2019).

Numerous molecular markers have been developed and applied for analyses of genetic diversity and relatedness (Mondal, 2020). All marker systems have their strengths and weaknesses (Rahman *et al.,* 2021). Among the molecular markers techniques that are particularly promising are Restriction Fragment Length Polymorphism (RFLPs), Amplified Fragment Length Polymorphism (AFLPs), Random Amplified Polymorphic DNA (RAPDs), Microsatellites and Polymerize Chain Reactions (PCR) based DNA markers such as Sequence Characterized Amplified Regions (SCARs) or Sequence Tagged Sites (STS), Single Nucleotide Polymorphism (SNP), Simple Sequence Repeats (SSR) and Inter Simple Sequence Repeats (ISSR). These markers are the most extensively used and have great potential in studying plant genetics and genomics (Fowler, 2016).

Most genetic variation studies defines a good genetic marker by being of highly polymorphic, highly reproducible, requires low quantity of genomic DNA template and being co-dominant (Wani *et al.,* 2020). All these make SSRs an ideal genetic marker for various studies most notably on genetic diversity (Grumet *et al.,* 2017), DNA fingerprinting (Litz *et al.,* 2020), and phylogenetics (Shanker, 2018).

2.9.1 Use of Simple Sequence Repeats (SSRs) marker in plant improvement

Simple Sequence Repeats (SSRs) markers are also known as microsatellites (Singh & Gupta, 2017). These are short tandem segments of DNA that contain a core motif which consist of a few nucleotides that are normally 1-6 base pairs in length (Shahabadi *et al.,* 2017). This core motif can be repeated up to a maximum of 60 times in tandem (Shahabadi *et al.,* 2017). The number of tandem repeats at a specific locus can vary between two individuals and it is possible to screen for these differences using primer that anneal to the flanking regions of the specific microsatellite (Shahabadi *et al.,* 2017).

The generated PCR products can be size-fractionated on a polyacrylamide or agarose gel, allowing the discrimination of alleles with a different size of up to one base pair because of their high mutability (Oginni & Fasina, 2018). Microsatellites are thought to play a significant role in gene evolution by creating and maintaining genetic variation (Jighly *et al.,* 2017). Microsatellite markers have several advantages that make them excellent molecular markers to use (Sme, 2017). Microsatellite markers requires low quantities of genomic DNA template, are highly polymorphic, highly reproducible and represent co-dominant marker in a locus to distinguish heterozygotes from homozygotes (Margeta *et al.,* 2019).

Microsatellites (SSRs) are widely used as a tool in plant biotechnology and breeding programs (Fliege *et al*., 2022). Simple sequence repeats markers were used in marker-assisted selection and genomics approaches to broaden the narrow genetic diversity of elite oilseed rape (OSR) (*Brassica napus* L.) breeding materials (Havlickova *et al.,* 2014). Specific oilseed rape targeted genes in breeding goals like oil yield, disease resistance and seed quality were achieved (Havlickova *et al.,* 2014). According to Havlickova *et al.* (2014), molecular marker (SSRs) technologies resulted in the transition of open pollinated line to hybrid varieties of oilseed rape (*Brassica napus* L.) in the market.

2.9.2 Use of Inter Simple Sequence Repeats (ISSR) in plant improvement

Inter Simple Sequence Repeats are semi-arbitrary markers amplified by polymerase chain reaction (PCR) in the presence of one primer complementary to a target microsatellite (Singh & Kumar, 2020). Each band corresponds to a DNA sequence delimited by two inverted microsatellites (Spealman *et al.,* 2019). The ISSR markers do not require genome sequence information, lead to multilocus, have highly polymorphous patterns, and produce dominant markers (Varma, 2022). Inter Simple Sequence Repeats PCR is a fast, inexpensive genotyping technique based on variations in the microsatellites' regions. The ISSR markers have been used in genetic diversity studies in different crop plants (Algady *et al.,* 2021). The ISSR markers are also suitable for identification and DNA fingerprinting (Panwar *et al.,* 2018). This method has several benefits over other techniques except for SSRs markers (Panwar *et al.,* 2018). It was proven reproducible and quick for characterizing many cultivars like poplar (Beser *et al.,* 2021).

Inter-simple sequence repeat (ISSR) exploits the existence of many copies of highly conserved repetitive sequences throughout the genomes of many plants (Yadav & Kumar, 2018). The ISSR markers have been widely used for cultivar identification in many species like sorghum, banana, potato, Arabidopsis and sunflower (Kumar *et al.,* 2021). The ISSR

markers have also been used for genetic diversity analysis and validation of mango genotypes (Al-Khayri *et al.,* 2018). The mango varieties grown in different regions like Australia were analyzed, and amplification patterns revealed that ISSR-PCR were applied to different mango cultivars (Al-Khayri *et al.,* 2018).

2.9.3 Application of Random Amplified Polymorphic DNA (RAPDs) in plant biodiversity studies

Random Amplified Polymorphic DNA techniques is a PCR-based method that involves binding a short single arbitrary primer of approximately 10bp to arbitrary sequences in the genome under relatively low conditions with annealing temperatures as low as 36 ºC (Kumar *et al.,* 2022). A fragment is amplified when the primer binds on both ends of the genomic DNA strands but in the opposite orientation and amplifiable distance between the two primer binding sites (Forsythe, 2020). Unfortunately, this technique has some disadvantages; it is very sensitive to reaction conditions and low reproducibility, and the primers can amplify DNA fragments from every type of genome (Singh, 2020).

The RAPDs markers were used to characterize four Pistacia species and determine the relationships among the species (Al-Khayri *et al.,* 2020). Arbitrary 23 sequence primers generated a total of 248 DNA fragments, and out of 248 bands, 139 bands were polymorphic at the inter-specific level (Al-Khayri *et al.,* 2020). The RAPDs data analysed using a parsimony data interface revealed relationships among four Pistacia species (Al-Khayri *et al.,* 2020).

2.9.4 Using Restriction Fragment Length Polymorphism (RFLPs) in plant improvement

Restriction Fragment Length Polymorphism was the first DNA-based marker to be developed (Singh & Gupta, 2017). A variant of the hybridization-based method is polymerase chain reaction/Restriction Fragment Length Polymorphism (PCR/RFLP), also known as cleaved amplified polymorphic sequence (CAPS) (Taylor & Granger, 2020). This method is much easier and faster and is based on amplifying a specific locus in a genome, followed by the restriction endonuclease digestion of the amplicon using a particular restriction enzyme (Buckingham, 2019). Agarose or polyacrylamide gel electrophoresis can be used to reveal differences in fragment size after staining the gel with ethidium bromide or silver nitrate (Misra, 2017).

This method is less laborious than the original one and provides results faster (Thi-Trung-Thu & Quang-Binh, 2016). A negative aspect of the PCR/RFLP method is that it

requires the presence of mutation, which can be recognized by restriction endonuclease for at least one allele, and the availability of PCR primers for the amplification reaction (Pass *et al.,* 2017). Unfortunately, many point mutations do not result in a restriction site, and the polymorphisms cannot be detected by this approach (Kumar, 2020).

Restriction Fragment Length Polymorphism (RFLP) markers were used to determine the genome coverage in the species *Oryza sativa* (Chukwu *et al.,* 2019). Seventy varieties of rice and ten rice restriction fragment length polymorphism markers were used in the study (Chukwu *et al.,* 2019). According to Chukwu *et al.* (2019), polymorphism was detected for all probes, and 58 of 70 varieties tested were uniquely distinguished from one another by combining all probe enzyme combinations. Homozygous variant alleles within the population were found to be 26% of the varieties, as reported by Chukwu *et al.* (2019).

2.9.5 Amplified Fragment Length Polymorphism (AFLPs) in plant improvement

The Amplified Fragment Length Polymorphism technique is used to simultaneously determine numerous amplified DNA restriction fragments (Srivastava *et al.,* 2021). This technique has several advantages; it requires a minimal amount of genomic DNA, no preliminary genome sequence data is necessary for primer construction, markers are randomly distributed throughout the genome, and many bands are generated that potentially provide a large number of polymorphisms (Srivastava *et al.,* 2021). It also has disadvantages; it requires extremely pure and high molecular weight genomic DNA, co-migration of a non-allelic fragment of the same size can occur (Parine, 2017), and AFLP markers are dominant; hence it is not possible to distinguish between the homozygous state of the dominant allele and the heterozygote (Tronsmo *et al.,* 2020).

Amplified Fragment Length polymorphisms (AFLPs) were used on Indian Cycas species to reveal the genetic variation of six species collected from eleven populations (Wu *et al.,* 2020). The study used two sets of primers with four selective nucleotides (Wu *et al.,* 2020). According to Wu *et al.* (2020), 78% polymorphism was found, and the study was instrumental in identifying Indian Cycas species, which has been stated as a highly endangered species. The AFLP markers were used in identifying species within the genus Caladium (Araceae) using 17 primers on two species of *Caladium* (*C. bicolour* and *C. schomburgkil*) and six cultivars of *C. bicolour* (Deng, 2018). According to Deng (2018), the AFLP markers distinguished the two species by their unique and different banding patterns. The AFLPs markers were also used to access the genetic relationship among twenty *Curcuma* species from Thailand (Sihanat *et al.,*

2020). According to Sihanat *et al.* (2020), Amplified Fragment Length Polymorphism showed 98.54% highly polymorphisms with 617 bands ranging between 48 and 80 bands.

CHAPTER THREE

OPTIMIZING *In-vitro* REGENERATION PROTOCOL FOR VANILLA *(Vanilla spp.)* IN KENYA

Abstract

In-vitro regeneration is useful for rapid multiplication of quality planting materials. This technology has not been exploited on a large scale for vanilla production in Kenya. An experiment was conducted to develop efficient protocol for rapid multiplication of vanilla planting materials in Kenya. Shooting and rooting were established using varying concentration levels of kinetin (0, 0.4, 0.8, 1.2, 1.6, 2.0 mg L^{-1}) and indole-3-acetic acid (IAA) (0, 0.4, 0.8, 1.2, 1.6, 2.0 mg L^{-1}) on stem nodal segments of vanilla. Trials were laid in a Completely Randomized Design with five replications. Data was subjected to analysis of variance at $p \leq 0.05$ level of significance using general linear model (GLM) procedure of Statistical Analysis System (SAS) and means separated by Tukeys' Honestly Significant Difference Test at 5%. Treatment with kinetin at 1.2 mg L^{-1} had the best performance on the following parameters; number of plants with shoots (4.2±0.2), shoots per plant (2.6±0.4), number of nodes (2.4±0.4) and shoot length (11.0±0.6 cm). Applying IAA at 1.2 mg L^{-1} had the highest number of plants with number of roots (6.8±1.0). Indole-3-acetic acid at 1.6 mg L^{-1} recorded the highest mean number of roots per plant (4.2±0.6). Treatment with IAA 2.0 mg L^{-1} resulted in the longest root length 4.6±0.6 cm. Kinetin at 1.2 mg L^{-1} and IAA at 2.0 mg L^{-1} were considered the best treatments for rapid mass multiplication of vanilla in Kenya.

3.1 Introduction

Vanilla species belong to the Orchidaceae family, which has about 25,000 species (Parizaca, 2019). Vanillas are native to the tropical rain forests of south-eastern Mexico (Grijp, 2021) and are the only orchids commercially cultivated for vanilla beans (Teoh, 2019). The main cultivated vanilla species are *V. planifolia* Andrews, *V. pompona* Schiede and *V. tahitensis* J.W. Moore (Briddon *et al.,* 2019). Botanically, vanilla species produce pods that contain vanillin content (Yulianto & Paramita, 2019). Vanilla species are perennial climbing orchids (Teoh, 2019). Vanilla species; *Vanilla planifolia, Vanilla pompona* and *Vanilla tahitensis* are diploid with 2n=32 (Briddon *et al.,* 2019).

The pods have a natural flavouring substance called vanillin (Adawiyah *et al.,* 2019), which is obtained as an extract from cured vanilla beans (Havkin-Frenkel & Belanger, 2018). Vanillin is the world's second most expensive spice after saffron (Khan, 2020). Vanilla flavour is universally used in flavouring food, perfume, beverages, cosmetics and pharmaceuticals industries (Prescott & Monteleone, 2015). Vanilla has a natural flavour made up of many aromatic compounds having sweet fragrances (Larsson & Dauncey, 2018). Vanilla compounds have antimutagenic (Aeschbacher, 2018), anticarcinogenic (Pouramini & Hensel, 2022), and antimicrobial (Moller & Gallage, 2017) properties.

Vanilla species were introduced in Kenya from neighbouring Uganda and Tanzania in 1954 (Ponomarev & Logunov, 2020). According to FAO (2018), in 2016, vanilla crop production in Kenya was 15 tons yr $^{-1}$. The level of vanilla production in Kenya was very low as compared to other countries like Reunion, producing 2,304 tons yr $^{-1}$ and Madagascar, having 2,926 tons yr $^{-1}$. The major markets for vanilla beans are European countries and the United States (Conybeare, 2017). Kenya has favourable climatic conditions for cultivating vanilla crops (Waititu *et al.,* 2020), and there are both local and export markets for the crop (Oyugi, 2018).

Vanilla farmers in most developing countries like Kenya have abandoned production of vanilla crop because of production constraints (Chauvin *et al.*, 2017). Highly specialized skills in terms of experience are required in the production and processing of vanilla crops (Chauvin *et al.*, 2017). Vanillas are propagated using stem cuttings of mature vines, though this method is very slow, labour intensive and takes a long time to establish (Smith *et al.,* 2018). Vegetative propagation of vanilla retards the growth and development of the mother plant (Martinkova & Klimesova, 2022). Vanilla stem cuttings serve as a means for the spread of varied diseases (Wiering *et al.,* 2020).

Shortage of vanilla planting materials, production and processing constraints in Kenya are partly drawbacks to the broader dissemination and cultivation of the vanilla crop. Tissue culture techniques have emerged as an alternative method for the mass production of planting materials within a relatively short period (Robinson, 2020). The *in-vitro* regeneration technology can save the situation of vanilla production in Kenya.

According to Daneshvar (2018), vanilla has been propagated through direct and indirect organogenesis. Recently, vanilla species have been propagated using tissue culture techniques showing significant advantages compared to the conventional method (Wodrich, 2020). A number of studies on *in-vitro* mass multiplication of vanilla species have been reported in other countries through the culture of stem nodes (Chambers, 2019), root tips (Dolce *et al.,* 2020), shoot tips (Isidro-Adolfo *et al.,* 2019) and callus culture (Park, 2021) but limited information is documented on *in-vitro* regeneration production of vanilla crop in Kenya.

The present study was undertaken to determine the suitable levels of kinetin and indole-3-acetic acids concentration levels supplemented with MS medium (Skoog & Murashige, 1962) on stem nodal segments of vanilla species grown in Kenya.

3.2 Materials and methods

3.2.1 Materials sampling and Experimental site

Purposive network sampling was done in Busia county (Latitude 0° 47' 11.113" N and Longitude 34° 24' 26.339" E) (Figure 4). This was because there are few vanilla farmers and agricultural institutions conducting research on vanilla in Kenya. Vines were sampled, collected and planted under the shade net at Egerton University (Latitude 0° 22' 11.0"S and Longitude 35°55' 58.0"E) for micropropagation. *In-vitro* experiments were conducted in the Tissue Culture Laboratory at the Kenya Agricultural and Livestock Research Organization-Njoro, located at the latitude of 0° 20' North and longitude of 35°56' East at an elevation of 2120 above the sea level.

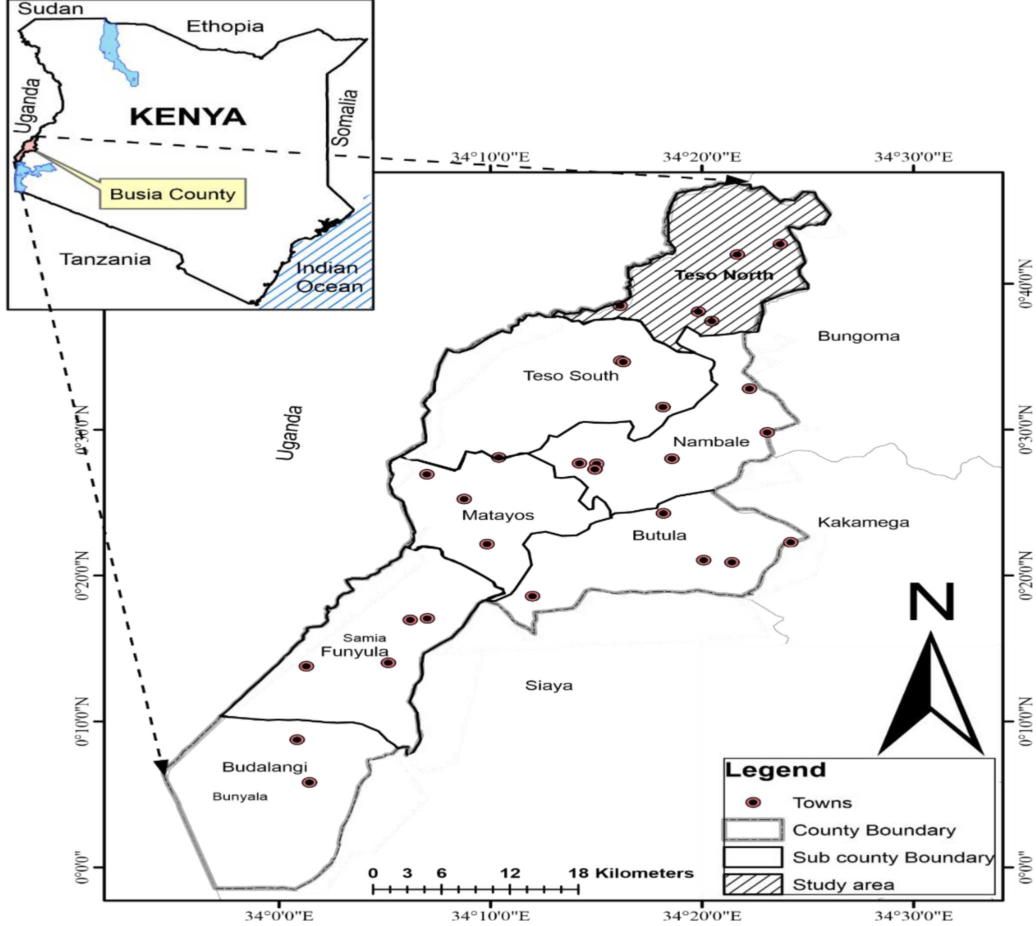


Figure 4: Map of the experimental site, Busia County, Kenya

3.2.2 Surface and equipment sterilization

A laminar airflow cabinet was prepared for use by switching it on for 15 minutes, its surfaces were sterilized with 70% ethanol, followed by exposure to UV light for 30 minutes. All metallic tools were placed in an aluminium foil and sterilized in an autoclave for 30 minutes

at 121 °C. During their use in the cabinet, tools were frequently dipped in 70% ethanol, followed by heat sterilization in a steribead sterilizer maintained at 25 °C for 30 seconds. All glassware and vessels were washed in hot water at 55 °C into which few drops of liquid detergent had been added. Glassware were rinsed in distilled water three times, followed by a final rinse with water to which a few drops of commercial bleach (JIK®) containing sodium hypochlorite had been added. All these were carried out in a clean dust free washing room. The glassware were then dried in the oven at 60 °C.

3.2.3 Ex-plants collection

Nodal segments were used as the ex-plants. The newly, young growing vines with two to three nodes were excised from healthy mother plants in the research field at Egerton University, placed in a beaker containing tap water, and transported to the plant tissue culture laboratory at KALRO, Njoro, Kenya.

3.2.4 Ex-plants surface sterilization

Ex-plants were surface sterilized according to Sharma (2011), with minor modifications which involved cutting ex-plants into 2.0 cm long sections with a node. Initial washing was done by thoroughly washing under running tap water for 30 minutes to remove soil. Ex-plants were rinsed four times with distilled water, followed by soaking in a fungicide solution of Benlate® (two drops in 100 ml of distilled water) for an hour. Ex-plants were also washed in a solution of Tween 20 (wetting agent - two drops in 100 ml of distilled water) for 5 minutes and were rinsed five times with distilled water to remove any traces of detergents.

The sterilized ex-plants were again treated with fungicide 0.5% Caberndezim for 8 minutes under aseptic conditions and washed eight times with distilled water to remove traces of fungicide. The above step was followed by transferring the ex-plants to the laminar hood. The ex-plants were sterilized a second time by dipping them in 25% commercial JIK® for 25 minutes, then rinsed three times in distilled water to completely get rid of sterilizing agent. Finally, ex-plants were immersed in 70% ethanol for 30 seconds. After decontamination, ex-plants were rinsed three times with distilled water in a laminar hood. The wounded sites exposed to sterilizing agents were trimmed properly and subsequently inoculated into culture media.

3.2.5 Nutrient media composition

Initiation culture media contained Murashige and Skoog (MS) media premix (Skoog & Murashige, 1962), vitamins (0.1 g L^{-1} myo-inositol, 0.15 g L^{-1} ascorbic acid, 0.15 g L^{-1} citric acid), Antioxidant (1.0 g L^{-1} polyvinylpyrrolidone 'PVP'), 5.0 ml L^{-1} fungicide (Benlate®), 0.2 g L^{-1} bactericide (Ampicillin trihydrate), carbon and energy source (sucrose 30 g L^{-1}) and solidifying agent (gelrite 2.5 g L^{-1}).

Kinetin and IAA at concentrations at 0, 0.4, 0.8, 1.2, 1.6, 2.0 mg L^{-1} were included during shooting and rooting processes.

3.2.6 Preparation of nutrient media and sterilization

The culture media were prepared by making concentration stock solutions which were diluted to the required concentration before use. The MS media premix stock solution was prepared by dissolving 4.40 g L^{-1} in distilled water and stored in a refrigerator at 2 – 4 °C until used. Kinetin and IAA stock solutions were prepared according to their molecular weights. Kinetin with molecular weight of 215.2 grams was dissolved in one litre of sodium hydroxide (1N NaOH). The suitable working concentration of kinetin range from 0.1 - 5.0 mg L^{-1}. To prepare one litre stock solution of kinetin, 0.005 gram of kinetin was dissolve in one litre of distilled water. Indole –3– acetic acid has a molecular weight of 175.2 grams and it was dissolved in one litre of sodium hydroxide (1N NaOH). The working concentration of IAA ranged from 0.01 - 3.0 g L^{-1} and 0.006 gram of IAA was used to prepare one litre stock solution of IAA.

Murashige and Skoog culture media were dispensed in glass containers sealed with plastic closures and sterilized by autoclaving for 30 minutes at 15 Pa and 121 °C. Vitamins (myo-inositol, ascorbic acid and citric acid) and phytohormones (kinetin, indole –3– acetic acid) were sterilized by using millipore filter papers of 0.2 µm pore diameter. This solution of compounds was added after the media was autoclaved and cooled but not solidified.

3.2.7 *In-vitro* conditions and inoculation

All media were adjusted to a 5.8 pH with 1 N NaOH prior to autoclaving. Nine sterilized ex-plants were inoculated onto the surface of solidified nutrient media under aseptic conditions for culture initiation. The cultures were incubated in the growth tissue culture room at 26 ±1 °C air temperatures, 50 to 60 % relative humidity and a 16-hour photoperiod under the illumination of 20 µmol $m^{-2}s^{-1}$ photons provided by fluorescent light. Roots were removed

from grown plantlets in initiation culture media without phytohormones after four weeks and transferred to shooting media.

3.2.8 Multiplications of shoots

After four weeks of shooting, elongated shoots with nodes were cut into individual nodal segments 2.0 cm long. Nine nodal segments were inoculated onto each fresh media containing kinetin at 0, 0.4, 0.8, 1.2, 1.6, and 2.0 mg L^{-1}. Shoots were re-inoculated onto fresh medium for further multiplication after four weeks. These sub-cultures were done for optimum multiplication at four weeks intervals over 56 days. Based on the number of shoots per plant at a given kinetin treatment level, newly developed shoots from the best performed kinetin treatment level were transferred to the rooting media of IAA at varying concentration levels of 0, 0.4, 0.8, 1.2, 1.6, 2.0 mg L^{-1}.

3.2.9 Experimental design, layout and data collection

The experiments were laid out in a completely randomized design (CRD) as a two-factor experiment with five replications.

Data was recorded on a number of shoot parameters at the final stage of shoot multiplication. Number of plants with shoots (number of plants having newly developed shoots) was recorded two weeks after the last stage of shoot multiplication. Number of shoots per plant (newly developed shoots per plant) and leaves (newly developed leaves), data were recorded after eight weeks. Additionally, the number of nodes (newly developed nodes) and shoot length (cm) (emerged shoots, measured from the point of emergence to the tip using a linear meter) were also recorded after eight weeks.

Rooting parameters were recorded after transferring the shoots to rooting media, where eight shoots were placed in each rooting bottle. The transferred shoots were selected on the basis of their good performance depending on kinetin concentration level. Number of plants with roots (number of plants having newly developed roots) and number of roots per plant (newly developed roots per plant) were recorded after three weeks of rooting. Root length (cm) (measuring the roots from the point of emergence to the tip using a linear meter) data recorded after five weeks.

Dead plants were determined by counting the number of plants which could not survive under kinetin and IAA treatments. Data for dormant plants was determined by measuring plants which could not initiate either shoots or roots. Recorded data for contaminated media was based on fungi and bacteria according to Mesay *et al.* (2015). Dead

plants, dormant plants and contaminated media data were recorded throughout the *in-vitro* regeneration processes.

3.3 Data analysis

Data were subjected to analysis of variance (ANOVA) at $p \leq 0.05$ level of significance using the general linear model (GLM) procedure of the Statistical Analysis System (SAS) program version 9.1 (SAS Institute, Cary Inc, 2001). Treatment means were separated by Tukeys' Honestly Significant Difference test at $p \leq 0.05$.

The linear statistical model used for the experiment was:

$$Y_{ij} = \mu + T_i + \varepsilon_{ij}$$

i=1, 2, 3, 4, 5, 6 j=1, 2, 3, 4, 5

Where;

$\mathbf{Y_{ij}}$ – An individual observation for i^{th} levels of phytohormone concentration and j^{th} replication, $\boldsymbol{\mu}$ - overall mean, $\mathbf{T_i}$ - effect due to i^{th} levels of phytohormone concentration, $\boldsymbol{\varepsilon_{ij}}$ - random error component associated with each observation.

3.4 Results and discussion

Vanilla stem nodal ex-plants were inoculated onto MS media without phytohormones for culture initiation. Initial experiments exhibited more contamination, especially where mature ex-plants were used. Younger ex-plant showed limited contamination, and this observation is in agreement with that of Furukawa (2019). Mature ex-plants showed more contamination probably because their tissues may harbour bacteria and fungi contaminants that were more difficult to eliminate.

All ex-plants exhibited direct shoot organogenesis. This means that adventitious shoots have the ability to protrude directly from the ex-plants in the free-plant growth regulator culture media. Similar observation was reported by Patil *et al.* (2017) who demonstrated direct and indirect shoot organogenesis using internodes of *Abutilon ranadei* Wooder.

Shoots induction was derived from vanilla stem nodal ex-plants cultured on MS media supplemented with kinetin concentration levels. The effects of kinetin at varying concentration levels were observed as shown in Table 2.

Table 2: Effect of kinetin concentration levels on the shooting of vanilla plantlets

No.	Plant Growth Regulator (mg L^{-1}) Kinetin	Number of plants with shoots	Number of shoots per plant	Number of leaves	Number of nodes	Shoot length (cm)
1	0	2.2±0.60c	1.2±1.0c	2.6±0.6a	1.2±0.8b	4.2±0.2c
2	0.4	2.6±0.20c	2.0±0.6a	3.0±0.2a	1.4±1.0b	4.4±3.8c
3	0.8	2.8±1.4bc	2.2±0.4ab	3.2±0.0a	1.6±0.8ab	8.2±0.2b
4	1.2	4.2±0.2a	2.6±0.4a	3.0±0.2a	2.4±0.4a	11±0.6a
5	1.6	4.0±1.4ab	1.8±0.8a	3.0±0.2a	2.0±0.4ab	10.4±0.6a
6	2.0	2.0±0.8c	1.6±1.0b	3.2±0.0a	1.4±1.0b	8.4±2.6b

Means followed by the same letter in a column are not significantly different at $p \leq 0.05$, according to Tukey's HSD test.

3.4.1 Number of plants with shoots

The ex-plants showed varied successes in shooting depending on the concentration levels of kinetin in the media after two weeks of shooting treatment. The number of plants with successfully established shoots varied significantly between 2.0 and 4.2 at 2.0 mg L^{-1} and 1.2 mg L^{-1} respectively (Table 2). MS media containing 1.2 mg L^{-1} (4.2) and 1.6 mg L^{-1} (4.0) levels were not significantly different at 5%. Kinetin at 0 mg L^{-1} (2.2), 0.4 mg L^{-1} (2.6), 0.8 mg L^{-1} (2.8) and 2.0 mg L^{-1} (2.0) were not significantly different from each other, however, they were significantly different from treatment at 1.2 mg L^{-1} (4.2) and 1.6 mg L^{-1} (4.0) except for 1.6 mg L^{-1}. Treatment at 1.6 mg L^{-1} (4.0) was not significantly different from concentration at 0.8 mg L^{-1} (2.8) at 5% level (Table 2 and Figure 5).

These findings indicated that stem nodal ex-plants cultured on MS media with 1.2 mg L^{-1} of kinetin performed significantly better with 4.2 plants with shoots as compared to other concentration levels (Table 2). Kinetin at 1.2 mg L^{-1} may be the optimum concentration level for the production of vanilla shoots in Kenya. However, the optimum concentration of kinetin is species dependent (Foo *et al.,* 2020). Similar observation was made by Abu-Romman *et al.* (2015) on cucumber (*Cucumis sativus*) when kinetin concentration of 1 mg L^{-1} was used. According to Abu-Romman *et al.* (2015), the highest number of cucumber (*Cucumis sativus*) with shoots recorded at 1.0 mg L^{-1} (7.93 shoots). Generally, cytokinins including kinetin are widely known as shoot promoting agents in many *in-vitro* cultured organs (Li *et al.,* 2022).

The present study indicated that kinetin at 2.0 mg L^{-1} gave the lowest number of plants with shoots (2.0) (Table 2). The lowest number of plants with shoot occurred because the level may be beyond the optimum ratio for shoot formation. The results are in agreement with those made by Satish *et al.* (2016), who reported that higher concentration of kinetin beyond the optimum concentration level induced necrosis and reduction in shoot formation of finger millet (*Eleusine coracana* L.). When the plant growth regulator (GPRs) is higher than the surrounding plant cells, such plant cells experiences mechanical stress (Arif *et al.,* 2021). According to Arif *et al.* (2021), higher GPRs concentration causes cells at the lateral parts of the wing disc of the plant to stop growing, as central plant cells experience mechanical stress.

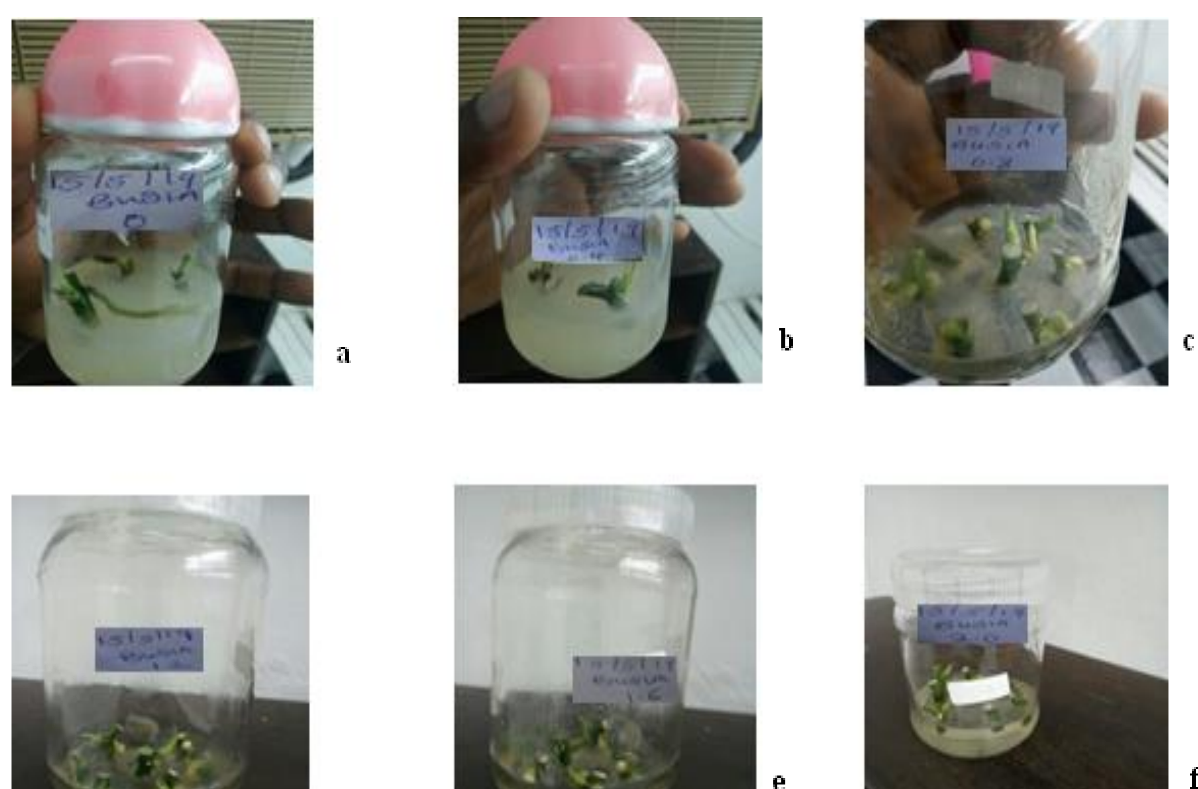


Figure 5: Plants with the shoot at varied kinetin concentration levels after two weeks of shooting of the vanilla plantlets (a – 0 mg L^{-1}, b – 0.4 mg L^{-1}, c – 0.8 mg L^{-1}, d – 1.2 mg L^{-1}, e – 1.6 mg L^{-1} and f – 2.0 mg L^{-1} concentration levels)

3.4.2 Number of shoots per plant

The mean values on number of shoots per plant ranged from 1.2 (0 mg L^{-1}) to 2.6 (1.2 mg L^{-1}). Kinetin concentration at 0.4 mg L^{-1} , 0.8 mg L^{-1} , 1.2 mg L^{-1} and 1.6 mg L^{-1} were not significantly different from each other. They were however significantly different from kinetin concentration levels of 0 mg L^{-1} and 2.0 mg L^{-1} except for 0.8 mg L^{-1} which was not significantly different from 2.0 mg L^{-1} treatment (Table 2). Plants inoculated on MS media supplemented with 1.2 mg L^{-1} kinetin produced significantly higher value on shoots regeneration per plant (2.6 shoots) as compared to plants inoculated on MS media that were not supplemented with kinetin (1.2 shoots) and at kinetin level 2.0 mg L^{-1} (1.6 shoots) after eight weeks.

In this study, kinetin treatment at 2.0 mg L^{-1} yielded 1.6 shoots. These results are in contrast with those of Erawati *et al*. (2021), who recorded the highest number of shoots per *Vanilla planifolia* ex-plant (6.00 shoots) from the medium supplemented with 2.0 mg L^{-1}

Kinetin after 56 days. This could be caused by genotypic differences of vanilla species studied. It was observed in the present study that MS media without hormone as well as higher hormone concentrations were ineffective in shoot regeneration of vanilla ex-plants (Table 2).

The present results are in agreement with those of Abu-Romman *et al*. (2015), who demonstrated the effect of kinetin on shoots regeneration of cucumber (*Cucumis sativus*). Abu-Romman *et al*. (2015) reported that increasing kinetin concentration to 2.0 mg L^{-1} reduced the rate of regeneration to 4%. This explains the relationship of plant growth hormonal dose with physiological response of the plants. Physiological response increases significantly until the plant reaches the saturation level. However, below or above the saturated concentration point of plant growth regulator, regardless the type of cytokinin will result to decline in physiological response (Abu-Romman *et al*., 2015).

Many research studies have reported higher proliferation rate at low concentration level of BAP as compared to kinetin. In the present study, the highest number of shoots per ex-plant under MS media supplemented with kinetin recorded were however lower than that recorded by Arafah *et al.* (2021) using 1.0 mg L^{-1} BAP on *Solanum tuberosum*. Nair (2019) also observed that the proliferation rate of turmeric ex-plants grown on the media supplemented with Kinetin was generally lower than BAP. This may be attributed to the fact that BAP has been widely documented as a more effective cytokinin for the induction of shooting in plant tissue culture (Park, 2021).

3.4.3 Number of leaves

The number of leaves counted after 8 weeks of shooting were not significantly different at different kinetin concentration levels. The values ranged between 2.6 (0 mg L^{-1}), 3.2 (0.8 mg L^{-1} and 3.2 (2.0 mg L^{-1}) (Table 2, Figure 6). Kinetin levels 0.8 mg L^{-1} and 2.0 mg L^{-1} produced shoots with highest mean number of leaves (3.2±0.0) while the least number was recorded for kinetin level 0 mg L^{-1} (2.6±0.6).

Number of leaves regenerated is an indicator of plant survival (Vynnycky & Papadopoulos, 2021). Results in this study is in contrast with those of Adugna *et al.* (2020) who recorded highest mean number of leaves per explant of 7.97±4.18, after 4 weeks where MS premix media contained 0.5 mg L^{-1} kinetin on *Moringa stenopetala.* This suggests that treatment levels and time could be species related.

Premix MS media with kinetin levels of 0.8 mg $L^{-1}$and 2.0 mg L^{-1} (3.2±0.0) gave the most suitable survival environment for vanilla plantlets initially (3 weeks) but at 8 weeks (Figure 6), the plantlets had stunted growth. Based on the results, stunted growth may have

occurred due to higher concentration level of kinetin that inactivated the growth hormones in plantlets (Winnicott, 2016). The present results concurred with those of Ferrando *et al.* (2018), who observed that higher concentrations of cytokinin increased leaf production initially but the plantlets became stunted in the culture after 4 weeks of shooting.

Figure 6: Number of leaves of vanilla crops in Kenya at varied kinetin concentration levels after 8 weeks (a – 0 mg L^{-1}, b – 0.4 mg L^{-1}, c – 0.8 mg L^{-1}, d - 1.2 mg L^{-1}, e – 1.6 mg $L^{-1}$and f – 2.0 mg L^{-1})

3.4.4 Number of nodes

The number of nodes recorded under MS media supplemented with kinetin ranged from 2.4 (1.2 mg L^{-1}) to 1.2 (0 mg L^{-1}) after 8 weeks of shooting (Table 2). The current results indicated that kinetin influenced growth response of vanilla species. However, kinetin concentration level of 1.2 mg L^{-1} was significantly different from 0 mg L^{-1}, 0.4 mg L^{-1} and 2.0 mg L^{-1} concentration levels except at 0.8 mg L^{-1} and 1.6 mg L^{-1} which were not significantly different at 5% (Table 2).

Premix MS media with kinetin concentration at 1.2 mg L^{-1} (2.4) gave the highest mean value as compared to 0 mg L^{-1} (control) and 2.0 mg L^{-1}. The number of nodes increased with the increasing concentration of kinetin up to the saturation point when the number of nodes started to decline. These results are in concurrence with those of Hesar *et al.* (2011), who reported on the effect of kinetin on number of nodes of *Matthiola incana* species. According to Hesar *et al.* (2011), MS media supplementation of 0.5-2 mg L^{-1} kinetin evaluated on nodal segments of *Matthiola incana*, revealed that the maximum number of nodes observed was at 2 mg L^{-1} (4.64). Hesar *et al.* (2011) noted that the number of nodes increased with the increase of kinetin concentration. The highest number of nodes recorded in *Matthiola incana* species was higher than the number recorded in vanilla species in this study, indicating that kinetin effects is species dependent. The number of nodes regenerated is important because it is a measure of how rapid vanilla plants have multiplied and it informs how many plantlets can be produced from the initiated plantlets in case of nodal propagation.

3.4.5 Shoot length

Shoot length varied significantly from 4.2 cm (0 mg L^{-1}) to 11.0 cm (1.2 mg L^{-1}) (Table 2 and Figure 7). Premix MS Media supplemented with 1.2 mg L^{-1} (11±0.6 cm) and 1.6 mg L^{-1} (10.4±0.6 cm) were not significantly different from each other. They were however significantly different from kinetin concentration at 0 mg L^{-1} (4.2±0.8), 0.4 mg L^{-1} (4.4±3.8), 0.8 mg L^{-1} (8.2±8.2) and 2.0 mg L^{-1} (8.4±2.6) at 5% level of significant (Table 2). Kinetin concentration at 1.2 mg L^{-1} (11±0.6 cm) performed best in enhancing shoot elongation in vanilla shoots (Table 2). Media supplemented with kinetin at any concentration performed better than media without kinetin (0 mg L^{-1}). This finding highlights the importance of using plant growth regulators to increase efficiency of vanilla *in-vitro* regeneration. According to Kameni *et al.* (2021), kinetin is made up of adenine that carries a Furan-2-ylmethy substituent at the exocyclic amino group that promotes cell division, plant growth and elongation. The present observation underlined the important role of cytokinins in initiating the regeneration response in the nodal explants in orchid species as was suggested by Hussain *et al.* (2017).

Generally, the results obtained here clearly indicated that shoot initiation and proliferation increased with increase in kinetin concentration up to 1.2 mg L^{-1} and then gradually decreased when kinetin concentration level was increased. This is supported by evidence that high levels of cytokinins have deleterious effects on shoot length in vanilla (Erawati *et al.,* 2020).

Figure 7: Shoots length representative at varied Kinetin concentration levels after 8 weeks (a - 0 mg L^{-1}, b - 0.4 mg L^{-1}, c - 0.8 mg L^{-1}, d - 1.2 mg L^{-1}, e – 1.6 mg L^{-1}, f – 2.0 mg L^{-1})

3.4.6 Rooting of Shoots

In-vitro shoots were separated and transferred to the rooting media to obtain complete plantlets. Solid MS media fortified with different concentration levels of IAA and auxin were used for rooting. The effects of different concentration levels of IAA (0.0 mg L^{-1}, 0.4 mg L^{-1}, 0.8 mg L^{-1}, 1.2 mg L^{-1}, 1.6 mg L^{-1} and 2.0 mg L^{-1}) on the root formation on vanilla shoot (Table 3).

Table 3: Effect of IAA concentration levels on rooting of vanilla shoots

No.	Plant Growth Regulator ($mg\ L^{-1}$) IAA	Number of plantlets with roots	Rooted plantlets %	Number of roots per plantlet	Root length (cm)
1	0	2.4±1.2bc	10.17	2.4±0.6c	2.0±0.6c
2	0.4	3.4±0.2b	14.41	2.6±0.8c	1.8±0.8c
3	0.8	3.6±3.2b	15.25	3.0±0.4bc	2.6±1.4c
4	1.2	6.8±1.0a	28.81	3.6±0.2ab	4.0±0.4ab
5	1.6	5.8±1.0a	24.58	4.2±0.6a	3.6±1.0b
6	2.0	1.6±2.0c	6.78	3.4±0.8b	4.6±0.6a

Results show mean ± standard error (SE) of treatments. Different letters within a column indicate significant differences at $p < 0.05$ level.

3.4.7 Number of plants with roots

Results observed after 3 weeks of rooting indicted that indole-3-acetic acid concentration levels at 1.2 mg L^{-1} (6.8±1.0) and 1.6 mg L^{-1} (5.8±1.0) were not significantly different at 5% but they were different from concentration levels at 0 mg L^{-1} (2.4±1.2), 0.4 mg L^{-1} (3.4±0.2), 0.8 mg L^{-1} (3.6±3.2) and 2.0 mg L^{-1} (1.6±2.0) on the effects on number of plants with roots. Treatments 0 mg L^{-1} (2.4±1.2), 0.4 mg L^{-1} (3.4±0.2), 0.8 mg L^{-1} (3.6±3.2) were not significantly different but were different from 2.0 mg L^{-1} (1.6±2.0) except 0 mg L^{-1} (2.4±1.2) which was not different from 2.0 mg L^{-1} (1.6±2.0) on number of rooted plants at 5% level of significant (Table 3 and Figure 8).

A significantly higher percentage of rooted plants was achieved on MS media fortified with 1.2 mg L^{-1} IAA (28.81%) than 2.0 mg L^{-1} IAA (6.78%) after 3 weeks, (Table 3). Significantly lower percentage of rooted plantlets was observed on MS media that was not supplemented with IAA (10.17%). The lowest percentage on MS media supplemented with 2.0 mg L^{-1} IAA (6.78%), (Table 3). In other studies using NAA (an auxin) Adugna *et al.* (2020), reported that the least mean number of rooted shoots were produced on growth regulator free media (0 mg L^{-1}) NAA (0.26 ±0.57) and 2.0 mg L^{-1} (0.27±0.45) of *Moringa stenopetala*. These results showed that IAA and NAA increased their effects on roots to certain saturated point, followed by a decline.

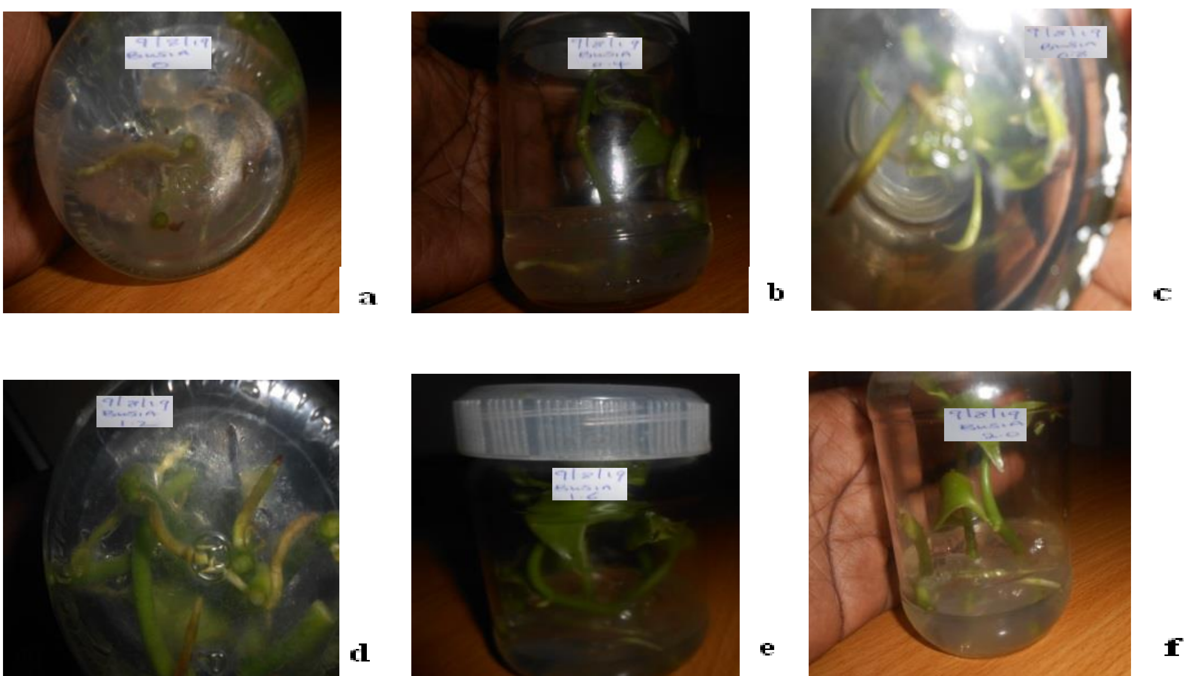


Figure 8: Images of the effects of different concentrations of IAA on rooting of vanilla shoots, 3 weeks after inoculation (a – 0 mg L^{-1}, b – 0.4 mg L^{-1}, c – 0.8 mg L^{-1}, d – 1.2 mg L^{-1}, e – 1.6 mg L^{-1} and f – 2.0 mg L^{-1})

3.4.8 Number of roots per plant

The results recorded after 3 weeks of rooting indicated that shoots grown on MS media supplemented with 1.6 mg L^{-1} IAA produced roots (4.2±0.6). These were significantly different from other treatments (Table 3). Shoot buds grown on MS media that was not fortified with any IAA resulted in least number of roots per plant (2.4±0.6) (Table 3 and Figures 8). Despite that IAA at 2.0 mg L^{-1} concentration level gave lowest percentage of rooted plants (6.78%), 2.0 mg L^{-1} IAA concentration level had the highest root length 4.6±0.6cm as compared to other treatments (Table 3).

These findings are comparable with those of Thapa (2020) who reported 2.9 and 2.4 roots per plant of *Pisidium guajava* on media supplemented with IBA and NAA respectively. Thapa (2020) noted that shoots cultured on media supplemented with IBA alone was observed to have increased in mean number of roots per shoot at a higher concentration (2.0 mg L^{-1}) whereas the less effect was observed for NAA. These findings confirmed that auxins including IAA have positive effect on rooting under tissue culture conditions (Biniari *et al.,* 2018).

Khan *et al.* (2017) also reported that response of shoots to rooting was dependent on the combination and concentrations level of auxins used. The combination of 1.0 mg L^{-1} IBA

+ 0.5 mg L^{-1} IAA was the best medium for rooting. The effect of IBA and IAA for root induction and proliferation has also been reported in medicinal plants (Kumar, 2018).

The current findings are in contrast to those of Erawati *et al*. (2020), who reported that root induction in *Vanilla planifolia* was strongly stimulated by a growth regulator free MS medium as compared to that MS media with IBA and NAA. Auxin free medium gave the higher number of roots, percentage of rooting and root length (Erawati *et al*., 2020). This may be due to the less effect of NAA and IBA as compared to the present study that involved the use of IAA on vanilla crops.

3.4.9 Root length

Results obtained after 5 weeks indicated that the highest value achieved for root length was 4.6±0.6 at 2.0 mg L^{-1} IAA and the least value 1.8±0.8 at 0.4 mg L^{-1} (Table 3). Based on the results, IAA concentration level 0 mg L^{-1}, 0.4 mg L^{-1} and 0.8 mg L^{-1} were not significantly different but were different from concentration level at 1.2 mg L^{-1}, 1.6 mg L^{-1} and 2.0 mg L^{-1} at 5% level of significance. Concentration levels at 1.2 mg L^{-1} and 1.6 mg L^{-1} IAA were not significant different but 1.6 mg L^{-1} was significant different from 2.0 mg L^{-1}, (Table 3).

The observations recorded indicated that IAA concentration at 2.0 mg L^{-1} (4.6±0.6) was the highest value obtained on mean root length. The roots elongated with the increased IAA concentration, was however not uniform as observed in Table 3. These observations are similar to those of Adugna *et al.* (2020), who reported the highest mean root length (0.87 ± 1.22 cm) at 1.0 mg L^{-1} when NAA was increased from 0 mg L^{-1} to 1.0 mg L^{-1} *in-vitro* culture of *Moringa stenopetala*. The NAA increment from 1.0 mg L^{-1} to 2.0 mg L^{-1} in a culture of *Moringa stenopetala*, resulted in a reduction in mean root length from 0.84 ±0.54 to 0.14 ± 0.24 cm (Adugna *et al.*, 2020). This revealed that suitable *in-vitro* concentration conditions for Moringa root elongation is at 1.0 mg L^{-1} NAA. The variations between IAA and NAA effects may be species dependent and the type of plant growth regulators used.

3.4.10 Micropropagation constraints

Table 4: Constraints of a combined concentration levels of kinetin and IAA on vanilla plants throughout the *in-vitro* experiments

No.	Phytohormones (mg L^{-1})		Dead plants	Dormant plants	Contaminated
	Kinetin	IAA			
1	0	0	2 .8±1.0b	4.8±1.6bc	8.2±6.6b
2	0.4	0.4	3.8±8.2b	3.4±3.0c	3.8±2.6d
3	0.8	0.8	9.6±2.4a	5.6 ±0.8b	6.0±0.4c
4	1.2	1.2	9.2±2.8a	6.4±4.4b	6.4±1.8c
5	1.6	1.6	12±2.4a	9.8±1.0a	6.4±1.8c
6	2.0	2.0	9.4±2.6a	10.8±1.0a	14.8 ±6.6a

Different letters within a column indicate significant differences at *$p<0.05$*.

The limiting factors of kinetin and IAA on common plant tissue culture are; dead plantlets, dormancy and contamination (Table 4 and Figure 9). Highest plantlets mortality (12±2.4) was recorded for MS supplemented with 1.6 mg L^{-1} of Kinetin and IAA as a combination, while MS without hormones (0 mg L^{-1}) resulted in least mortality (2 .8±1.0) which was significantly different from other treatments. The results of dead plantlets indicated that the higher the concentration, the more negative effects on the survival of vanilla plantlets. Plants that were dormant in culture media were significantly higher (10.8±1.0) in MS media supplemented with 2.0 mg L^{-1} Kinetin and IAA and least (3.4±3.0) in MS supplemented with 0.4 mg L^{-1} kinetin and IAA.

Contamination is one of the major bottlenecks in vanilla tissue culture (Herman, 2017). In this study, observation was on an endogenous fungal contaminant that was resistant to the surface sterilization and to the broad-spectrum fungicide used (Benlate® SP fungicide contain Benomyl Methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate). Plantlets grown in culture media fortified with 2.0 mg L^{-1} showed significantly higher contamination (14.8 ±6.6) while those cultured on MS media supplemented with 0.4 mg L^{-1} kinetin and IAA showed the least (3.8±2.6) contamination. This suggests that contamination in the media could also be due to the plant growth regulators.

The success in tissue culture depends on the effectiveness of the sterilization methods used on the ex-plants prior to the culture initiation (Park, 2021). It is also important to screen for diseases using ELISA method so that disease free materials can be micropropagated. It is essential that all the materials used in the plant tissue culture be sterilized to kill microorganisms (Yadav, 2019). According to Franco (2021), sterilization of plant tissue culture minimizes contamination to 5%. Contaminants of microorganisms such as viruses, bacteria, yeast and fungi are the reason for plants losses during *in-vitro* culture of plants regeneration (Thomas & Franco, 2021). The disinfectants widely used are sodium hypochlorite which dates back to the mid-18th century (Maffettone *et al*., 2018).

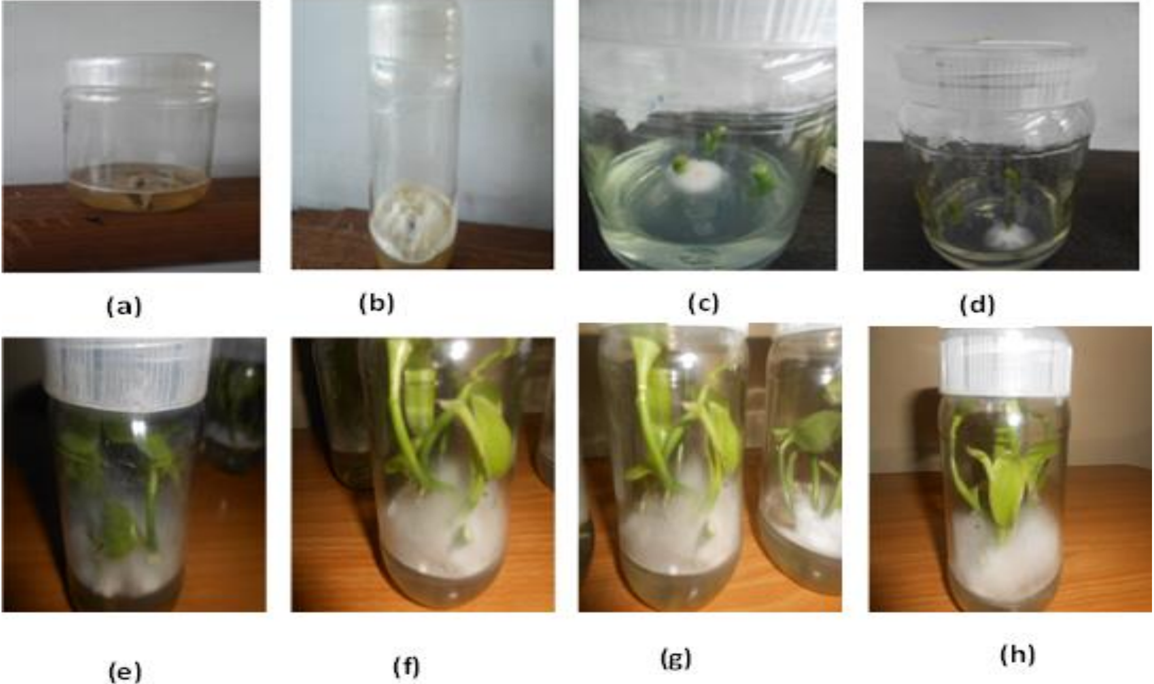


Figure 9: Forms of constraints during Micropropagation (a, b – dead plants, c, d – dormant, and e, f, g, h – contaminated).

3.5 Conclusion and recommendation

The present study confirmed that *in-vitro* regeneration protocol could be used for rapid multiplication method of producing *Vanilla planifolia* Andrews crops in Kenya. The study demonstrated that premix MS media supplemented with kinetin and indole-3-acetic acid had varied effects on shooting and rooting of vanilla depending on the concentration used. Premix MS media supplemented with kinetin at 1.2 mg L^{-1} was the most suitable for *in-vitro* regeneration of vanilla shoot induction while indole-3-acetic acid at 2.0 mg L^{-1} was preferable for roots differentiation and elongation on shoot plants.

Kinetin at 1.2 mg L^{-1} and indole-3-acetic acid at 2.0 mg L^{-1} are considered the best for *in-vitro* rapid mass multiplication of vanilla plant and can be recommended for use at large scale production of clean planting materials in Kenya.

CHAPTER FOUR

MORPHOLOGICAL DIVERSITY OF KENYAN VANILLA (*Vanilla spp.*) ACCESSIONS

Abstract

Several countries have successfully bred vanilla crops based on variations in morphological traits. However, such programmes in Kenya are limited by lack of information on phenotypic diversity. The objective of this study was to characterize vanilla accessions from Bungoma, Busia, Kwale, Mombasa and Kilifi counties in Kenya using morphological variations. Morphological traits analyses were based on eighteen qualitative and nine quantitative characteristics. Qualitative and quantitative data were analyzed using STATA software (version 7, edition 5.0). Factorial analysis for qualitative traits clustered the samples into two, (I - Inland western counties) and (II - Coastal counties). Five factor analyses for quantitative traits was strongly negative for overall stem height (-0.1441) and aerial root length (-0.1329), and strongly positive for number of flowers, leaf petiole length, stem internode length, leaf blade width and leaf blade length accounting for 80.12%. The highest uniqueness was recorded for aerial root length (0.825) and overall stem length (0.734) indicating that these traits were more variable. The least uniqueness was recorded for leaf blade width (0.1099), leaf petiole length (0.1754) and stem internode length (0.180), implying that these traits were more uniform in among the accessions.

4.1 Introduction

Vanilla species are the only orchids commercially grown for their pods (Chase *et al.,* 2017). Orchids comprise 7% of flowering plants and are considered one of the most prominent families among flowering plants (Rao & Rajasekharan, 2019). *Vanilla* is derived from the Spanish name *vainilla*, which means "little pod" and refers to the long and slender fruit (Sanchez-Galindo *et al.,* 2018). *Vanilla pompona* Scheide, *Vanilla tahitensis* and *Vanilla planifolia* Andrews (syn. *V. fragrans*) are the main species commercially grown for natural vanillin flavour production (Briddon *et al.,* 2019). Vanilla species are in the Orchidaceae family and belong to Plant Kingdom (Havkin-Frenkel & Belanger, 2018) and are native to Mexico South Eastern parts of the country (Wodrich, 2020).

Brunschwig *et al.* (2017) investigated the comparative vegetative anatomy and systematics of the species of vanilla and found that vegetative anatomical characters have some phylogenetic value. The vanilla genus has 16 (x=16) chromosomes whereas, *Vanilla pompona, Vanilla planifolia,* and *Vanilla tahitensis* are diploid with 2n=32 (Briddon *et al.,* 2019). Vanilla species are cultivated in tropical climates where rainfall is evenly distributed between 150 to 300mm annually (Romps, 2020). Excessive rainfall cause mildew and root rot diseases in vanilla crops, while drought conditions result in a small number of flowers and low yield (Gaznayee & Al-Quraishi, 2019). Suitable temperatures for vanilla crops are between 24 °C and 30 °C (Nany & Grisoni, 2021). The vanilla plant is usually cultivated on various types of soils, from sandy loam to laterites (Mohamed & Aly, 2019).

Phenotypic characterization differentiates one crop from another (Doussoh *et al.,* 2018). The common phenotypic descriptors used in individualizing plant species are mature plant height, leaf length, leaf width, root type and flower colour (Bahar *et al.,* 2018). Phenotypic traits are influenced by agricultural practices and environmental factors (Gamrat *et al.,* 2021).

Vanillin is present in the vanilla bean at 20 grams per kilogram dry weight (Adawiyah *et al.,* 2022). It contains other compounds like vanillic acid, sugar, fats, cellulose, minerals, water, *p*-Hydroxybenzaldehyde and *p*-Hydroxybenzyl methyl-ether (Kranz & Jahns, 2014). Natural vanillin has antimutagenic, anticarcinogenic and antimicrobial properties (Ravindran, 2017). The antimutagenic property has the ability to reduce chromosomal damage caused by X-ray and ultraviolet (UV) light (Cassar *et al.,* 2020). The anticarcinogenic property provides a natural defence mechanism against cancer, deactivate carcinogens and block the means by which carcinogens act (Soloneski & Larramendy, 2021), while antimicrobial property prevent

the spread of microorganisms such as protozoa, bacteria, virus and fungi like mildew and molds (Singh, 2020).

Adawiyah *et al.* (2022) reported that natural vanillin is extracted from cured vanilla beans and is universally used in flavouring food, pharmaceutical, beverage, cosmetic and perfumery industries. Production of vanilla crop acts as a source of income for small-scale farmers and earns foreign exchange for most tropical countries in the world (Wilson, 2021). The global market for the vanilla bean is projected to record 3.9% growth during the forecast period 2020-2025 (FAO, 2020). About 20 countries including Kenya import natural vanilla, of which America, Germany, France, the United Kingdom and Japan are the leading importers (Tenedios *et al.,* 2018).

According to Oyugi (2018), vanilla is one of the orphaned crops in Kenya and was introduced in Kenya from neighbouring countries like Uganda and Tanzania in 1954. The vanilla crop was believed to have come from Madagascar and Reunion through Christian missionaries in Uganda (Nany & Grisoni, 2021). Since its introduction, vanilla species found in Kenya have not been characterized. This hinders the breeding programme, dissemination and cultivation of the crop in Kenya. Limited or no information on available vanilla species found in Kenya has been documented. Determining morphological variations in crop species is essential for crop improvement (Maqbool *et al.,* 2021). Therefore, assessing the extent of diversity in vanilla species found in Kenya was necessary. The study was conducted to determine the diversity of vanilla species found in Kenya using qualitative and quantitative vanilla descriptors.

4.2 Materials and methods

4.2.1 Experimental sites

The *in-situ* evaluation was conducted in five select counties in Kenya; Bungoma county (Latitude 0° 45' 16.799" N and Longitude 34° 30'09.270" E), Busia county (Latitude 0° 47' 11.113" N and Longitude 34° 24' 26.339" E), Kwale county (Latitude 4° 09' 58.223" S and Longitude 39° 34' 18.127" E), Kilifi county (Latitude 4°16' 53" S and Longitude 39° 44' 41.306" E) and Mombasa county (Latitude 4° 02' 12.7608" S and Longitude 39° 40'10.4556" E), (Figure 10). The counties were selected purposively because they host institutions and farmers who grow vanilla in Kenya.

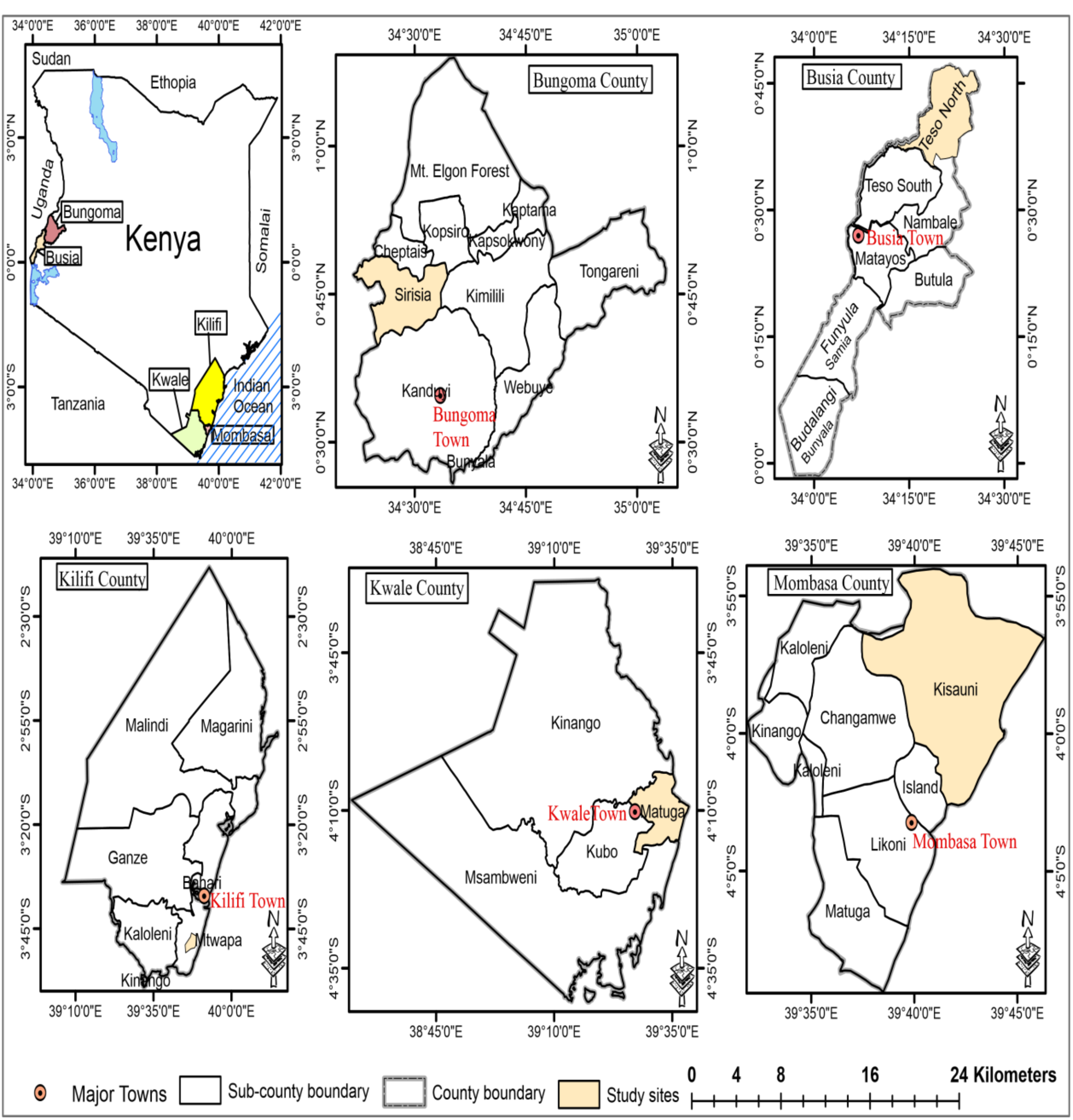


Figure 10: Map of Kenya showing the physical location of the experimental sites

4.2.2 Morphological traits

Eighteen qualitative and nine quantitative morphological traits were used to determine variation level among vanilla accessions in five counties (Table 5). Phenotypic characterization (Vegetative, flowering and maturing traits) was done according to vanilla descriptor (IPGRI, 2001).

Table 5: The qualitative and quantitative traits according to IPGRI (2001) used to characterize vanilla accessions from five counties of Kenya

SNO.	Code	Trait	Phenotypic scale
			Qualitative traits
1	LBC	Leaf Blade Colour	1=white yellow, 2=light green, 3=dark green
2	LBV	Leaf Blade Variegation	1=absent, 2=present.
3	PLBS	Plant Leaf Blade Symmetry	1=symmetric/slightly asymmetric, 2=moderate asymmetric, 3=strongly asymmetric.
4	LBB	Leaf Blade Base	1= clasping, 2=tapering.
5	LBSA	Leaf Blade Shape of Apex	1=obtuse, 2=acute, 3=acuminate.
6	LBCM	Leaf Blade Conspicuousness of the main vein	1 = weakly visible, 2=slightly visible, 3=clearly visible.
7	LBTS	Leaf Blade Transverse Section	1=flat/slightly concave, 2=moderately concave, 3=strongly concave.
8	LBS	Leaf Blade Shape	1=narrow ovate, 2=medium ovate, 3=elliptic, 4=obovate, 5=oblong.
9	SC	Stem Colour	1=light green, 2=medium, 3=dark green.
10	SSCS	Stem Shape in Cross Section	1=round, 2=round to angular, 3=angular.
11	SV	Stem Variegation	1=absent, 2=present.
12	SST	Stem Surface Texture	1=smooth, 2=medium, 3=rough.
13	FS	Fruit Shape	1= ovate, 2 = oblong, 3 = obovate.
14	FST	Fruit Surface Texture	1= smooth, 2= medium, 3= rough.

15	FTSS	Fruit Transversal Section Shape	1=circular, 2=elliptic, 3=medium ovate, 4=broad ovate, 5=trullate, 6=triangular.
16	OH	Overall Health	1=dead, 2=stressed, 3=well-nourished and health.
17	OLV	Overall Leaf Vigour	1=no new leaf growth, 2=leaf growth development.
18	PAR	Plant Aerial roots	1=long, strong aerial roots, 2=short, strong aerial roots 3=long, weak aerial roots, 4=short, weak aerial roots.
		Quantitative traits	
1	OSL	Overall stem length (m)	Stem length measured from the proximal end (base end) to the distal end (apical end).
2	LAR	Length of Aerial roots (m)	Lengths of the root from the root base (at the point of emergence) to the tip.
3	NOF	Number of flowers	The grown flowers from sampled vanilla plants were counted, and the average number recorded.
4	LPL	Leaf petiole length (cm)	The length of the petiole from the point of attachment to the leaf blade base.
5	SIL	Stem internode length (cm)	The lengths of the internode between the nodes.
6	LBW	Leaf blade width (cm)	Measured across between the margins at the broadest point.
7	LBL	Leaf blade length (cm)	Leaf blade length from the base of the blade to the apex.
8	SC	Stem circumference (cm)	A string was used to determine stem circumference.
9	FL	Fruit length (cm)	Measure from the point of attachment to the tip.

4.3 Statistical analyses

Statistical analyses were computed using STATA software (version 7, edition 5.0) for qualitative and quantitative data.

4.3.1 Qualitative traits

Results of qualitative traits were presented in a frequency distribution table. Factorial and phylogenetic analyses were also done.

4.3.2 Quantitative traits

Quantitative data were presented in analysis of variance (ANOVA) table. Pearson's simple correlations were used to compare the degrees of association in the traits. The quantitative data were automatically standardized using Pearson's simple correlations (to avoid inflating the impact of variables with high variances on the results) before they were used as input for Principal Component Analysis (PCA) and cluster analysis.

Statistical model for quantitative data:

$Y_{ij} = \mu + \alpha_i + \beta_{j(i)} + \varepsilon_{ij}$

Where,

Y_{ij} - an individual observation for i^{th} county and j^{th} vanilla accession, μ - overall mean, α_i - effect of ith county, $\beta_{j(i)}$ - effect due to j^{th} vanilla accession nested within the ith county, ε_{ij} - random error component associated with each observation.

4.4 Results and discussion

4.4.1 Results of qualitative traits analysis

The overall health of vanilla in the five counties was fair as most of the plants sampled (52.5%) exhibited well-nourishing characteristics, while 47.5% were stressed (Table 6). All the vanilla sampled in Kwale (26) and Mombasa (21) counties showed better overall health, while all the vanilla accessions in Busia (17) were stressed. However, all five counties' vanilla accessions showed good overall leaf vigour characterized by leaf growth and development.

Stem colour was mainly medium green for all counties except Mombasa, where all accessions had dark green stems. Vanilla accessions in Bungoma (8) and Busia (17) had variegated stems, while those from coastal counties (Mombasa (21), Kwale (26) and Kilifi (4)) did not show variegation on the stems. Most counties had vanilla with a medium stem surface texture and a flat to slightly concave transverse section with a round cross-section shape.

All vanilla accessions had narrow ovate-shaped, moderately asymmetric, non-variegated leaf blades with acute apexes. The colour of the leaf blade was either light or dark green, and either clasping or tapering bases were observed. All types of aerial roots were observed in the vanilla accessions. However, most accessions had either long or short, strong aerial roots. Fruits observed on vanilla accessions were few and scanty. However, all the observed fruits were oblong with smooth surface texture and a trullate transversal section shape (Table 6).

Cota *et al.* (2021) discovered that most of the species have similar habitat and exhibit strong similarities in morphological traits. However, this discovery is comparable with the results found in this study, where most accessions in all counties had either long or short strong aerial roots despite being in varied agro-ecological zones. According to Pausic *et al.* (2018), orchid species are highly variable in their morphological traits based on varied agro-ecological zones. The current results are aligned to those of Pausic *et al.* (2018), as the Inland Western counties (Bungoma and Busia) showed a variation in variegated stems as opposed to the non-variegated stems from coastal counties (Mombasa, Kwale and Kilifi).

Table 6: Frequency distribution table of qualitative morphological traits of vanilla accessions from five counties in Kenya

Plant Traits/ Site		Bungoma (%)	Busia (%)	Kwale (%)	Mombasa (%)	Kilifi (%)	Total (%)
Overall health	*Stressed*	62.5	100	0	0	75	47.5
	Well-nourished	37.5	0	100	100	25	52.5
Overall Leaf Vigour	*leaf growth development*	100	100	100	100	100	100
Stem Colour	*Medium green*	65.5	100	76.9	0	75	63.05
	Dark Green	37.5	0	23.1	100	0	36.95
Stem Variegation	*Present*	100	100	0	0	0	40
	Absent	0	0	100	100	100	60
Stem Surface Texture	*Medium (smooth/rough)*	100	100	100	100	100	100
Leaf Blade Transverse Section	*flat/slightly concave*	65.5	100	100	100	100	93.1
	moderately concave	37.5	0	0	0	0	6.9
Stem Shape in Cross section	*Round*	37.5	100	100	100	100	86.9
	Round to angular	65.5	0	0	0	0	13.1
Plant Aerial roots	*Long, strong*	37.5	76.5	88.5	0	0	43
	Short, strong	12.5	17.5	11.5	100	100	48.3
	Long, weak	37.5	0	0	0	0	7.5
	Short weak	12.5	6	0	0	0	3.7
Leaf Blade Colour	*Light green*	87.5	100	53.8	0	75	43.2
	Dark green	12.5	100	46.2	100	25	56.8
Leaf Blade Base	*tapering*	100	100	100	0	0	60

	Clasping	0	0	0	100	100	40
Plant Leaf Blade Symmetry	*moderate asymmetric*	100	100	100	100	100	100
Leaf Blade Variegation	*Absent*	100	100	100	100	100	100
Leaf Blade Shape of Apex	*Acute*	100	100	100	100	100	100
Leaf Blade Shape	*Narrow ovate*	100	100	100	100	100	100
Leaf Blade Conspicuousness of main vein	*Weakly visible*	0	0	100	100	100	60
	Slightly visible	100	100	0	0	0	40
Fruit Shape	*Oblong*	0	17.5	26.9	0	0	8.9
Fruit Surface Texture	*Smooth*	0	17.5	26.9	0	0	8.9
Fruit Transversal Section Shape	*Trullate*	0	17.5	26.9	0	0	8.9

4.4.2 Factorial analysis

Factorial analysis grouped the samples into two clusters, I and II. Cluster I contained vanilla accessions from inland western counties (Bungoma and Busia), while cluster II contained accessions from coastal counties (Mombasa, Kwale and Kilifi). The factorial analysis showed that diversity existed among accessions within and between clusters. Cluster I showed a higher level of diversity than cluster II as shown in Figure 11.

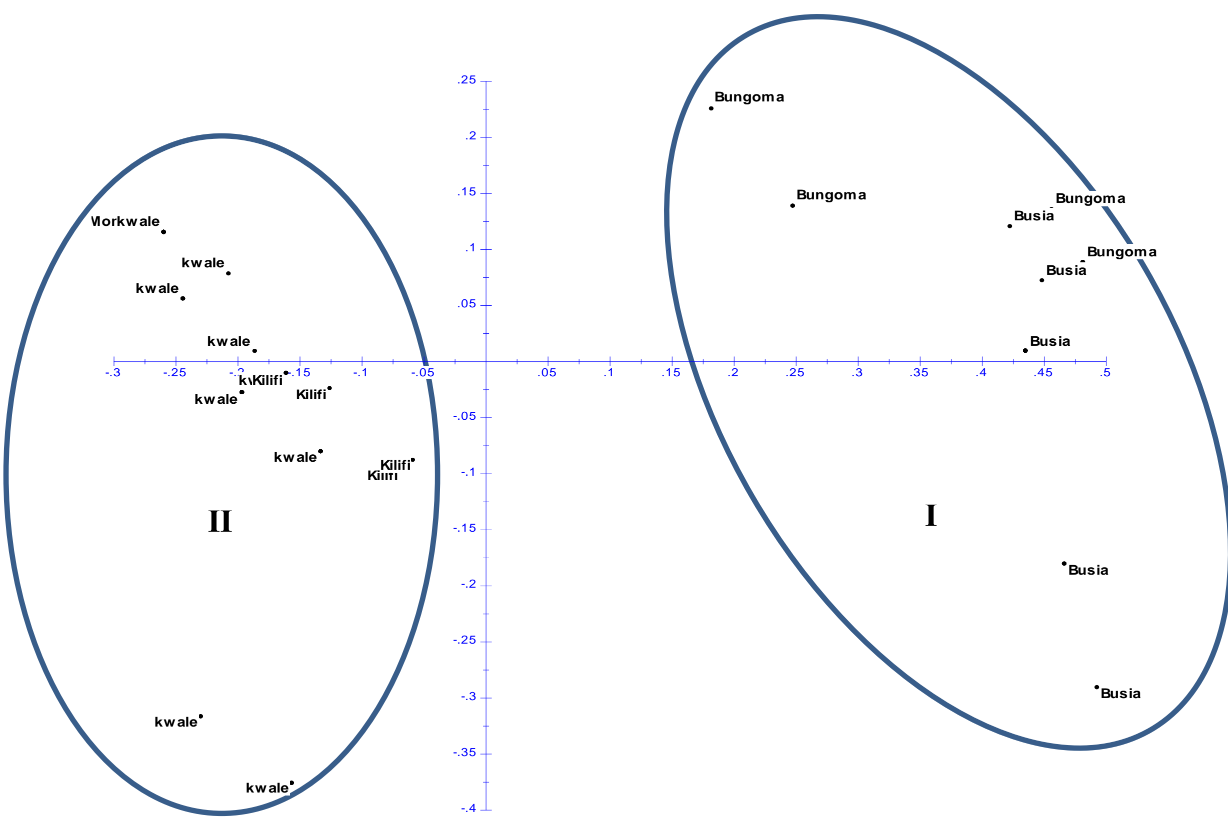


Figure 11: A factorial analysis based on qualitative traits of vanilla accessions from five counties of Kenya

The accessions in cluster I showed poor overall health, variegated stems and light green leaf blades with a weak visible main vein. Cluster II was characterized by well-nourished accessions with non-variegated stems and dark green leaf blades with slightly visible main veins. Chanda *et al.* (2021) explained that morphological characterization is a broad subject that includes botanical or taxonomy descriptors. It distinguishes one plant from another or accessions against the others (Chanda *et al.,* 2021). Morphological descriptors involved in Chanda *et al.* (2021) study are plant height, panicle length, leaf length and width, flower colour and root type. Chanda *et al.* (2021) findings were in-line with those of the current study, where poor overall health, variegated stems and light green leaf blades with a weakly visible main vein, well-nourished accessions with non-variegated stems and dark green leaf blades with slightly visible main veins were used to cluster the accessions from five counties into two clusters.

4.4.3 Cluster Analysis

The coded qualitative traits were used to construct a complete linkage dendrogram presented in Figure 12. The accessions were grouped into two major clusters A and B, and five sub-clusters (AI, AII, BI, BII and BIII) based on the county of origin. Cluster A comprised accessions from Bungoma and Busia counties, while cluster B comprised accessions from Mombasa, Kwale and Kilifi. Sub-cluster AI comprised accessions from Busia county. Busia county AI sub-cluster was based on all accessions having good overall health and medium green stem colour. Sub-cluster AII was made up of all accessions from Bungoma county and a few from Busia county. These accessions had mixed characteristics; stressed and good overall health, variegated stems, dark and light green leaf blades and slightly visible leaf blade conspicuousness of the main vein.

Sub-cluster BI comprised most of Kwale county's accessions and was characterised by a tapering leaf blade base. All accessions from Mombasa county and one from Kwale county were grouped in sub-cluster BII because they all had well-nourished overall health, dark green coloured stems and leaf blades. Sub-cluster BIII consisted of more accessions from Kilifi and three from Kwale characterised by accessions with mixed traits; stressed and good overall health, light green and dark green leaf blades colour.

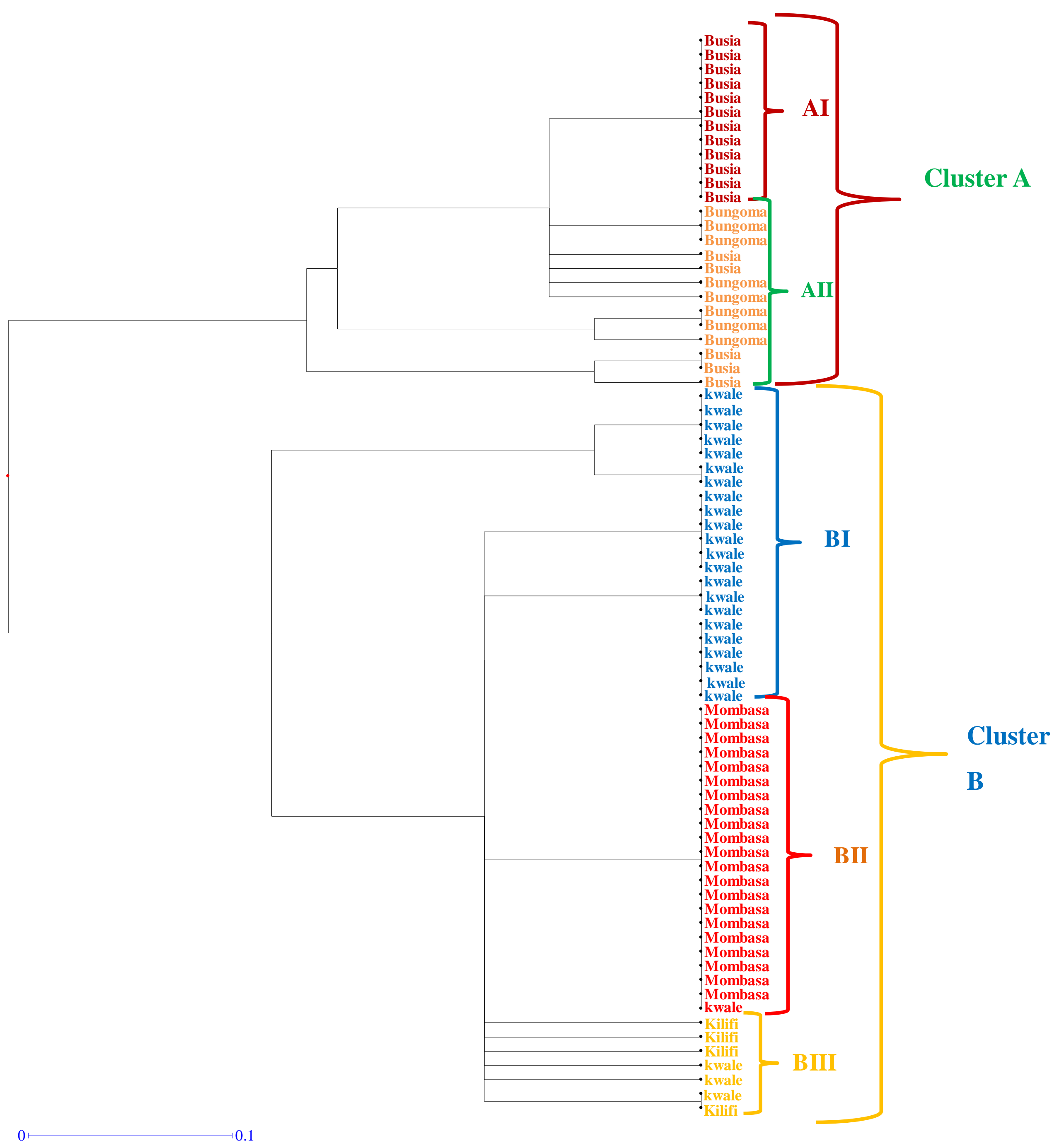


Figure 12: A dendrogram of vanilla accessions based on qualitative traits characterized from five counties (Bungoma, Busia, Kwale, Kilifi and Mombasa) in Kenya

Vanilla is cultivated in many tropical countries (Carbajal-Valenzuela *et al*., 2022). Carbajal-Valenzuela *et al*. (2022) discovered that the success of the vanilla crop depends on the proper management of light because it is the environmental factor with the most significant influence on plant photosynthesis, growth survival and ultimately the ability to acclimation to different habitats. According to Ji *et al.* (2018), vanilla is a hemi-epiphyte orchid that faces seasonal water stress throughout its life cycle under natural conditions, which explains its CAM photosynthetic pathway meaning that this species faces different light environments.

The results of the current study validate those of Petrauskas *et al.* (2020), where vanilla accessions from Bungoma county and a few from Busia counties had the characteristic of mixed stressed and good overall health, variegated stems, dark and light green leaf blades, and slightly visible leaf blade conspicuousness of main vein meaning that these phenotypic traits exhibited depended on proper management of light and seasonal water stresses that prevails in vanilla species throughout its life cycle under normal conditions.

4.4.4 Results of quantitative traits analysis

On overall stem height, Kwale county accessions were significantly longer (22.78) than vanilla accessions from the other counties (Table 7). Stem circumference was significantly larger in accessions from Busia (2.74), Bungoma (2.66) and significantly smaller in accessions from Kwale (2.37), Mombasa (2.32) and Kilifi (2.21) (Table 7). Stem internode length was significantly highest in accessions from Busia (11.05) and shortest in Kilifi (2.18). Aerial root length was significantly longer in accessions from Kwale (15.78) and significantly shorter in Mombasa (1.86) (Table 7).

Vanilla accessions from Busia and Bungoma had significantly wider leaf blades (4.52 and 4.44, respectively) with longer petioles (1.50 and 1.50) than those from Kwale, Mombasa and Kilifi (Table 7). Bungoma and Busia counties had significantly longer leaf blades (11.95 and 13.82 respectively). Vanilla in Busia (0.59) and Bungoma (0.38) had significantly more flowers than the other counties, where none was observed, and there was no significant difference in fruit length across the counties (Table 7).

De-Camargo *et al.* (2018) studied descriptions of new species in the Brazilian Amazon. During their survey for the taxonomy of orchids in Para State, they found that specimens in the Para State resembled some Amazonian types despite having no designated holotype. The discoveries of the current study correspond with the study of Krahl *et al.* (2020), where vanilla accessions from Busia and Bungoma were similar to each other; they had significantly wider leaf blades with longer petioles and many flowers. According to Krahl *et*

al. (2020), *Vanilla odorata and Vanilla labellopapillata* differ by stem internode length, where *Vanilla odorata* range from 7–10.5cm long while *Vanilla labellopapillata* range was 12.6–16.2cm long despite resembling each other.

The current research concurred with those of Krahl *et al.* (2020) in the manner that accessions from Busia county had the highest stem internode length (11.05cm) as compared to those from Kilifi county (2.18cm) despite showing similarity. The results of the current study also agreed with the report of Brunschwig *et al.* (2017). They reported that *Vanilla labellopapillata* (12.6–16.2 cm) differs from *Vanilla tahitensis* based on the internode length (5.0 cm).

Table 7: Quantitative traits of vanilla accessions from five counties in Kenya

Site	OSH (m)	ARL (m)	NOF	LPL (cm)	SIL (cm)	LBW (cm)	LBL (cm)	SC (cm)	FL (cm)
Bungoma	2.82b	5.14b	0.38a	1.50a	9.96a	4.44a	11.95a	2.66ab	0.00a
Busia	4.99b	2.46b	0.59a	1.50a	11.05a	4.52a	13.82a	2.74a	3.41a
Mombasa	1.71b	1.86b	0.00a	0.68c	4.63b	2.06c	7.05b	2.32b	0.00a
Kwale	22.78a	15.78a	0.00a	1.05b	4.65b	2.53b	8.84b	2.37b	4.73a
Kilifi	4.49b	4.69b	0.00a	0.70bc	2.18c	2.25bc	2.10b	2.21c	0.00a
Pr(>F)	0.00***	0.01*	0.08	4.9e-14***	2e-16***	<2e-16***	<2e-16***	0.00**	0.05

Key: OSL- Overall Stem Height, ARL- Aerial Root Length, NOF- Number Of Flowers, LPL- Leaf Petiole Length, SIL- Stem Internode Length, LBW – Leaf Blade Width, LBL-Leaf Blade Length, SC-Stem Circumference, FL- Fruit Length

4.4.5 Correlations analysis of quantitative traits of vanilla accession from different counties in Kenya

Overall stem height was significant (*p*=0.05) and positively correlated with length of aerial roots (r = 0.35) and negatively correlated with stem internode length (r = -0.19) and leaf blade width (r = 0.16) (Table 8). Aerial root length was positively significantly correlated with flower length and a strong negative correlation with stem internode length (-0.14), leaf blade width (-0.17) and leaf blade length (-0.10). There was a strong positive correlation between the number of flowers and leaf petiole length (0.22), stem internode length (0.29), leaf blade width (0.34), leaf blade length (r = 0.30) and stem circumference (r = 0.14) (Table 8). Leaf blade

width was significant and was positively correlated with leaf blade length (r = 0.84) and stem circumference (r = 0.43) (Table 8).

Table 8: Linear correlation table of the quantitative traits of vanilla accessions characterized from five counties in Kenya

	OSH (m)	ARL (m)	NOF	LPL (cm)	SIL (cm)	LBW (cm)	LBL (cm)	SC (cm)	FL
OSH (m)									
ARL (m)	0.35*								
NOF	-0.07	-0.02							
LPL (cm)	-0.01	-0.02	0.22*						
SIL (cm)	-0.19*	-0.14*	0.29*	0.67*					
LBW (cm)	-0.16*	-0.17*	0.34*	0.70*	0.80*				
LBL (cm)	-0.07	-0.10*	0.30*	0.72*	0.85*	0.84*			
SC (cm)	-0.06	0.02	0.14*	0.38*	0.55*	0.43*	0.56*		
FL	1.00	0.35*	-0.07	-0.01	-0.19	-0.16	-0.07	-0.06	

Key: OSH- Overall Stem Height, ARL- Aerial Root Length, NOF- Number Of Flowers, LPL- Leaf Petiole Length, SIL- Stem Internode Length, LBW – Leaf Blade Width, LBL-Leaf Blade Length, SC-Stem Circumference, FL- Fruit Length. Significant at $p<0.05$*, 0.01**, 0.001***

Strong positive correlations existed between stem internode length and leaf blade length (r = 0.85) and leaf blade width and leaf blade length (r = 0.84) while strong negative correlations were observed between overall stem height and stem internode length (r = -0.19) and leaf blade width (r = -0.16).

4.4.6 Factor Analysis of quantitative traits of vanilla accessions in Kenya

The overall stem height and aerial root length of the five factors accounted for all the variations observed in the vanilla accessions (Table 9). The overall stem height (-0.1441) and aerial root length (-0.1329), which were strongly negative significant and positive for all the other traits that accounted for 80.12% of all the variance observed. The highest uniqueness was recorded for aerial root length(0.825) and overall stem height (0.734), showing that these traits were more variable, while the least uniqueness was recorded for leaf blade width (0.1099), leaf petiole length (0.1754) and stem internode length (0.180), showing that these traits were more uniform in the among the accessions.

Table 9: Un-rotated factor loadings for the variables of vanilla accessions based on factor analysis

Variable	Factor1	Factor2	Factor3	Factor4	Factor5	Uniqueness
OSH (m)	-0.1441	0.7109	0.0585	0.0395	0.0157	0.7337
ARL (m)	-0.1329	0.3140	0.3815	0.0644	-0.0159	0.8251
NOF	0.3283	0.0933	-0.0868	0.2166	0.0635	0.4063
LPL cm	0.7493	0.1360	0.0100	0.0830	-0.0820	0.1754
SIL cm	0.9010	0.0732	0.0355	-0.0779	-0.0086	0.180
LBW cm	0.8891	0.0301	-0.1092	0.1261	0.0048	0.1099
LBL cm	0.9371	0.0989	-0.0032	-0.0432	0.0150	0.6044
Eigen value	3.5030	1.0012	0.2884	0.1297	0.0124	-
Df	2.5020	0.7127	0.1587	0.1173	0.0785	-
Proportion	0.8012	0.2290	0.0660	0.0297	0.0028	-
Cumulative	0.8012	1.0302	1.0962	1.1258	1.1287	

Key: OSH- Overall Stem Height, ARL- Aerial Root Length, NOF- Number Of Flowers, LPL-Leaf Petiole Length, SIL- Stem Internode Length, LBW – Leaf Blade Width, LBL-Leaf Blade Length, SC-Stem Circumference, FL- Fruit Length

4.4.7 Principal Component Analysis

Principal component (PC) analysis was used to cluster the variance in the multiple crop variables into different components so that the first principle component would be the independent cause of the most available variance and the following components justify gradually less variance of the variations. Principal component analysis (PCA) of the nine quantitative traits is presented in Table 10. The fifth PCs had Eigen values > 0.5, and they explained 88.9% of the total variation for quantitative morphological traits of the accessions. Explained variance by the first principle component was 42.4% while component 2 explained 18.3%; the two (Comp1 ‘0.424’. and Comp2 ‘0.183’) most significant components explained 60.7% of the total variance. Aerial root length, overall stem height and fruit length loaded highly in PC1, accounting for 42.4% of the total variation of the vanilla accessions.

Table 10: Eigen Values, Standard deviation, Proportion of variance and Cumulative proportions of morphological traits that contributed to the five PCs

Traits	Comp.1	Comp.2	Comp.3	Comp.4	Comp.5
ARL(m)	0.082	0.378	0.761	0.187	0.271
FL	- 0.054	0.592	-0.488	0.159	-0.142
LBL(cm)	- 0.479	0.079	-0.028	0.043	0.051
LBW(cm)	- 0.464	-0.014	-0.068	-0.077	0.194
LPL(cm)	- 0.412	0.125	-0.002	0.034	0.453
NOF	- 0.206	-0.109	0.366	-0.838	-0.286
OSH(cm)	0.084	0.684	0.049	-0.137	-0.105
SC(cm)	- 0.324	0.045	0.199	0.447	-0.751
SIL(cm)	- 0.468	-0.040	-0.000	0.088	0.046
Eigen Values	3.822	1.637	1.038	0.879	0.626
Standard deviation	1.955	1.279	1.019	0.938	0.791
The proportion of Variance (%)	0.424	0.183	0.115	0.098	0.070
Cumulative Proportion (%)	0.424	0.607	0.722	0.820	0.889

Key: Comp - component, OSH- Overall Stem Height, ARL- Aerial Root Length, NOF-Number Of Flowers, LPL- Leaf Petiole Length, SIL- Stem Internode Length, LBW – Leaf Blade Width, LBL-Leaf Blade Length, SC-Stem Circumference, FL- Fruit Length

Principle component analysis revealed that very small variances in quantitative traits exist between vanilla accessions from select counties in Kenya. Most quantitative traits had very small variances except for overall stem height, aerial root length and fruit length, which showed significant variances (Figure 13).

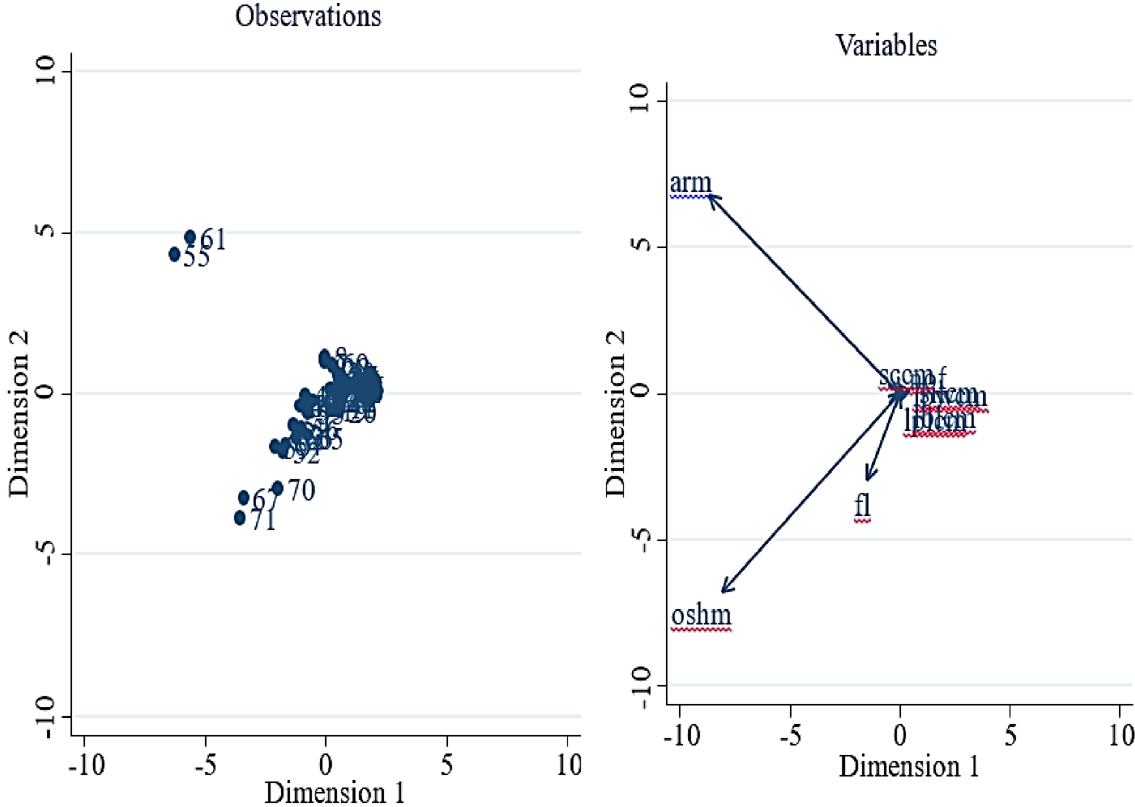


Figure 13: Two-panel Bi-plots of the first two Principle components of the vanilla accessions

Cluster analysis using complete linkage hierarchical clustering grouped the accessions into three phylogenetic groups (I, II and III) (Figure 14). Group I, contained all the accessions from Mombasa, Kwale, Busia, Bungoma and Kilifi. Group II contained one accession from Bungoma and three accessions from Busia, while Group III contained three accessions from Busia and seven from Kwale. No significant pattern could be deduced from the clustering of accessions in this study. Quantitative traits did not have enough resolution to distinguish the vanilla accessions into respective counties of origin.

Cluster analysis of quantitative traits of vanilla accessions from five counties in Kenya

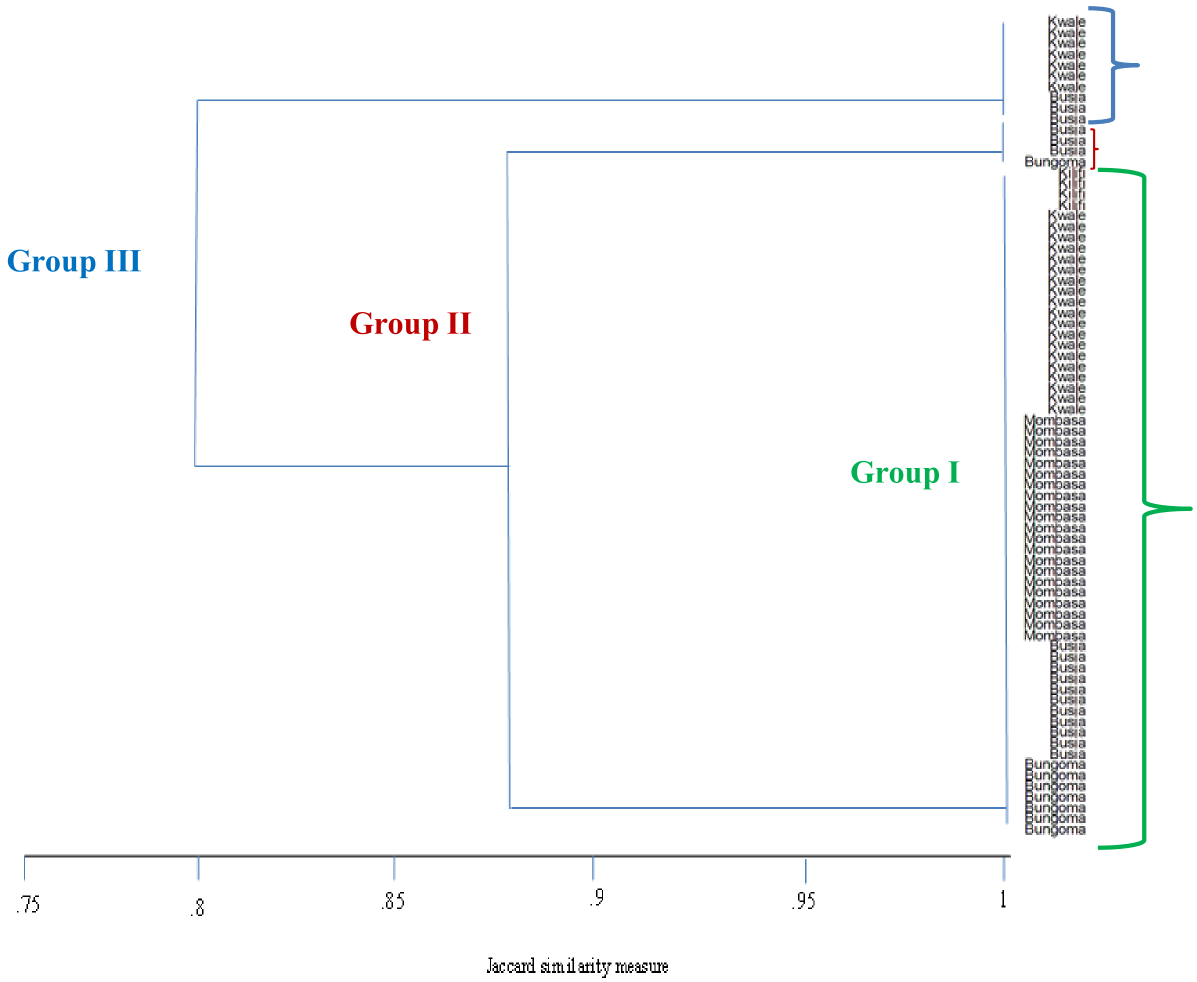


Figure 14: Dendrogram based on quantitative traits- Group I (Mombasa, Kwale, Busia, Bungoma & Kilifi), Group II (1 Bungoma & 3 from Busia), Group III (3 Busia & 7 Kwale)

4.5 Conclusion and recommendation

Morphological markers grouped accessions into different clusters irrespective of their geographical areas in which they were found growing showing that selecting accessions for crop improvement on the basis of morphological traits alone may be inadequate. Complementing molecular techniques in crop selection was needed.

CHAPTER FIVE

DIVERSITY ASSESSMENT OF VANILLA *(Vanilla spp.)* ACCESSIONS IN FIVE COUNTIES OF KENYA USING SIMPLE SEQUENCE REPEATS (SSRs) MARKERS

Abstract

Diversity assessment of vanilla (*Vanilla spp.*) in Kenya is the critical strategy for germplasm conservation, utilization and improvement. Production of the vanilla crop in Kenya is limited partly due to inadequate knowledge of genetic diversity. This study was carried out to characterize 76 vanilla accessions from five counties of Kenya using 14 microsatellite DNA markers. POPGENE version 1.32 was used to compute a variety of factors. Amplicons ranged between 1 and 4. A total of 27 (96.43%) alleles were observed, ranging from 1.00 to 2.00, with a mean of 1.93. Effective allele values ranged from 1.00 to 1.99, with a mean of 1.63. Gene diversity ranged from 0 to 0.50 with a mean of 0.35, the mean shannon information index was 0.50, and Polymorphic information content was 0.375 less than the expected value >0.6 and ranged from 0 to 0.38 with a mean of 0.35. Jaccard's similarity coefficient ranged from 0.08 to 1.00, averaging 0.54. The unrooted phylogenetic tree was constructed in DARwin 6.0.8 using the Unweighted Pair Group Method with Arithmetic Mean, clustering the samples into three main clusters (**A** 99.6%, **B** 98.96 % and **C** 100%) and six sub-clusters (A1, A2, B1, B2, B3 and C1). Vanilla accessions grown in Kenya have a broad genetic background but low genetic diversity. Results inform the need to introduce more molecular markers and vanilla species as sources of genetic variation for breeding.

5.1 Background of the study

Vanillas are tropical climbing orchids that belong to the Orchidaceae family (Teoh, 2019). The Orchidaceae family comprises more than 25000 species distributed in 800 genera (Laman & Beehler, 2020). Vanilla species originated from tropical forests of south-eastern Mesoamerica (Havkin-Frenkel & Belanger, 2018). In the nineteenth century, vanilla species were introduced in tropical countries like Asia and Africa from the original Mexican cultivated stock (Ellestad *et al.,* 2022). Christian missionaries introduced vanilla species in Kenya (Chambers, 2019). According to Nany (2021), Madagascar has been vanilla's leading producer since its introduction. The natural vanilla global market was projected to increase by 1.3% per year from 2016 to 2025 based on demand for natural vanilla in the food, perfumery, cosmetic and medicinal sectors (Gu *et al.,* 2017).

Orchid species are grown for their flowers, and vanilla is the only genus of the orchid family whose species produces a commercially important flavour (Thammasiri, 2020). Brunschwig et *al.* (2017) studied the comparative vegetative anatomy and systematics of the species of vanilla and found that vegetative anatomical characters have some phylogenetic value. The chromosomal number of the vanilla genus is 16 (x=16) (Briddon *et al.,* 2019).

The vanilla flavour is an expensive spice traded in the global markets after saffron (Kafi *et al.,* 2018). According to Oyugi (2018), the vanilla crop has a substantial monetary value. A farmer can earn sixteen thousand or more per vine when managed well (Oyugi, 2018). The high monetary value is on account of the crop's uses, including flavouring food, drinks, soaps, ointments, perfumes and cosmetics (William-Shurtleff, 2021). A vine of vanilla can bear up to 80 beans. One vanilla bean measuring 10-12 centimetres long can fetch KES. 200 and a hectare can accommodate over 200 vines (Hernandez & Guenette, 2018). The vanilla venture can act as a source of farmer income and earn the country foreign exchange.

According to Shabbir (2018), the vanilla crop is well grown in other countries such as Uganda, Comoros and Madagascar on both small and large scales. Most parts of Kenya have favourable climatic conditions for cultivating the vanilla crop, and both local and export markets are ready upon its maturity. The major markets for vanilla beans include European countries and the United States (Shabbir, 2018).

Vanilla planifolia Andrews (syn. *V. fragrans*), *Vanilla tahitensis* and *Vanilla pompona* Scheide are commercially cultivated species for producing natural vanilla flavour (Briddon *et al.,* 2019). The natural vanilla flavour is made up of a large number of aromatic compounds with sweet fragrances (Moller & Gallage, 2017). Vanillin flavour is found to have

antimutagenic, anticarcinogenic and antimicrobial properties (Swamy, 2020). Antimutagenic property has the ability to reduce chromosomal damage caused by X-ray and ultraviolet (UV) light (Cassar *et al.,* 2020). Anticarcinogenic property affects a family of DNA-PK inhibitors (Pollard & Curtin, 2018), while antimicrobial property acts against the yeasts (Baspinar *et al.,* 2018).

Limited knowledge of genetic diversity is the major bottleneck in wider breeding programmes, hybridization and dissemination of vanilla crops in developing countries (Cowling, 2020) like Kenya. According to Oyugi (2018), vanilla is one of the orphaned crops in Kenya, and limited information on available vanilla accessions found in Kenya is documented. Studies of genetic variation in crop species are an essential tool for germplasm conservation and crop improvement (Nunes, 2021). Assessing the extent of diversity in vanilla accessions found in Kenya was necessary. The information forms the base for future vanilla crop improvement programmes. The objective of the present study was to determine the genetic diversity among vanilla accessions from five counties of Kenya using simple sequence repeat markers.

5.2 Materials and methods

5.2.1 Experimental site

A total of 76 vanilla accessions were collected *in-situ* from five different Agro-ecological zones in Kenya; Bungoma county (Latitude 0° 45' 16.799" N and Longitude 34° 30' 09.270" E), Busia county (Latitude 0° 47' 11.113" N and Longitude 34° 24' 26.339" E), Kwale county (Latitude 4° 09' 58.223" S and Longitude 39° 34' 18.127" E), Kilifi county (Latitude 4°16' 53" S and Longitude 39° 44' 41.306" E) and Mombasa county (Latitude 4° 02' 12.7608" S and Longitude 39° 40' 10.4556" E) (Figure 15). Genotyping was done in the Marker Assisted Breeding Laboratory at KALRO-FCRI, Njoro, in Nakuru county, Kenya.

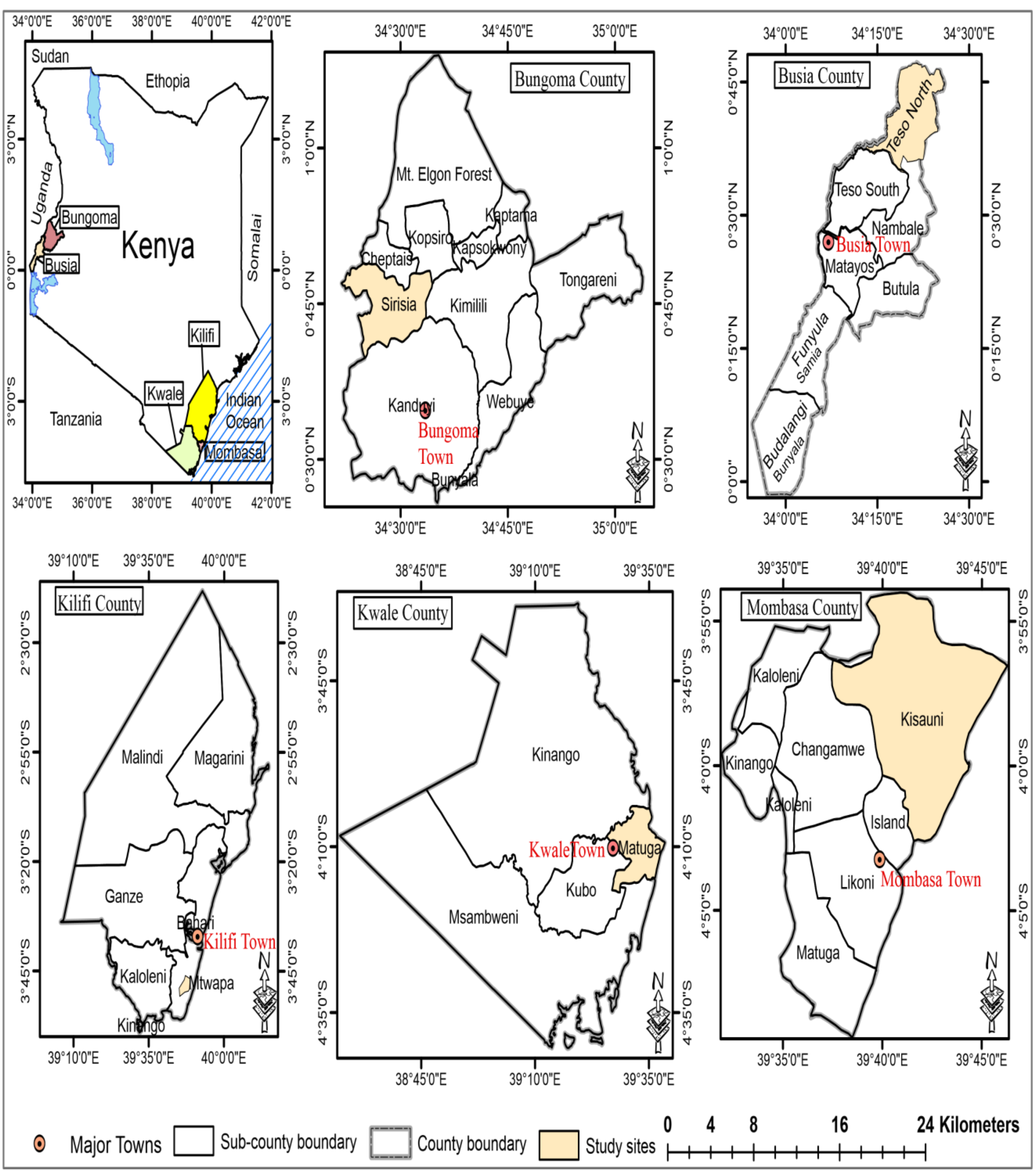


Figure 15: Map of the experimental sites

5.2.2 Collection of plant accessions

The purposive network sampling method was done *in-situ* due to fewer vanilla farmers in Kenya. Global Positioning System (GPS) data were taken in each study county. Young vanilla leaves were sampled based on the morphological distinction of the crops and stored in labelled falcon tubes containing silica gel (Xtrack) for DNA extraction. Plants with similar features growing in the ecologically distinct county were assumed to be different; such plants were sampled and used for analysis. All plant materials sampled were coded with slight modifications, as described by Oyoo *et al.* (2015).

Coding modifications, according to the description of Oyoo *et al.* (2015), involved reflection of the county and the number of accessions collected. Example; 'BGM 1, BGM 2, BGM 3,………BGM 8'.

5.2.3 Total Nucleic Acid Extraction

Total DNA was extracted from dried young plant leaf samples using the CTAB protocol, according to Ibrahim (2011), with slight modifications. Modifications to Ibrahim (2011) involved the introduction of a preliminary cleaning stage to remove phytates using TAE buffer and increased centrifugation time for initial stages from 10000rpm for 5 minutes to 10 minutes, which ensured that cell debris and the proteins were well decanted and hence minimized contamination. Final centrifugation time was reduced in the last precipitation from 14000rpm for 10 minutes to 5 minutes and washing stages from 80% to 70% ethanol to avoid pelleting carryover impurities. Also, precipitation time was increased from the reported 20 minutes to 18 hours of Ibrahim (2011). This allowed ample time for DNA to precipitate and increased the concentration of DNA recovery.

5.2.4 DNA Quantification

The purity and concentration of DNA samples were determined using Nanodrop-spectrophotometry and Agarose gel electrophoresis. DNA samples were run in a 1% agarose gel containing Ethidium bromide staining dye at a voltage of 100 Volts and a current of 400mA for 30 minutes and visualized on a UV Trans illuminator.

5.2.5 Selection of SSR markers and PCR genotyping

A total of 14 sets of SSR markers (Table 11), selected from earlier publications according to Bory *et al*. (2008), were amplified using the PCR process. The SSR markers were selected based on; coverage and distribution in all the linkage groups, High PIC values (>0.6)

and the maximum number of alleles detected. The primers for SSR were synthesized by Inqaba Biotech, South Africa.

Polymerase chain reaction (PCR) was done in a 96 universal gradient, 2720 thermal cycler (Applied Biosystems) in 20µl final volume containing DNA template (20ng) 2.0µl, Primer Forward (10µmol/L) 0.5µl, primer Reverse (10µmol/L) 0.5µl, 10 x PCR buffer 2.0µl, 10mM dNTPs (2.5mM each) 1.6µl, $MgCl_2$ (2.5mM) 1.2µl, Taq DNA polymerase (HiMedia) (5 U/µl) 0.25 U (0.05µl) and dd H_2O 12.15µl. The amplification conditions for the PCR profile were: initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 seconds of denaturation, specific annealing temperature for each SSR primer for 1 min, extension at 72°C for 2 min and final extension at 72°C for 10 min and infinite time at 4°C for Storage. The PCR amplicons were mixed with 5µl bromophenol blue DNA loading dye and run in a 2% agarose gel containing 3µl ethidium bromide staining dye in a 1x Sodium Borate (SB) buffer at the voltage of 80 Volts and a current of 400mA for 1 hour and visualized in a UV Trans illuminator.

Table 11: List of primer sequences of 14 microsatellite loci in the vanilla orchid (F-forward primer and R - reverse primer)

SSR locus	Primer sequences	size (bp)	Annealing T(°C)
mVplCIR002	F-TGGATGTGCATTTGTG	222	53– 60
	R-CGCATTCATTCACTTGT		
mVplCIR003	F-TATAGATGCACACGAGC	349	53 – 60
	R- TCACATCCCTACATGC		
mVplCIR005	F-TTTGCTTGAACGTATGTC	259	53 – 60
	R- GCAAACATAGAAATGCAC		
mVplCIR010	F-GCACATAAATACCTTACACC	346	53 – 60
	R- GTTCACGTCAGTGTGCT		
mVplCIR015	F-AGTGTCTTTGTGTGCCT	280	53 – 60
	R-TAGATAGTAAACCCATACTCAC		
mVplCIR016	F- TATGTGTGAGAGGGTGC	320	53 – 60
	R- CAATTAGTCACATCCATAAAC		
mVplCIR019	F- AAGTGCCCAATCTATC	222	53 – 60
	R-TGGATTCACCATGAC		
mVplCIR022	F-CAAAACACAAGGAAATGC	197	53 – 60
	R-TGCAAGCCCACAAGT		
mVplCIR025	F-GTGTAGCGGTTCATACAA	231	53 – 60
	R-CATTCATGGAAGTGGAG		
mVplCIR026	F-GCACATACATGCTTATTG	223	53 – 60
	R-CATGTTCTTATTTGAGTGG		
mVplCIR028	F-AACATGCACAAGAAAG	190	53 – 60
	R-TTTATGCACCTTGTTAG		
mVplCIR031	F--ATTTCCTCCCTCACTGTA	346	53 – 60
	R-AATCTCAGGTGCTATTGG		
mVplCIR047	F-CATGCTTACATCTTTGTGTT	301	53 – 60
	R-TAATGGACATGCACACTC		
mVplCIR050	F-CTATGTGCGCTTTGG	224	53 – 60
	R-CACTCAAGAACATGCAAC		

Bory *et al*. (2008)

5.2.6 Scoring of the markers

Scoring of marker alleles of SSRs was done manually from the gel images. A simple numerical scoring method was used where one was used to represent the presence of the expected band while 0 was used to represent the absence of the band.

5.2.7 Phylogenetic analysis

The utility of markers was quantified in terms of the number of amplicons per primer, percent polymorphism and polymorphic information content (PIC). [The PIC values of individual primers were calculated based on the formula PIC = 2 × F (1- F), where F is the frequency of the bands].

Genetic variation at each locus was characterized in terms of the number of alleles, observed heterozygosity (HO), expected heterozygosity (HE), Shannon's diversity index (I), gene flow (Nm), and gene differentiation coefficient (Gst) using the genetic analysis packages POPGENE Version 1.32 (Victoria-Garcia & Eugenia-Barrandeguy, 2021). Gene diversity (GD) and the polymorphic information content (PIC) were measured by calculating the shared allele frequencies (Martinez, 2019) using PowerMarker 3.25 (Sunseri & Mercati, 2021).

Nei's gene diversity for the whole sample (Ht) was calculated to estimate the genetic diversity across the counties (Onder & Mercan, 2020), while genetic diversity within the populations (Hs) was used to determine diversity (Linlokken, 2018) within counties. The proportion of genetic diversity among populations (Gst =1-(HS/Ht) was also calculated for each primer combination and all the primers. These estimates were partitioned into respective county population groups using the POPGENE software package Version 1.32.

Phylogenetic analysis was carried out to estimate evolutionary relationships among the vanilla accessions. All phylogenetic analyses were done in DARwin 6.0.8 using binary data from the gel image marker scores. Single data dissimilarity was calculated and factorial coordinates calculated from the resulting dissimilarity data, used to determine the segregation of individual samples. The unrooted phylogenetic tree was constructed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) agglomerative hierarchical clustering method (Hibbert, 2017) from distance matrices based on genetic distances.

5.3 Results and discussion

5.3.1 Quality and Quantity of DNA

The quality and quantity of the isolated DNA were suitable for PCR. Sample DNA concentrations ranged from 62.5 to 3421.7ng/µl, while sample purity was between 1.71 and 2.09 absorbance. All the primers produced the expected product sizes. Some samples showed no bands (indicating the absence of the target loci), one band (scored as homozygous at the amplified loci), and others multiple bands (scored as heterozygous at the amplified loci). Representative gel images are shown in Figure 16.

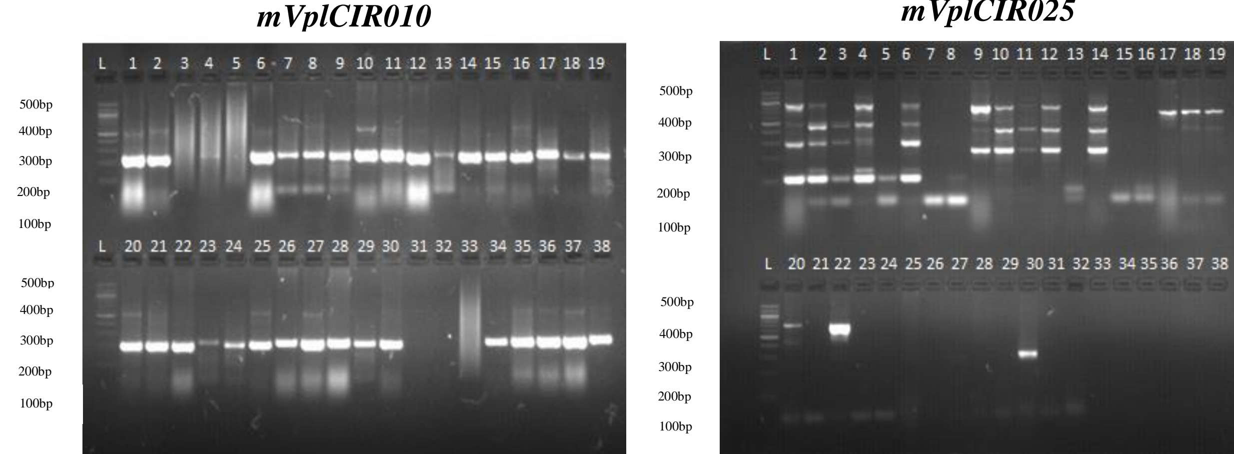


Figure 16: Gel images of L - Ladder (100bp), single and multiple bands of primers (mVplCIR010, mVplCIR025 respective), (1 – 8 Bungoma), (9 – 25 Busia), (26 – 38 Mombasa) samples

5.3.2 Polymorphism of the markers

Fourteen SSR primers used in the study resulted in amplified fragments ranging in size from 100 to 500 bp (Figure 16). The utility of markers was quantified in terms of the observed number of alleles, effective alleles, gene diversity and shannon's information index (Aravanopoulos, 2018). The number of amplicons per primer ranged from 1 to 4, with only one marker giving one allele (mVplCIR031). The total effective alleles were 22.76, and the number of alleles per primer ranged from 1.00 to 1.99, with a mean of 1.63. The observed number of alleles was 27.00, while the number per primer ranged from 1 to 2, with a mean of 1.93. Based on the results, the effective number of alleles was lower than the observed number of alleles (Table12), showing a strong geographic differentiation in the population, informing that the observed genetic diversity was mainly due to geography rather than the original evolution.

Gene diversity indices ranged from 0 to 0.50, while the mean shannon information index was 0.5, suggesting that the vanilla accessions studied had less genetic diversity. This finding shows that vanilla generally has a narrow genetic background, as supported by the findings in India (Ravindran, 2017), Mexico (Havkin-Frenkel & Belanger, 2018), and Reunion Island (Pearlstine, 2022). These authors worked in different regions with different technologies and observed the results within the same range. Polymorphic information content of the markers ranged from 0 (mVplCIR031) to 0.375 (mVplCIR025). The primer with 0 PIC value was monomorphic, showing that it could not differentiate accessions, while the primer with the highest PIC had the highest resolution. This is an indication that the markers used revealed little polymorphisms between the accessions.

A total of 27 (96.43%) alleles were identified, and out of the 14 markers used in this study, 13 were polymorphic, while one (mVplCIR031) was monomorphic. This indicated that alleles with high frequencies contributed more to the effective number of alleles. This frequency is higher than that reported by Gigant (2012), who observed 57.83% of alleles using 19 similar primers on Comoros Island. This may be due to the large sampling areas used in the present study, and perhaps the study used more effective sample collection strategies. Besse *et al.* (2004) identified 76.3% of alleles using RAPD markers in Mexico. This shows that SSR markers have better resolution in distinguishing vanilla accessions than RAPDs. However, the polymorphic alleles identified in this study are significantly lower than Hu *et al*. (2019), who used SNP markers. The SNP markers are generally more effective than SSR markers. Primer mVplCIR031 was the most frequently observed allele 1.0, while mVplCIR025 was the least frequently observed marker because it had a 0.5 chance of being observed, as shown in Table 12. This indicates that marker mVplCIR031 showed the least variation among the accessions studied, while marker mVplCIR025 was the most variable.

Table 12: Summary statistics of genetic diversity indices of vanilla accessions studied in five counties in Kenya

Locus	Sample Size	na*	ne*	h*	I*	PIC	M
mVplCIR002	76	2.000	1.877	0.467	0.660	0.364	0.605
mVplCIR003	76	2.000	1.084	0.077	0.169	0.135	0.921
mVplCIR005	76	2.000	1.980	0.495	0.688	0.333	0.697
mVplCIR010	76	2.000	1.685	0.406	0.596	0.375	0.513
mVplCIR015	76	2.000	1.857	0.462	0.654	0.366	0.592
mVplCIR016	76	2.000	1.400	0.286	0.460	0.339	0.684
mVplCIR019	76	2.000	1.929	0.482	0.675	0.353	0.645
mVplCIR022	76	2.000	1.944	0.486	0.679	0.349	0.658
mVplCIR025	76	2.000	1.707	0.414	0.605	0.375	0.500
mVplCIR026	76	2.000	1.442	0.306	0.485	0.349	0.658
mVplCIR028	76	2.000	1.995	0.499	0.692	0.320	0.724
mVplCIR031	76	1.000	1.000	0.000	0.000	0.000	1.000
mVplCIR047	76	2.000	1.178	0.151	0.284	0.231	0.842
mVplCIR050	76	2.000	1.685	0.406	0.596	0.375	0.513
Mean	**76**	**1.929**	**1.626**	**0.353**	**0.517**	**0.350**	**0.638**

* **na** = Observed number of alleles, * **ne** = Effective number of alleles (Aravanopoulos, 2018), * **h** = gene diversity (Onder & Mercan, 2020), * **I** = Shannon's Information index (Caliskan, 2012), **PIC** = Polymorphic Information content, **m** = Major allele frequency

5.3.3 Gene diversity

The average estimated haplotype diversity in the entire population (Ht= 0.37) was higher than the weighted average of estimated haplotype diversities in the subpopulations (Hs=0.11), and the differentiation among populations was high (Gst=0.71). The highest Ht was observed at locus mVplCIR022 (Ht=0.50), indicating that this was the most polymorphic and informative locus within the population. The lowest haplotype diversity in the population was observed at locus mVplCIR031 (Ht=0.00), showing that this locus was monomorphic and no diversity information could be deduced from it. All the other loci showed varied levels of genetic diversity ranging from 0.11 to 0.5 (Table 13).

There was low genetic diversity within subpopulations (Hs), ranging from 0.00 to 0.19. The highest diversity within subpopulations was at locus mVplCIR019 (Hs = 0.19),

indicating that this locus had the highest resolution in segregating vanilla accessions from different counties and the lowest at locus mVplCIR031 (Hs= 0.00) showing that this locus was not able to differentiate vanilla accessions based on the counties of origin (Table 13).

The present study's mean among the populations was high (Gst = 0.71). The highest Gst was recorded at locus mVplCIR002 (Gst = 0.97), while no Gst was calculated for locus mVplCIR031 because the primer was monomorphic and therefore had zero Hs and Ht values. This study's mean estimated gene flow was high (Nm = 0.21). The highest Nm was recorded at locus mVplCIR003 (Nm = 2.56), while no gene flow was estimated at locus mVplCIR031 because of the nil Gst value (Table 13).

In autogamous plant species, efficient gene dispersion is ensured by seeds, while pollen is the source of gene diversity in allogamous species (Ma & Ellison, 2020). As such, populations of autogamous species are more strongly differentiated, but less variable than the populations of allogamous species, which are less differentiated but more variable (Ma & Ellison, 2020). In the study, the calculated GST value in Kenyan *Vanilla spp.* (0.71) was higher than that reported by Gigant (2012) in Guadalupe for autogamous *Vanilla spp*. such as *V. humblotii* (Gst = 0.520), *V. barbellata* (Gst = 0.558) and *V. claviculata* (Gst = 0.623). The strong differentiation but less variability is characteristic of self-fertilized plants as it is often in vanilla, exhibiting genetic variability among populations rather than within, with high Gst and low Hs. (Ma & Ellison, 2020).

Therefore, the results confirmed autogamy as the major mating system in *Vanilla spp.* in Kenya. The high estimated gene flow (Nm = 0.21) observed in this study can be explained by inbreeding. This was demonstrated by Campagne *et al.* (2012) in *Vanilla Mexicana* populations using reproductive biology experiments. This shows that vanilla accessions in Kenya have low genetic diversity due to constant inbreeding and informs the need to introduce new accessions as sources of genetic variation for breeding and hybridization purposes.

Table 13: Nei's gene diversity indices for subdividing the populations

Locus	Ht	Hs	Gst	Nm*
mVplCIR002	0.477	0.015	0.969	0.016
mVplCIR003	0.105	0.087	0.164	2.547
mVplCIR005	0.470	0.042	0.912	0.049
mVplCIR010	0.424	0.100	0.765	0.154
mVplCIR015	0.491	0.187	0.620	0.307
mVplCIR016	0.303	0.059	0.806	0.121
mVplCIR019	0.480	0.189	0.607	0.324
mVplCIR022	0.500	0.147	0.705	0.209
mVplCIR025	0.440	0.200	0.545	0.417
mVplCIR026	0.361	0.089	0.753	0.164
mVplCIR028	0.461	0.087	0.810	0.117
mVplCIR031	0.000	0.000	***	***
mVplCIR047	0.177	0.140	0.209	1.887
mVplCIR050	0.436	0.156	0.642	0.279
Mean	**0.366**	**0.107**	**0.708**	**0.207**
St. Dev	**0.026**	**0.004**	-	-

Ht = whole sample, **Hs** = within populations and **Gst** = degree of differentiation among the populations and **Nm*** = estimate of gene flow from Gst

5.3.4 Genetic of vanilla among distance counties

The highest genetic distance and lowest genetic similarity were recorded between Kwale and Kilifi counties (0.78, 0.46), while the lowest genetic distance and highest genetic similarity were between Busia and Bungoma (0.07, 0.94). Vanilla accessions in Busia and Bungoma showed 94% identity showing that they are very close genetically and could have shared a common source since the counties border each other. In comparison, accessions collected from Kilifi showed the least similarity to those from Kwale at 46% identity (Table 14). This means that accessions from Kilifi are genetically different and could not have shared the source with other counties.

Table 14: Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

County	Bungoma	Busia	Mombasa	Kwale	Kilifi
Bungoma	***	0.937	0.501	0.464	0.775
Busia	0.065	***	0.510	0.576	0.712
Mombasa	0.691	0.673	***	0.751	0.673
Kwale	0.769	0.552	0.286	***	0.459
Kilifi	0.254	0.340	0.397	0.780	***

Analysis of molecular variance (AMOVA) was used to partition among and within the genetic variation of the vanilla accessions. A total of (0.947) 94.7% of the variation was observed among the accessions collected within the counties. In comparison, the variation revealed within populations was (0.053) 5.3% (Table 15), indicating that *Vanilla spp.* grown in Kenya have very little diversity, and some variations observed may be due to microclimatic conditions.

Table 15: Analysis of molecular variance (AMOVA) for the 76 vanilla accessions from 5 select counties of Kenya

Source	DF	SS	MS	E. Var.	Var. %
Among groups	4	17.196	4.299	0.708	94.7%
Within groups	71	0.962	0.014	0.107	5.3%
Total	75	18.158		0.815	100%

DF= Degree of freedom, **SS** = sum of squares, **MS**= expected mean squares, **E. Var.** = Estimated variance, **Var. %**= Variation percentage

5.3.5 Factorial analysis

Factor analysis was used to describe variability among the vanilla accessions and group them into clusters. The accessions were segregated into three distinct groups, **A**, **B** and **C,** according to their counties of origin (Bungoma and Busia **'A'**, Kwale and Mombasa **'B'** and Kilifi **'C')**. Accessions from Busia and Bungoma counties were aggregated together **(A)** into indistinct group showing that vanilla species in these areas are closely genetically related. Cluster **B** was the most inclusive, containing accessions from Mombasa and Kwale, indicating that vanilla accessions in these counties have similar genetic makeups, perhaps due to the same sources**.** However, some accessions from Busia county were closely related to accessions from Mombasa and Kwale. Moreover, they were found at the intersection of the two clusters, **A** and **B (**Figure 17). This may be attributed to the fact that farmers or

researchers may have transported the accessions to these counties. Accessions from Kilifi county were segregated alone, showing that they were genetically distinct from other counties, as shown in Figure 17.

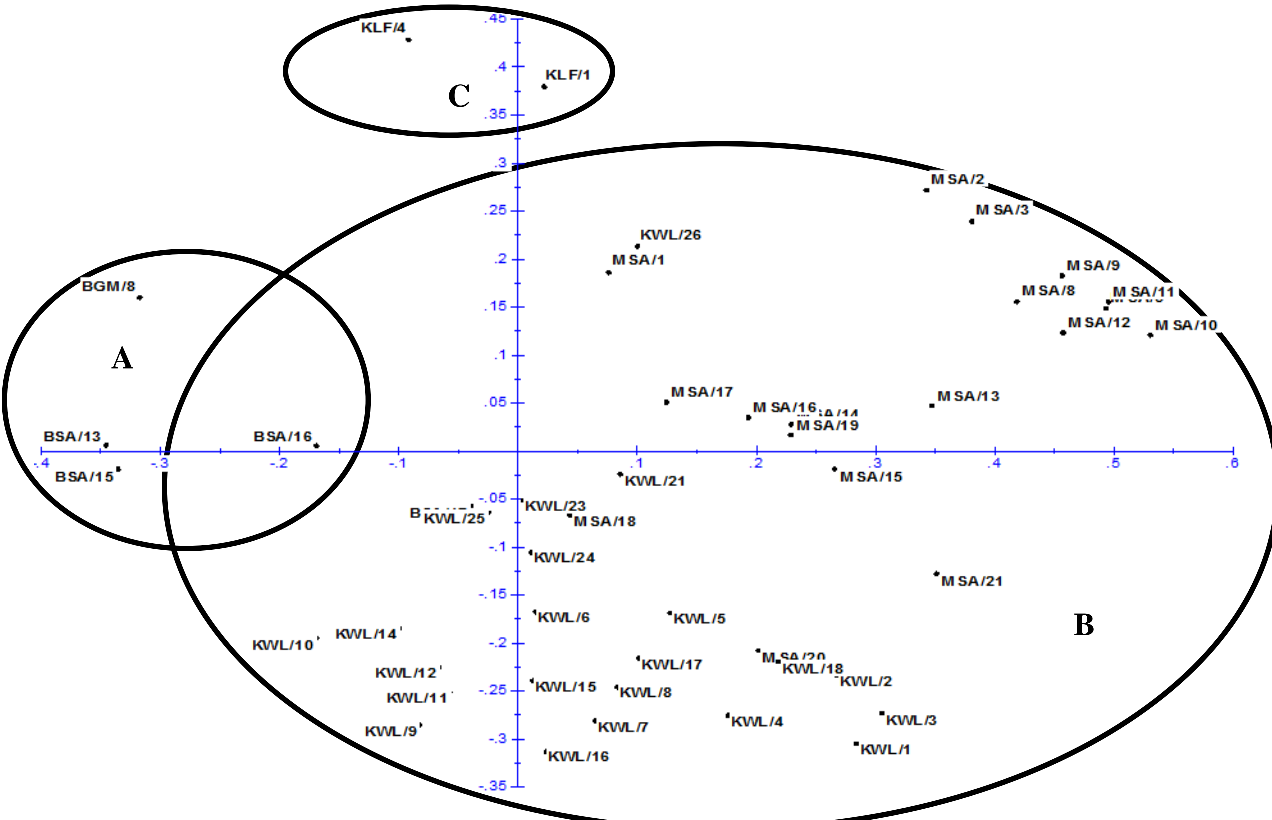


Figure 17: Factorial plot showing variability among the vanilla accessions, BGM-Bungoma, BSA-Busia, MSA- Mombasa, KWL-Kwale, KLF-Kilifi

5.3.6 Phylogenetic Trees

The primers clustered the samples into three main clusters (**A, B** and **C**) according to the county of origin and six sub-clusters (**A1, A2, B1, B2, B3** and **C1**), as shown in Figure 18. Most accessions within a sub-cluster were probably duplicates, meaning they were genetically identical. The genetic closeness of vanilla accessions may result from asexual reproduction, the most common mode of propagation and geographical location closeness. This observation is supported by Mulpuri *et al.* (2019). They observed that plant species' clonal propagation limits the crop's genetic variability, especially in countries where the crop was introduced. Low genetic diversity may also occur because of successfully establishing a few well-adapted genotypes which are rapidly propagated despite multiple introductions (Selechnik *et al.,* 2019).

Cluster **A** comprised accessions from Bungoma (**A1**) and Busia (**A2**) counties. These were the accessions with the least diversity, averaging 99.6% similarity index. Vanilla accessions from Mombasa, Kwale and a few from Busia (**B1, B2** and **B3**) clustered together in

cluster **B**. They showed the highest level of diversity among the accessions ranging from 98.96% to 100%, while vanilla accessions from Kilifi clustered on their own as **C** with a 100% similarity index. The Jaccard's similarity coefficient ranged from 0.083 to 1.00, with an average of 0.54 among all the 76 accessions used. The genetic relationship among the accessions was presented in the dendrogram constructed from the gel marker.

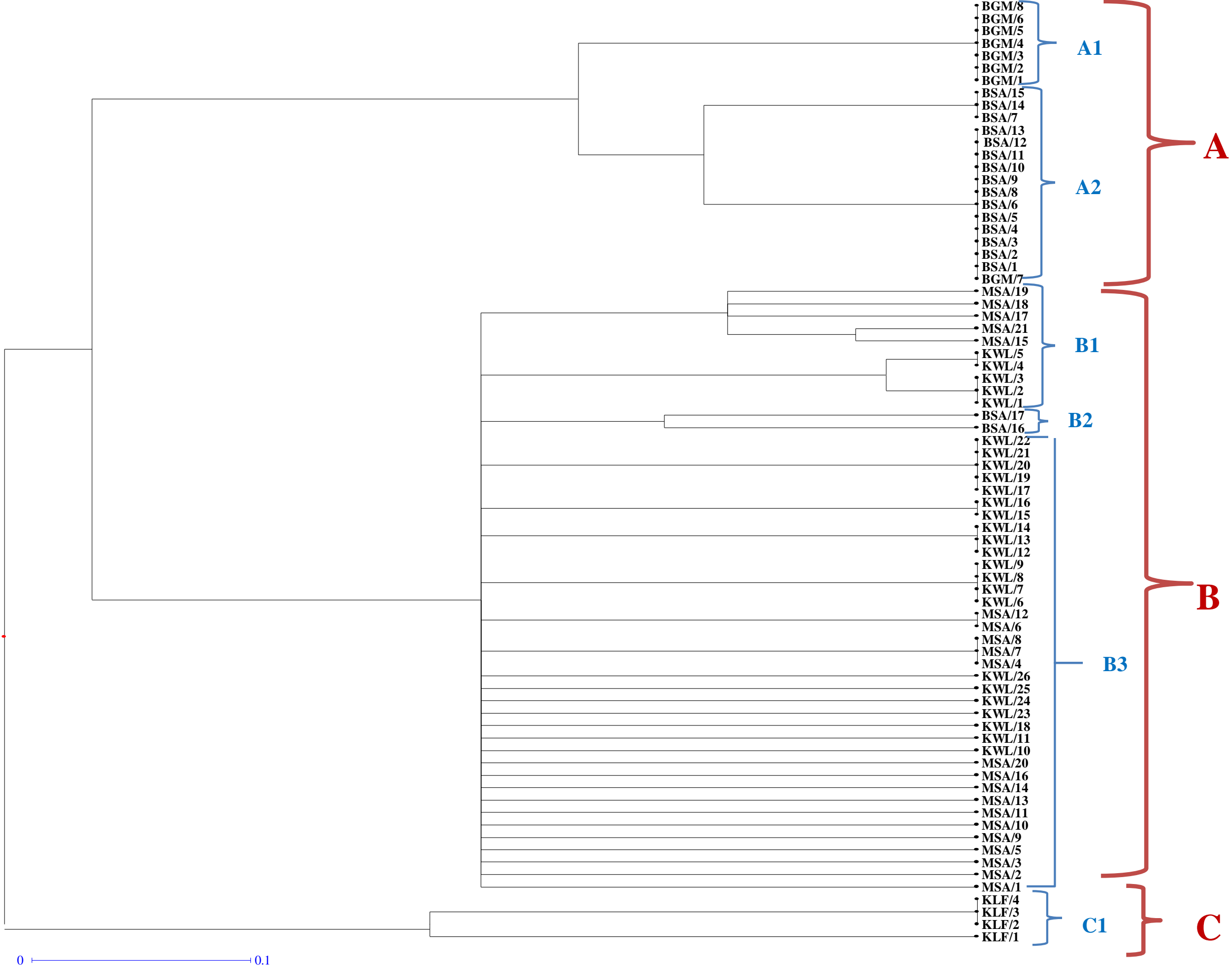


Figure 18: A dendrogram showing the relationship among the vanilla accessions BGM-Bungoma, BSA-Busia, MSA- Mombasa, KWL-Kwale, KLF-Kilifi

5.4 Conclusion and recommendation

Limited information has been documented on vanilla crops in Kenya. The use of microsatellites to characterize vanilla species grown in Kenya established that accessions found in Kenya have low genetic diversity, and the highest polymorphic information content recorded was 0.375, which was less than the expected value of >0.6. The SSR markers used were less polymorphism, hence not suitable for characterizing vanilla accession found in Kenya.

The molecular study lays the basis for introducing more markers and germplasm to establish variations in vanilla accessions found in Kenya.

CHAPTER SIX

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 General discussion

Plant biotechnology is essential for crop regeneration and improving food security worldwide. The vanilla crop has economical value in food, beverages, cosmetics, perfumery and medicinal industries. Vanillin flavour contains various valuable compounds, such as antimutagen, that can reduce chromosomal damage caused by x-ray and ultra-violet light (Uysal *et al.,* 2019). Anticarcinogenic property affects a family of DNA-PK inhibitors (Pollard & Curtin, 2018), while antimicrobial property acts against the yeasts (Molina *et al.,* 2020).

The study evaluated the effect of different concentration levels of kinetin and indole-3-acetic acid on nodal stem segments of vanilla (*Vanilla spp.*) species from Busia county in Kenya and assessed the extent of diversity in vanilla species found in Kenya. *In-vitro* regeneration, morphological characterization and genotyping experiments were done in the study.

Generally, different concentration levels of phytohormone induce bud break and root initiation at varying degrees in vanilla plants. Shoot induction and root initiation are influenced by the concentration level of phytohormones and the type of phytohormone used. The present study evaluated the effects of different concentration levels of kinetin on vanilla shooting and indole-3-acetic acid on vanilla rooting.

The study found that among the phytohormone levels evaluated (0.0, 0.4, 0.8, 1.2, 1.6, 2.0 mg L^{-1}), kinetin at 1.2 mg L^{-1}gave the highest number of shoots per plant and shoot length, while IAA at 2.0 mg L^{-1}gave the most increased root length (chapter 3). Li *et al.* (2022) noted that cytokinins are shoot promoting agents in many *in-vitro*-cultured plant organs. Ahmad (2018) gave a related report to the present results of using cytokinins, including kinetin that induced shoot break and multiplication. Based on the current results, a higher degree of performance depends on the optimum level of kinetin concentration. A higher concentration level of kinetin beyond its optimum level was reported to induce necrosis and reduction in shoot formation (Endres, 2017). According to Foo *et al.* (2020), the optimum concentration of cytokinin is species dependent.

The present study shows that IAA at 2.0 mg L^{-1}gave the highest root length compared to other concentration levels. The best results of root length recorded in this study contrast with

Izzati *et al.* (2013). They reported that root induction in *Vanilla planifolia* was strongly stimulated by a growth regulator-free MS medium as compared to that MS media with IBA and NAA. According to Izzati *et al.* (2013), an auxin-free medium gave a higher number of roots, percentage of rooting and root length. This may be due to the less effect of NAA as compared to the present study that involved the use of IAA on vanilla crops.

Determining variation in crop species is an essential tool for germplasm conservation and crop improvement (Nunes, 2021). The studies assessed vanilla species found in Kenya using morphology and DNA markers. The current morphological markers study revealed that vanilla accessions found in Kenya are not significantly different though cluster analysis grouped accessions into two clusters and five sub-clusters. Based on the results, vanilla accessions from different counties were morphologically similar (chapter 4). This may result from the aspects of environmental factors and accessions being transported from one county to another. According to Bai (2017), phenotypic traits are influenced by environment. Morphological markers failed to cluster accessions into respective counties of origin, hence were supplemented with DNA markers.

Genotyping was done using 14 SSRs markers to determine the level of diversity in vanilla accessions found in Kenya (chapter 5). According to Farsangi *et al.* (2018), the utility of the markers was quantified based on the number of amplicons per primer, percent polymorphism, and polymorphic information content. Based on the results, the recorded polymorphic information content (PIC) value was 0.375. The results revealed that vanilla accessions found in Kenya have low genetic diversity.

6.2 Conclusions

i. MS media supplemented with 1.2 mg L^{-1}concentration levels of kinetin had more effects on shoot induction, the number of shoots per plant, number of nodes and shoot length. MS media containing indole-3-acetic acids at 2.0 mg L^{-1}positively affected the elongation of roots on generated shoots.
ii. Geographical differences had a highly significant effect on phenotypic traits, both qualitative and quantitative. Cluster analysis based on morphological traits failed to group vanilla accessions into respective counties of origin.
iii. The present study established that accessions found in Kenya have low genetic variation. The highest polymorphic information content value recorded was 0.375, the value was lower than the expected value of >0.6.

6.3 Recommendations

i. Kinetin at 1.2 mg $L^{-1}$and Indole-3-acetic acids at 2.0 mg $L^{-1}$are considered the best for *in-vitro* mass multiplication of vanilla crops. This *in-vitro* regeneration protocol can be applied in various research institutions to produce vanilla crops on both small and large scale in Kenya and worldwide.

ii. Morphological markers grouped accessions into different clusters showing their importance in selecting accessions for crop improvement, but its performance was not efficient. A morphological study should be complemented by genetic diversity studies using molecular markers.

iii. The molecular study lays the basis for introducing more DNA markers and germplasm to establish diversity in vanilla accessions found in Kenya.

6.4 Areas for further research

i. Further studies should consider other phytohormones 'cytokinins and auxins' on vanilla accessions found in Kenya.

ii. Further research should be done using other molecular markers, which are more polymorphism on vanilla accessions found in Kenya.

REFERENCES

Aarti, P., Pratibha S., & Shagufta, K. (2018). Standardization of protocol for *in-vitro* micropropagation of *Phyllanthus niruri*: An important medicinal plant. *Pharmaceutical and Biosciences Journal*, *6*(4), 42-47.

Abdin, M. Z., Kiran, U., Kamaluddin, & Ali, A. (2017). *Plant biotechnology: Principles and applications*. Springer Singapore.

Abu-Romman, S. M., Al-Hadid, K. A., & Arabiyyat, A. R. (2015). Kinetin is the most effective cytokinin on shoot multiplication from cucumber. *Journal of Agricultural Science*, *7*(10), 159. http://dx.doi.org/10.5539/jas.v7n10p159

Adawiyah, D. R., Reri, P. Y. D., & Lioe, H. N. (2022). *The effect of bean size and curing process on aroma profile and vanillin/glucovanillin content of Indonesian cured vanilla beans*: *Alcohol* (Vol. 100, p. 10). Scitepress, Indonesia.

Adugna, A. Y., Feyissa, T., & Tasew, F. S. (2020). Optimization of growth regulators on *in-vitro* propagation of *Moringa stenopetala* from shoot explants. *BMC Biotechnology*, *20*(1), 1-10.

Aeschbacher, H. U. (2018). Mutagenic and antimutagenic compounds in beverages. In *Mutagens in food: Detection and prevention* (pp. 181-191). CRC Press.

Ahmed, S., Duval, A., & Meyer, R. (2019). *Botany at the bar: The art and science of making bitters*. Shambhala Publications.

Alamgir, A. N. (2017). *Cultivation of herbal drugs, biotechnology and in-vitro production of secondary metabolites, high value medicinal plants, herbal wealth, and herbal trade: Progress in drug research* (pp. 379-452). Springer.

Algady, W., Weyell, E., Mateja, D., Garcia, A., Courtin, D., & Hollox, E. J. (2021). Genotyping complex structural variation at the malaria-associated human glycophorin locus using a PCR-based strategy. *Annals of Human Genetics*, *85*(1), 7-17.

Al-Khayri, J. M., Jain, S. M., & Johnson, D. V. (2020). *Advances in plant breeding strategies: Nut and beverage crops* (Vol. 4). Springer Nature.

Al-Khayri, J., Jain, S. M., & Johnson, D. V. (2018). *Advances in plant breeding strategies: Fruits* (pp. 727-771). Springer.

Arafah, D. L., Hernawati, D., & Nuryadin, E. (2021). The effect hormone BAP (6-benzyl amino purine) on the growth of potato axillary shoots (*Solanum Tuberosum* L.) *in - vitro. Journal Biology Tropic*, *21*(3), 641-647.

Aravanopoulos, F. A. (2018). *Genetics and genomics of forest trees*. MDPI.

Arif, Y., Singh, P., Bajguz, A., & Hayat, S. (2021). *Phytocannabinoids biosynthesis in angiosperms, fungi, and liverworts and their versatile role: Plants* (Vol. 10, No. 7, p. 1307). MDPI.

Arifuzzaman, M., Sultana, S., Hossain, M. S., Saifullah, S., Al-Naser, R., Alim, S., & Hasan, M. J. (2016). Paraphernalia of growth regulators during *in-vitro* micro-propagation of grapevine (*Vitis vinifera* L.) from shoot tips and nodal segments. *Scholars Journal of Agriculture and Veterinary Sciences*, *3*(4), 326-331.

Askun, T. (Ed.). (2018). *Fusarium: plant diseases, pathogen diversity, genetic diversity, resistance and molecular markers*. BoD–Books on Demand. https://doi.org/10.5772/intechopen.76507

Ayemele, A., Muafor, F., & Levang, P. (2017). Indigenous management of palm weevil grubs (*Rhynchophorus phoenicis*) for rural livelihoods in Cameroon. *Journal of Insects as Food and Feed*, *3*(1), 43-50.

Bahar, E., Yusoff, A. M., & Rasyad, A. (2018). *Effects of ethylene on plant height, leaf width, leaf length and number of productive branches in chili* (*Capsicum Annuum* L.) *under enviromental and climatical conditions of Rokan Hulu Riau Indonesia*: In *AIP conference proceedings* (Vol. 2030, No. 1, p. 20300). AIP Publishing LLC.

Bai, S. N. (2017). Reconsideration of plant morphological traits. From *A structure-based perspective to a function-based evolutionary perspective: Frontiers in plant science* (Vol. 8, p. 345). Frontiers.

Baspinar, Y., Kotmakci, M., & Ozturk, I. (2018). Antimicrobial activity of phytosphingosine nano-emulsions against bacteria and yeasts. *Celal Bayar University Journal of Science*, *14*(2), 223-228.

Bernauer, S., Schopf, M., Khinast, J., & Hardiman, T. (2021). *Scale-up of aerated industrial multistage rushton impeller bioreactors with complex rheology.* Authorea Preprints. https://doi.org/10.22541/au.162241730.02586689/v1

Beser, N., Mutafcılar, Z. Ç., & Hasancebi, S. (2021). *Diversity analysis of the rice cultivars (Oryza sativa L.) by utilizing SSRs rice diversity by SSRs*: *Journal of food processing and preservation* (Vol. 45, No. 2, p. 15232). Wiley Online Library.

Besse, P., DaSilva, D., Bory, S., Duval, M. F., Grisoni, M., & Lebellec, F. (2004). RAPD genetic diversity in cultivated vanilla: *Vanilla planifolia* and relationships with *Vanilla tahitensis* and *Vanilla pompona*. *Plant Science, 167*(2), 379–385.

Biniari, K., Bouza, D., Daskalakis, I., & Stavrakaki, M. (2018). *The effect that indolebutyric acid (IBA) and position of cane segment have on the rooting of cuttings from grape vine root stocks and from cabernet franc (Vitis vinifera L.) under conditions of a hydroponic culture system*: *Scientific horticulture* (Vol. 227, pp. 79-84). Elsevier.

Boone, C., Kaila, H., & Sahn, D. E. (2022). Posh spice or scary spice. *The impacts of Madagascar's vanilla boom on household well-being: The impacts of Madagascar's vanilla boom on household well-being.* Available at SSRN. https://doi.org/10.2139/ssrn.4085179

Bory, S., Lubinsky, P., Risterucci, A. M., Noyer, J. L., Grisoni, M., Duval, M. F., & Besse, P. (2008). Patterns of introduction and diversification of *Vanilla planifolia* (Orchidaceae) in Reunion Island (Indian Ocean). *American Journal of Botany*, *95*(7), 805-815.

Briddon, R. W., Hammond, J., Mandal, B., Sastry, K. S., & Scott, S. W. (2019). *Vanilla species* (*V. planifolia* and *V. tahitensis*): *Encyclopedia of plant viruses and viroids* (pp.2660-2664). Springer India. https://doi.org/10.1007/978-81-322-3912-3_982

Brunschwig, C., Collard, F., Lepers-Andrzejewski, S., & Raharivelomanana, P. (2017). *Tahitian vanilla* (Vanilla ×tahitensis): *A vanilla species with unique features: Active ingredients from aromatic and medicinal plants*. BoD-Books on Demand. https://doi.org/10.5772/66621

Buckingham, L. (2019). *Molecular diagnostics: Fundamentals, methods and clinical applications*. F.A. Davis.

Caliskan, M. (2012). *The molecular basis of plant genetic diversity*. BoD - Books on Demand.

Campagne, P., Smouse, P.E., Varouchas, G., Silvain, J.F., & Leru, B. (2012). Comparing the van O osterhout and C hybicki B urczyk methods of estimating null allele frequencies for inbred populations. *Molecular Ecology Resources*. *12*(6), 975–982.

Carbajal-Valenzuela, I. A., Munoz-Sanchez, A. H., Hernandez-Hernandez, J., Barona-Gomez, F., Truong, C., & Cibrian-Jaramillo, A. (2022). Microbial diversity in cultivated and feral vanilla (*Vanilla planifolia*) orchids affected by stem and rot disease. *Microbial Ecology*, *84*(3), 821-833.

Cassar, J. R., Ouyang, B., Krishnamurthy, K., & Demirci, A. (2020). Microbial decontamination of food by light-based technologies. *Ultraviolet (UV) light, pulsed*

UV light (PUV), and UV light-emitting diodes (UV-LED): *Food engineering series* (pp. 493-521). Springer.

Chambers, A. H. (2019). Vanilla (*Vanilla spp*.) breeding. *Advances in plant breeding strategies: Industrial and food crops* (Vol. 6, pp. 707-734). Springer.

Chanda, S. C., Mia, M. A., Sagar, A., & Golam-Sarwar, A. (2021). Stem anatomical descriptors of four *Sesbania scop*: Species and their systematic implication. *Bangladesh Journal of Plant Taxonomy*, *28*(2), 441-449.

Chase, M. W., Christenhusz, M. J., & Mirenda, T. (2017). *The book of orchids: A life-size guide to six hundred species from around the world*. University of Chicago Press.

Chauvin, N. D., Porto, G., & Mulangu, F. (2017). *Agricultural supply chains, growth and poverty in sub-Saharan Africa: Market structure, farm constraints and grass-root institutions*. Springer.

Chukwu, S. C., Rafii, M. Y., Ramlee, S. I., Ismail, S. I., Oladosu, Y., Okporie, E., & Jalloh, M. (2019). Marker-assisted selection and gene pyramiding for resistance to bacterial leaf blight disease of rice (*Oryza sativa* L.). *Biotechnology and Biotechnological Equipment*, *33*(1), 440-455.

Cizkova, A., Burg, P., Zatloukal, P., & Vaidova, M. (2021). Organic mulch materials improve soil moisture in vineyard. *Soil Science Annual*, *72*(2), 1-6.

Conybeare, J. A. (2017). *United States foreign economic policy and the international capital markets: the case of capital export countries, 1963-1974*. Routledge.

Cota, M. M., Rando, J. G., Snak, C., & De Queiroz, L. P. (2021). Tricky morphology: A new species of *Chamaecrista* (Leguminosae) shows strong morphological convergence among not closely related species found in similar habitat. *Systematic Botany*, *46*(4), 962-972.

Cowling, W. A. (2020). *Genetic diversity in narrow-leafed lupin breeding after the domestication bottleneck*: *The lupin genome* (pp. 1-17). Springer

Cyriac, A., Thomas, T., & Thomas, T. D. (2020). Mass multiplication of *Gymnema sylvestre* (Retz.) R. BR. ex Schult: Through *in-vitro* shoot organogenesis from callus. *Plant Tissue Culture and Biotechnology*, *30*(1), 27-32.

Daneshvar, M. H., & Hesami, M. (2018). *In-vitro* adventitious shoot regeneration through direct and indirect organogenesis from seedling-derived hypocotyl segments of *Ficus religiosa* L.: An important medicinal plant. *Horticultural Science*, *53*(1), 55-61.

De Vos, C. M. (2020). *Complementarity, catalysts, compliance: The international criminal court in Uganda, Kenya, and the Democratic Republic of* Congo (Vol. 147). Cambridge University Press.

De-Camargo, S. L., Carvalho-Filho, F. D. S., & Esposito, M. C. (2018). The genus *Peckia Robineau-Desvoidy* (Diptera: Sarcophagidae) in the Brazilian Amazon: A new species, new records, descriptions of female terminalia and key to species. *Zootaxa*, *4483*(1), 1-35.

Deng, Z. (2018). *Caladium: Ornamental crops* (pp. 273-299). Springer, Cham.

Dharman, A. K., & Anilkumar, M. (2021). Micropropagation through indirect organogenesis in *Solanum capsicoides* all and assessment of clonal fidelity using ISSR markers. *In-vitro Cellular and Developmental Biology Plant*, *57*(3), 481-492.

Dolce, N. R., Medina, R. D., Terada, G., González-Arnao, M. T., & Flachsland, E. A. (2020). In-vitro propagation and germplasm conservation of wild orchids from South America. *Orchid biology: Recent trends and challenges* (pp. 37-94). Springer.

Doussoh, A. M., Dangou, J. S., Ahanhanzo, C., Houedjissin, S. S., & Assogba, A. K. (2018). Assessment of the phenotypic diversity of sweet potato landraces (*Ipomoea batatas* L.) cultivated in Benin using morphological descriptors. *International Journal of Plant and Soil Science*, *24*(1), 1-13.

El-Esawi, M. A. (2019). Introductory chapter. *Assessment and conservation of genetic diversity in plant: Diversity in plant species characterization and conservation*. IntechOpen. https://doi.org/10.5772/intechopen.86060

Elisha, S., Heiner, J. S., & Nagelhout, J. J. (2022). *Nurse anesthesia - E-book*. Elsevier Health Sciences.

El-Shemy, H. (2017). *Active ingredients from aromatic and medicinal plants*. BoD – Books on Demand.

Endres, P. (2017). Quality of care and self-reported health: Effects beyond individual level factors. *SSRN Electronic Journal*. https://doi.org/10.2139/ssrn.3094071

Erady, V., Mascarenhas, R. J., & Satpati, A. K. (2020). *Highly efficient and selective quantification of vanillin in food, beverages and pharmaceuticals using surfactant modified carbon paste sensor*: *Sensors international* (Vol. 1, p. 100023). Elsevier.

Erawati, D. N., Wardati, I., Humaida, S., & Fisdiana, U. (2020). Micropropagation of vanilla (*Vanilla planifolia* Andrews) with modification of cytokinins. In *IOP conference*

series: Earth and environmental science (Vol. 411, No. 1, p. 12009). IOP Publishing.

Erawati, D. N., Wardati, I., Humaida, S., Mawadah, Y., Ikanafi'ah, A., & Ryana, W. M. (2021). Shoots multiplication of vanilla (*Vanilla planifolia*) with benzyl amino purine and kinetin modification. In *IOP conference series: Earth and environmental science* (Vol. 672, No. 1, p. 12007). IOP Publishing.

Faisal, M., & Ahmad, N. (2018). *Thidiazuron: From urea derivative to plant growth regulator*. Springer.

Faisal, M., & Alatar, A. A. (2019). *Synthetic seeds: Germplasm regeneration, preservation and prospects*. Springer Nature.

FAO. (2018). *Production data 2016*. Retrieved on 10th June, 2022 from fao.org: http://www.fao.org.

FAO. (2020). *Production data 2018*. Retrieved on 12th April, 2021 from fao.org: http://www.fao.org.

FAO. (2020). *Production data 2019*. Retrieved on 24th June, 2022 from fao.org: http://www.fao.org.

FAO. (2022). *Herbs and spice production data.* Retrieved on 7th July 2022 from fao.org:http://www.fao.org.

Farsangi, F. J., Thorat, A. S., & Devarumath, R. M. (2018). Assessment of the utility of TRAP and EST-SSRs markers for genetic diversity analysis of sugarcane genotypes. *Cytology and Genetics*, *52*(6), 467-477.

Fathima-Rafieah, N., & Divakaran, M. (2021). *Genetic resources of the universal flavuor, vanilla. Herbs and spices new processing technologies*. IntechOpen https://doi.org/10.5772/intechopen.99043

Fernandes, S., Godoy-Lutz, G., Jochua, C., Urrea, C., Eskridge, K., Steadman, J. R., & Herr, J. R. (2021). Root and crown rot pathogens found on dry beans grown in Mozambique. *Tropical Plant Pathology*, *46*(3), 294-310.

Ferrando, A., Castellano, M. M., Lison, P., Leister, D., Stepanova, A. N., & Hanson, J. (2018). *Relevance of translational regulation on plant growth and environmental responses*. Frontiers Media SA.

Fliege, C. E., Ward, R. A., Vogel, P., Nguyen, H., Quach, T., Guo, M., & Diers, B. W. (2022). Fine mapping and cloning of the major seed protein quantitative trait loci on soybean chromosome 20. *The Plant Journal*, *110*(1), 114-128.

Foo, E., Plett, J. M., Lopez-Raez, J. A., & Reid, D. (2020). *The role of plant hormones in plant-microbe symbioses.* Frontiers Media SA.

Forsythe, S. J. (2020). *The microbiology of safe food*. John Wiley & Sons.

Fowler, B. A. (2016). *Molecular biological markers for toxicology and risk assessment.* Academic Press.

Furukawa, J. (2019). *Movement of cesium in model plants*. *Radiocesium dynamics in a Japanese forest ecosystem*, (pp. 141-147). Springer.

Gamrat, R., Stankowski, S., & Jaroszewska, A. (2021). *Morphological features of plants on ash settling ponds: Case study*. *Plants* (Vol. 10, No. 4, p. 616). MDPI.

Gaznayee, H. AAl-Quraishi, A. M., & Gaznayee, H. A. Al-Quraishi, A. M. (2019). Analysis of agricultural drought, rainfall, and crop yield relationships in Erbil province, the Kurdistan region of Iraq based on land sat time-series MSAVI2. *Journal of Advanced Research in Dynamical and Control Systems*, *11*(12), 536-545.

Gigant, R., Brugel, A., De Bruyn, A., Risterucci, A. M., Guiot, V., Viscardi, G., & Besse, P. (2012). Nineteen polymorphic microsatellite markers from two African Vanilla species: across-species transferability and diversity in a wild population of *V. humblotii* from Mayotte. *Conservation Genetics Resources*, *4*(1), 121-125.

Globe Newswire. (2022). *World research market data.* Retrieved on 7th July 2022 from wrm.org:http://www.worldresearchmarket.com.

Gonzalez, C. G., Mustafa, N. R., Wilson, E. G., Verpoorte, R., & Choi, Y. H. (2017). Application of natural deep eutectic solvents for the "green"extraction of vanillin from vanilla pods. *Flavour and Fragrance Journal*, *33*(1), 91-96.

Grijp, P. V. (2021). *Identity and development: Tongan culture, agriculture, and the perenniality of the gift*. BRILL.

Grumet, R., Katzir, N., & Garcia-Mas, J. (2017). *Genetics and genomics of cucurbitaceae*. Springer.

Gu, F., Chen, Y., Hong, Y., Fang, Y., & Tan, L. (2017). *Comparative metabolomics in vanilla pod and vanilla bean revealing the biosynthesis of vanillin during the curing process of vanilla* (Vol. 7, No. 1, pp. 1-9). AMB Express.

Gulyaeva, E. N., Ignatenko, R. V., & Galibina, N. A. (2020). Somaclonal variability of conifers in culture *in-vitro*. *Ecological Genetics*, *18*(3), 301-315.

Hakeem, K. R., & Aftab, T. (2021). *Medicinal and aromatic plants: Healthcare and industrial applications*. Springer Nature.

Hanci, F. (2019). Genetic variability in peas (*Pisum sativum* L.) from Turkey asssessed with molecular and morphological markers. *Folia Horticulturae*, *31*(1), 101-116.

Havkin-Frenkel, D., & Belanger, F.C. (2018). *Handbook of vanilla science and technology*. John Wiley & Sons.

Havlickova, L., Jozova, E., Rychla, A., Klíma, M., Kucera, V., & Curn, V. (2014). Genetic diversity assessment in winter oilseed rape (*Brassica napus* L.) collection using AFLP, ISSR and SSR markers. *Czech Journal of Genetics and Plant Breeding*, *50*(3), 216-225.

Hegele, S., Hegele, M., & Wunsche, J. N. (2021). *Low-cost gelling agents for tissue culture propagation of plantain:* In *IV international symposium on horticulture in Europe-SHE2021* (Vol. 1327, pp. 341-348). ISHS.

Herman, E. B. (2017). Plant tissue culture contamination. *Challenges and opportunities:* In *VI international symposium on production and establishment of micropropagated plants* (Vol. 1155, pp. 231-238). ISHS.

Hernandez, G., & Guenette, K. (2018). *Can faba bean physiological responses stem from contrasting traffic management regimes?*: *Agronomy* (Vol. 8, No. 10, p. 200). MDPI.

Hesar, A. A., Kaviani, B., Tarang, A., & Zanjani, S. B. (2011). Effect of different concentrations of kinetin on regeneration of (*'Matthiola incana'*). *Plant Omics*, *4*(5), 236-238.

Hibbert, D. B. (2017). Unweighted pair group method with arithmetic mean (UPGMA). *IUPAC Standards Online*.

Hill-Saya, B. (2020). *Like a tree planted by streams of water. Aaron McDuffie Moore* (pp. 39-46). UNC Press Books.

Hodgkin, T., & Brown, A. H. (2015). *Indicators of genetic diversity, genetic erosion, and genetic vulnerability for plant genetic resources. Sustainable development and biodiversity* (pp. 25-53). Springer.

Hu, Y., Resende Jr, M. F., Bombarely, A., Brym, M., Bassil, E., & Chambers, A. H. (2019). *Genomics-based diversity analysis of vanilla species using a Vanilla planifolia draft genome and genotyping-by-sequencing*: *Scientific reports* (Vol. 9, No.1, p. 3416). Springer Nature.

Hussain, A., Ullah, I., & Hasnain, S. (2017). *Microbial manipulation of auxins and cytokinins in plants*: *Methods in molecular biology* (Vol. 1569, pp. 61-72). Springer.

Ibrahim, R. (2011). A modified CTAB protocol for DNA extraction from young flower petals of some medicinal plant species. *Gene conserve*, *10*(40), 165–182.

Ingram, C., Liu, L., & Ng, N. (2020). *Falling leaves and new roots: Informed practice within the Sydney conservatorium of music's Chinese music ensemble*: In *Creative research in music* (pp. 73-81). Routledge

IPGRI (International Plant Genetic Resources Institute). (2001).The design and analysis of evaluation trials of genetic resources collections. *A guide for genebank managers*: *IPGRI technical bulletin* No. 4. Springer.

Isidro-Adolfo, P., Netzahualcoyotl-Mata, M. F., Gonzalez-Arnao, M. T., Iglesias Andreu, L. G., & Martinez-Castillo, J. (2019). *Assessment of genetic stability during cryopreservation of vanilla (Vanilla Planifolia) shoot-tips using ISSR markers*: *Cryobiology* (Vol. 91, p. 159). Elsevier.

Izzati, K. (2013). A simple and efficient protocol for the mass propagation of *Vanilla planifolia*. *American Journal of Plant Sciences*, *4*(9), 1685–1692.

Ji, M., Qin, H., Chen, H., & Wen, B. (2018). Seed germination requirements of *Ficus virens* (Moraceae) as adaptation to its hemi-epiphyte life form. *Polish Journal of Ecology*, *66*(1), 14-22.

Jighly, A., Al-Abdallat, A. M., & Alnemer, L. M. (2017). The role of microsatellites in streptophyta gene evolution. *Journal of Molecular Evolution*, *84*(2-3), 144-148.

Kafi, M., Kamili, A. N., Husaini, A. M., Ozturk, M., & Altay, V. (2018). *An expensive spice saffron (Crocus sativus* L.*): A case study from Kashmir, Iran, and Turkey. Global perspectives on underutilized crops* (pp. 109-149). Springer.

Kameni, S. L., Meutchieye, F., & Ngoula, F. (2021). Liquid storage of ram semen: associated damages and improvement. *Open Journal of Animal Sciences*, *11*(3), 473-500.

Khan, N. A., Khan, M. I., Ferrante, A., & Poor, P. (2017). *Ethylene: A key regulatory molecule in plants*. Frontiers Media SA.

Khan, N. T. (2020). Saffron: An ancient culinary spice. *Biomedical Journal of Scientific and Technical Research*, *28*(5), 21916-21918.

Khasim, S. M., Hegde, S. N., Gonzalez-Arnao, M. T., & Thammasiri, K. (2020). *Orchid biology: Recent trends and challenges*. Springer Nature.

Khedr, A. (2019). Management of soft rot disease, caused by *Erwinia carotovora* sub-species carotovora in potato tubers. *African Journal of Biological Sciences*, *15*(1), 211-218.

Khoyratty, S., Kodja, H., & Verpoorte, R. (2018). Vanilla flavor production methods. A review: *Industrial crops and products* (Vol. 125, pp. 433-442). Elsevier.

Kowsalya, A., Rojamala, K., & Muthukumar, T. (2017). *Comparative vegetative anatomy of South Indian Vandas (Orchidaceae): Flora* (Vol. 235, pp. 59-75). Elsevier.

Krahl, D. R., Krahl, A. H., Chiron, G., & Terra-araujo, M. H. (2020). First record of *Vanilla labellopapillata* (Orchidaceae: Vanilloideae) in the state of Amazonas, Brazil. *Acta Amazonica*, *50*(3), 260-262.

Kranz, S., & Jahns, L. (2014). High proportions of food recommended for consumption by United States dietary guidance contain solid fats and added sugar: Results from the national health and nutrition examination survey (2007-2008). *Nutrition Journal*, *13*, (23), 1-6.

Kumar, A. (2018). *Augmenting medicinal plant research through in-vitro and in silico approach*. GRIN Verlag.

Kumar, A. (2020). *A text book on the roles of biotechnology in biological sciences*. EduBubs Publishing House.

Kumar, A., Choudhury, B. I., Dayanandan, S., & Khan, M. L. (2022). *Molecular genetics and genomics tools in biodiversity conservation*. Springer Nature.

Kumar, A., Kumar, R., Shukla, P., & Pandey, M. K. (2021). *Omics technologies for sustainable agriculture and global food security* (Vol. 1). Springer Nature.

Kumar, N. (2018). *Biotechnological approaches for medicinal and aromatic plants: Conservation, genetic improvement and utilization*. Springer.

Laman, T., & Beehler, B. M. (2020). *New Guinea: Nature and culture of earth's grandest Island*. Princeton University Press.

Larsson, S., & Dauncey, E. A. (2018). *Plants that kill: A natural history of the world's most poisonous plants*. Princeton University Press.

Latheef, A., & Kavino, M. (2018). Variability in genotypes of Jamun (*Syzygium cumini* Skeels) for morphological and yield characters. *Madras Agricultural Journal*, *105*(10-12), 601-606.

Lee, B., Jung, S., & Hashimura, Y. (2019). Scale up of allogeneic cell therapy manufacturing in single use bioreactors: Challenges, insights and solutions. *Cytotherapy*, *21*(5), 1-16.

Li, M., Pu, Y., Meng, X., Chen, F., Dixon, R. A., & Ragauskas, A. J. (2022). Strikingly high amount of tricin lignin observed from vanilla (*Vanilla planifolia A.*) aerial roots. *Green Chemistry*, *24*(1), 259-270.

Li, Y., Hao, Z., Miao, S., Zhang, X., Li, J., Guo, S., & Lee, Y. (2022). Profiles of cytokinins metabolic genes and endogenous cytokinins dynamics during shoot multiplication *in-vitro* of phalaenopsis. *International Journal of Molecular Sciences*, *23*(7), 3755.

Limi, A. (2019). Location revisited. *Evidence from Antananarivo*: *World bank policy research working paper* (No. 8958). Madagascar. https://doi.org/10.1596/1813-9450-8958

Linlokken, A. N. (2018). *Genetic diversity in small populations*: In *Genetic diversity and disease susceptibility* (pp. 43-55). London. https://doi.org/10.5772/intechopen.76923

Litz, R. E., Pliego-Alfaro, F., & Hormaza, J. I. (2020). *Biotechnology of fruit and nut crops* (2nd edition.). CABI.

Lu, D. (2019). *DNA data storage made more reliable with silica coating*. *New scientist* (Vol. 242, No. 3232, p. 9). Unirioja.

Ma, Z., & Ellison, A. M. (2020). *Towards a unifying diversity area relationship (DAR) of species and gene diversity*. Biorxiv. https://doi.org/10.1101/2020.05.16.099861

Maffettone, R., Sarathy, S., Wen, Y., Penkarski-Rodon, E., Passalacqua, K., Neofotistos, P., Wobus, C., Santoro, D., & Gehr, R. (2018). Waste water disinfectant showdown: A techno-economic and life cycle assessment of sodium hypochlorite, peracetic acid, and performic acid. *Proceedings of the Water Environment Federation*, *12*(12), 2858-2866. https://doi.org/10.2175/193864718825135577

Maqbool, A., Bakhsh, A., & Aksoy, E. (2021). *Effects of natural variations on biofortification.* In *Wild germplasm for genetic improvement in crop plants* (pp. 115-138). Academic Press.

Marazzi, F., Sambusiti, C., Monlau, F., Cecere, S., Scaglione, D., Barakat, A., Mezzanotte, V., & Ficara, E. (2017). *A novel option for reducing the optical density of liquid digestate to achieve a more productive microalgal culturing: Algal research (*Vol 24, pp. 19-28). Elsevier.

Margeta, P., Skorput, D., Salamon, D., Mencik, S., Gvozdanovicc, K., Karolyi, D., Lukovic, Z., & Salajpal, K. (2019). 12-*plex* highly polymorphic microsatellite marker set for parentage analysis in Banija spotted pigs. *Journal of Central European Agriculture*, *20*(1), 50-54.

Mariska, I., & Fronika-Sianipar, N. (2020). *Micropropagation of Orthosiphon aristatus through indirect and direct organogenesis*. *Jurnal teknologi* (Vol. 82, No. 3). University Teknologi Malaysia. https://doi.org/10.11113/jt.v82.13887

Martinez, M. (2019). *Molecular advances in wheat and barley*. MDPI.

Martinkova, JKlimeosva, J., & Martinkova, J. Klimeosva, J. (2022). *Clonal growth, resprouting, and vegetative propagation of weeds: Persistence strategies of weeds* (pp. 200-218). Wiley Online Library,

Masih, M., & Jolly, J. (2017). Demand forecasting through market potential for gluten-free food industry in India and USA. *International Journal of Civil, Structural, Environmental and Infrastructure Engineering Research and Development*, *7*(3), 17-24.

Mesay, A., Derebew, B., & Digafie, T. (2015). Influence of rooting media and number of nodes per stem cutting on nursery performance of vanilla (*Vanilla planifolia* Andrews syn. *Vanilla* fragrans). *Journal of Horticulture and Forestry*, *7*(3), 48-56.

Misra, G. (2017). *Introduction to biomolecular structure and biophysics: Basics of biophysics*. Springer.

Mohamed, G., & Aly, A. (2019). Influence of fish culture water irrigation and nitrogen fertilization on cowpea growth and yield in clay loam and loam sandy soils. *Journal of Plant Production*, *10*(12), 949-956.

Molina, G., Gupta, V. K., Singh, B. N., & Gathergood, N. (2020). *Bio-processing for biomolecule production*. John Wiley & Sons.

Moller, B. L., & Gallage, N. J. (2017). Vanilla. *The most popular flavour*: *Biotechnology of natural products* (pp. 3-24). Springer.

Mondal, T. K. (2020). *Tea: Genome and genetics*. Springer Nature.

Motagi, B. N., Rao, M. S., & Mathad, A. (2020). Integrated and sustainable management of fungal diseases of chickpea. *Current status and challenges: Fungal biology* (pp. 73-91). Springer.

Mulpuri, S., Carels, N., & Bahadur, B. (2019). *Jatropha, challenges for a new energy crop: A sustainable multipurpose crop* (Vol. 3). Springer.

Nair, K. P. (2019). *Turmeric (Curcuma longa* L.*) and ginger (Zingiber officinale* Rosc.) *- World's invaluable medicinal spices: The agronomy and economy of turmeric and ginger*. Springer Nature.

Nair, P. K., Kumar, B. M., & Nair, V. D. (2022). *An introduction to agroforestry: Four decades of scientific developments*. Springer Nature.

Nany, F., & Grisoni, M. (2021). The beautiful hills: Half a century of vanilla (*Vanilla planifolia* jacks. ex Andrews) breeding in Madagascar. *Genetic Resources and Crop Evolution*, *68*(5), 1691-1708.

Nargar, K., & Chen, J. (2020). *Orchid genomics and developmental biology*. Frontiers Media SA.

Nhut, D. T. (2022). Some aspects of plant tissue culture. *Plant tissue culture*: *New techniques and application in horticultural species of tropical region* (pp. 1-23). Springer Singapore.

Nunes, A. C. (2021). ConservaGen software. *A useful tool for genetic conservation of germplasm*: *Crop breeding and applied biotechnology* (Vol. 21, No. 4). SciELO Brasil

Oginni, O., & Fasina, O. (2018). *Theoretical estimation of silo design parameters for fractionated loblolly pine grinds–moisture content and particle size effects: Industrial crops and products* (Vol. 123, pp. 379-385). Elsevier.

Onder, H., & Mercan, L. (2020). Comparison of Bray Curtis and NEI's genetic distance on mantel test for chicken diversity data. *Black Sea Journal of Engineering and Science*, *3*(3), 76-80.

Ortigao, J. A. R. (2020). *Soil mechanics in the light of critical state theories: An introduction.* CRC Press.

Oyoo, M. E., Muhammed, N., Githiri, S. M., Ojwang, P. O., Muniu, F. K., Masha, E., & Owuoche, J. (2015). *In-situ* morphological characterization of coconut in the coastal lowlands of Kenya. *African Journal of Crop Science,* 9(2):65–74.

Oyugi, Z. (2018). *Vanilla farming profit booster.* Retrieved on 14th August, 2018 from http:/www.farmbizafrica.com/profit booster/.

Panwar, S., Singh, K. P., Namita, Janakiram, T., Sonah, H., & Sharma, T. (2018). DNA fingerprinting in African Marigold (*Tagetes erecta* L.) genotypes using ISSR and URP markers. *Indian Journal of Horticulture*, *75*(1), 105-110.

Parine, N. R. (2017). *Genetic polymorphisms*. BoD – Books on Demand.

Parizaca, A. D. (2019). *Vanilla yanesha* (Orchidaceae), a new species of the membranaceous-leaved group from the central rainforest of Peru. *Willdenowia*, *49*(1), 5-9.

Park, S. (2021). *Callus induction*. *Plant tissue culture* (pp. 67-85). Frontier.

Park, S. (2021). *Haploid plants from anther culture*: *Plant tissue culture*, (pp. 111-121). Frontier.

Pass, H., Ball, D., & Scagliotti, G. (2017). *IASLC thoracic oncology E-book*. Elsevier Health Sciences.

Patil, S.S., Mane, J.J., Umdale, S.D., & Chavan, J.J. (2017). Direct and indirect shoot organogenesis strategies for propagation and conservation of *Abutilon ranadei* Wooder and Stapf - A critically endangered plant. *Advance Cell Science and Tissue Culture, 1*(1), 1-4.

Pausic, I., Dakskobler, I., Surina, B., & Dolinar, B. (2018). *Taxonomic revision and morphological analysis of Red Vanilla Orchid, Nigritella miniata (Crantz) Janchen 1960 (Orchidaceae- Orchideae) in the Julian and Dinaric Alps (Slovenia)* (No. 25, pp. 179-208). Wulfenia.

Pearlstine, E. V. (2022). *Scent: A natural history of fragrance*. Yale University Press.

Penkarski-Rodon, E. (2018). *Life cycle assessment for wastewater disinfection using sodium hypochlorite, peracetic acid or performic acid* (Doctoral dissertation, McGill University). Canada.

Perdani, A. Y., Paradisa, Y. B., Wahyuni, W., Indrayani, S., Sulistyowati, Y., & Cahyani, Y. (2021). Response of six chili varieties to anthracnose disease caused by *Colletotrichum acutatum* and C. gloeosporiodes. *Journal Hama Dan Penyakit Tumbuhan Tropika*, *21*(2), 144-150.

Petrauskas, G., Norkeviciene, E., Stukonis, V., & Kemesyte, V. (2020). Phenotypic traits for wild red clover seed yield under drought conditions. *Czech Journal of Genetics and Plant Breeding*, *56*(4), 140-149.

Pollard, J., & Curtin, N. (2018). *Targeting the DNA damage response for anti-cancer therapy*. Springer.

Ponomarev, A. V., & Logunov, D. V. (2020). New species and records of *Salticidae* (Aranei) from Turkmenistan and neighbouring countries. *Arthropoda Selecta*, *29*(1), 67-81.

Pouramini, P., & Hensel, G. (2022). *Herbs for health*. Cambridge Scholars Publishing.

Prescott, J., & Monteleone, E. (2015). *Consumer perceptions of food and beverage flavour*: In *Flavour development, analysis and perception in food and beverages* (pp. 369-385). Woodhead Publishing.

Radotic, K., & Pavlovic, M. (2017). *Shoot and root apical meristems*. *Animal and plant stem cells* (pp. 175-178). Springer, Switzerland.

Rahman, M., Zafar, Y., & Zhang, T. (2021). *Cotton precision breeding*. Springer Nature.

Raj, S., & Mohan, S. (2016). Evaluation of growth performance of biodiesel plant *Jatropha curcus* in fly ash-A waste for sustainable development. *The Journal of Solid Waste Technology and Management*, *42*(1), 1-4.

Rao, V. R., & Rajasekharan, P. (2019). *Conservation and utilization of horticultural genetic resources*. Springer.

Ravindran, P. N. (2017). *The encyclopedia of herbs and spices*. CABI.

Robinson, N. (2020). *Large-scale planting*. *The planting design handbook* (pp. 251-302). Routledge.

Rodrik, D. (2018). An African growth miracle. *Journal of African Economies*, *27*(1), 10-27.

Romps, D. M. (2020). *Theory of tropical moist convection: Fundamental aspects of turbulent flows in climate dynamics* (pp. 1-45). Oxford University Press.

Sabel, A., Bredefeld, S., Schlander, M., & Claus, H. (2017). Wine phenolic compounds. *Antimicrobial properties against yeasts, lactic acid and acetic acid bacteria*. *Beverages*, (Vol. 3, No. 3, p. 29). MDPI.

Sanchez-Galindo, M., Arevalo-Galarza, M. D., Delgado-Alvarado, A., Herrera-Cabrera, B. E., & Osorio-Garcia, C. (2018). Quality of green and cured vanilla (*Vanilla planifolia* Jacks. ex Andrews) fruit in relation to its age at harvest. *Revise Campaign Series Horticultural*, *24*(3), 203-213.

SAS Institute, Inc. (2001). *The SAS system: Version 9.0., SAS institute*. Inc., Cary, North Carolina.

Satish, L., Rency, A. S., Rathinapriya, P., Ceasar, S. A., Pandian, S., Rameshkumar, R., & Ramesh, M. (2016). Influence of plant growth regulators and spermidine on somatic embryogenesis and plant regeneration in four Indian genotypes of finger millet (*Eleusine coracana* L.). *Plant Cell, Tissue and Organ Culture (PCTOC)*, *124*(1), 15-31.

Schmidt, B. M., & Cheng, D. M. (2017). *Ethnobotany: A phytochemical perspective*. John Wiley & Sons.

Selechnik, D., Richardson, M. F., Shine, R., DeVore, J. L., Ducatez, S., & Rollins, L. A. (2019). *Increased adaptive variation despite reduced overall genetic diversity in a rapidly adapting invader* (Vol. 10, p. 1221). Frontiers.

Selina, W. (2022). *Sourcing food and agricultural produce from cooperatives data*. Retrieved on 7th July 2022 from selina.org:http://www.selinawamucii.com.

Sen, M. (2022). *Food chemistry: The role of additives, preservatives and adulteration*. John Wiley and Sons.

Shabbir, B. (2018). *World vanilla production.* Retrieved on 14th August, 2019 from http:/www.farmbizafrica.com/profit booster/.

Shahabadi, N., Asadian, A. A., & Mahdavi, M. (2017). Intercalation of a Zn (II) complex containing ciprofloxacin drug between DNA base pairs. *Nucleosides, Nucleotides and Nucleic Acids*, *36*(11), 676-689.

Shanker, A. (2018). *Bioinformatics: Sequences, structures, phylogeny*. Springer.

Sharma, P., & Sarma, K. P. (2011). *In-vitro propagation of Bambusa balcooa for a better environment.* In *International conferences on advances in biotechnology and pharmaceutical sciences (ICABPS'11)* (pp. 248-252). Bangkok.

Sihanat, A., Theanphong, O., & Rungsihirunrat, K. (2020). Assessment of phylogenetic relationship among twenty Curcuma species in Thailand using amplified fragment length polymorphism marker. *Journal of Advanced Pharmaceutical Technology and and Research*, *11*(3), 134-141.

Singh, B. P., & Gupta, V. K. (2017). *Molecular markers in mycology: Diagnostics and marker developments*. Springer.

Singh, B. P., Singh, G., Kumar, K., Nayak, S. C., & Srinivasa, N. (2020). *Management of fungal pathogens in pulses: Current status and future challenges*. Springer Nature.

Singh, D. P., Singh, A. K., & Singh, A. (2021). *Plant breeding and cultivar development*. Academic Press.

Singh, K. P. (2020). *Analysis of toxicants-induced alterations in DNA methylation by methylation-sensitive-Random amplified polymorphic DNA-polymerase chain reaction (MS-RAPD-PCR)*: *Molecular toxicology protocols* (Vol. 2102, pp. 213-224). Spinger.

Singh, R. V. (2020). *Microorganisms: Earth's tiny heroes and villains*. The Energy and Resources Institute (TERI).

Singh, S., & Kumar, D. (2020). *Protocols used in molecular biology*. Bentham Science Publishers.

Skoog, F., & Murashige, T. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum,* 15(3), 473–497.

Sme, N. (2017*). Distinction of saffron cod (Eleginus gracilis) from several other gadid species by using microsatellite markers*: *Supplementary Table 1. fishery bulletin* (Vol. 116, p. 1). University of Georgia, Athens, GA (United States)

Smith, N. J., Williams, J. T., Plucknett, D. L., & Talbot, J. P. (2018). *Tropical forests and their crops*. Cornell University Press.

Soloneski, S., & Larramendy, M. L. (2021). *Genotoxicity and mutagenicity: Mechanisms and test methods*. BoD – Books on Demand.

Soni, S., Vishwakarma, G., Singh, S. C., Kumar, S., Singh, R. K., Awasthi, P., & Gangwar, V. (2022). Commercial use of plant growth regulators in horticultural crops: An overview. *The Pharma Innovation Journal*, *11*(6), 112-119.

Sowbhagya, H. B. (2019). Value-added processing of by-products from spice industry. *Food Quality and Safety*, *3*(2), 73-80.

Spealman, P., Burrell, J., & Gresham, D. (2019). *Nanopore sequencing undergoes catastrophic sequence failure at inverted duplicated DNA sequences* (p. 852665). BioRxiv.

Srivastava, D. K., Thakur, A. K., & Kumar, P. (2021). *Agricultural biotechnology: Latest research and trends*. Springer Nature.

Starrantino, A., & Caruso, A. (2021). *In-vitro culture techniques to produce virus-free citrus plants:* In *Integrated pest control in citrus-groves* (pp. 393-398). CRC Press.

Stevens, H. M. (2019). *Biotechnology and plant biology*. Scientific e-Resources.

Sunseri, F., & Mercati, F., & Sunseri, F. (2021). *Genetic diversity assessment and marker-assisted selection in crops*. MDPI.

Swamy, M. K. (2020). *Plant-derived bioactives: Production, properties and therapeutic applications*. Springer Nature.

Takami, H. (2019). Molecular tools in microbial diversity. *Functional assessment tool for genomes and metagenomes, MAPLE system*: In *Microbial diversity in the genomic era* (pp. 117-136). Academic Press.

Tanwar, A & Bisen, R. (2018). Genetic diversity analysis in sesame (*Sesamum indicum* L.) germplasm based on morphological and quality traits. *Electronic Journal of Plant Breeding*, *9*(1), 9-17.

Taylor, M. K., & Granger, D. A. (2020). *Salivary bioscience: Foundations of interdisciplinary saliva research and applications*. Springer Nature.

Tekere, M., Jacob-Lopes, E., & Zepka, L. Q. (2019). *Microbial bioremediation and different bioreactors designs applied*: *Biotechnology and bioengineering* (pp. 1-19). Amazon. https://doi.org/10.5772/intechopen.83661

Tenedios, C., O'Leary, S., Capocci, M., & Desai, S. P. (2018). History of anaesthesia. *European Journal of Anaesthesiology*, *35*(3), 158-164.

Teoh, E. S. (2019). *The story of vanilla: Orchids as aphrodisiac, medicine or food* (pp. 109-130). Springer.

Thammasiri, K. (2020). *Orchid biology: Recent trends and challenges* (pp. 3-10). Springer.

Thapa, T., & Rawat, V. (2020). Effect of plant growth regulators (IBA & NAA) on propagation of guava (*Pisidium guajava* L.) by cutting – a review. *International Journal of Current Microbiology and Applied Sciences*, *9*(11), 278-284.

Thi-Trung-Thu, N., & Quang-Binh, T. (2016). Determining kcnj11 e23k polymorphism in a group of vietnamese population by restriction fragment length polymorphism method. *Journal of Science, Natural Science*, *61*(9), 177-184.

Thomas, P., & Franco, C. M. (2021). Intracellular bacteria in plants. *Elucidation of abundant and diverse cytoplasmic bacteria in healthy plant cells using in-vitro cell and callus cultures: Microorganisms* (Vol. 9, No. 2, p. 269). MDPI. https://doi.org/10.3390/microorganisms9020269

Tronsmo, A. M., Collinge, D. B., Djurle, A., Munk, L., & Yuen, J. (2020). *Plant pathology and plant diseases*. CABI.

Tyupin, V. (2021). Action time of explosion-produced seismic waves on guarded objects during large-scale blasting at open pit mines. *Mining Informational and Analytical Bulletin*, (4), 124-133.

Upadhyay, S. K., & Singh, S. P. (2021). *Bioprospecting of plant biodiversity for industrial molecules*. John Wiley & Sons.

Uysal, A., Gunes, E., & Durak, Y. (2019). Elucidation of biological properties of some commercial anthraquinones: Mutagenic/antimutagenic and antimicrobial activity approaches. *Journal of Research in Pharmacy*, *23*(2), 208-216.

Varma, A. (2022). *Biology and biotechnology of quinoa: Super grain for food security*. Springer Nature.

Verdi, R., Bettoni, J. C., Werner, S. S., Boff, M. I., & Boff, P. (2020). Effects of the phenological stage, type of cutting and plant growth regulators on the propagation by stem cutting of *Poiretia latifolia* Vogel, a Brazilian native medicinal plant. *Revise Colombia Scientific Horticulture*, *14*(2), 267-274.

Victoria-Garcia, M., & Eugenia-Barrandeguy, M. (2021). *The sensitiveness of expected heterozygosity and allelic richness estimates for analyzing population genetic diversity:* In *Genetic variation.* IntechOpen.

Volza. (2020). *Volza's data.* Retrieved on 25th June, 2022 from volza grow global: http://www.volza.org.

Vynnycky, E., & Papadopoulos, T. (2021). Estimates of the basic reproduction number for rubella using seroprevalence data and indicator-based approaches. *PLoS Computational Biology*, *18*(3), e1008858. https://doi.org/10.1371/journal.pcbi.1008858

Waititu, J. M., Mundia, C. N., & Sichangi, A. W. (2020). *Assessing distribution changes of selected native and alien invasive plant species under changing climatic conditions.* Nyeri County, Kenya.

Wani, S. H., Thakur, A. K., & Khan, Y. J. (2020). *Brassica improvement: Molecular, genetics and genomic perspectives*. Springer Nature.

Watanabe, Y., & Sakaguchi, J. (2017). Light perception in aerial tissues enhances DWF4 accumulation in root tips and induces root growth. *Scientific Reports*, *7*(1), 1-11.

Wiering, N., Gardner, G., Sheaffer, C. C., & Ehlke, N. J. (2020). Root and axillary shoot development of hairy vetch stem cuttings and cessation of flower development under a short photoperiod. *Crop Science*, *60*(5), 2386-2393.

William-Shurtleff, A. (2021). *History of soy nutritional research (1946-1989): Extensively annotated bibliography and sourcebook*. Soyinfo Center.

Wilson, R. T. (2021). *An overview of traditional small-scale poultry production in low-income, food-deficit countries: Annals agricultural crop sci*ence (Vol. 6, No. 3, p. 1077). Austin Publishing.

Winnicott, D. W. (2016). *The collected works of DW Winnicott* (Vol. 12). Oxford University Press.

Wodrich, K. H. (2020). *Introduction to South African indigenous orchids*: *Growing South African indigenous orchids* (pp. 1-36). London.

Wodrich, K. H. (2020). *Propagation of indigenous orchids from seed and by tissue culture*: *Growing South African indigenous orchids* (pp. 161-203). London.

Wu, X., Duan, L., Chen, Q., & Zhang, D. (2020). Genetic diversity, population structure, and evolutionary relationships within a taxonomically complex group revealed by AFLP markers: *A case study on Fritillaria cirrhosa D. Don and closely related species*: *Global ecology and conservation* (Vol. 24, p. 1323). Elsevier.

Yadav, P., Yadav, K. K., & Bhagyawant, S. S. (2022). *Molecular assessment of wild and cultivated Cicer species using ISSR markers*. *Ecological genetics and genomics* (Vol. 25, p. 100137). Elsevier.

Yadav, H. K., & Kumar, V. (2018). Assessment of genetic diversity in *Lepidium sativum* L. using inter simple sequence repeat (ISSR) marker. *Physiology and Molecular Biology of Plants*, *25*(2), 399-406.

Yadav, R. (2019). *Manual on general laboratory handling techniques in plant pathology*. GRIN Verlag.

Yahyah, H., Ginzky, H., Kasimbazi, E., Kibugi, R., & Ruppel, O. C. (2020). *Legal instruments for sustainable soil management in Africa*. Springer Nature.

Yang, J., Wang, Z., Jiang, Y., & Fei, S. (2019). *Genetics and genomics of polyploid plants*. Frontiers Media SA.

Yulianto, M., & Paramita, V. (2019). Response surface analysis on the microwave integrated-rumen based extraction of natural vanillin from cured vanilla pods. In *Journal of Physics*: *Conference Series*, (Vol. 1295, No. 1, p. 012016). IOP Publishing.

APPENDICES

Appendix A. ANOVA showing the effect of varied kinetin concentrations levels on number of plants with shoots of vanilla accessions from Busia county

Source of Variation	D.F	S.S	M.S	F	*p-value*
Treatment	5	21.37	4.274	3.7165	0.0124
Error	24	27.6	1.15		
Total	29	48.97			

Appendix B. ANOVA showing the effect of varied kinetin concentrations levels on number of shoots per plant of vanilla accessions from Busia county

Source of Variation	D.F	S.S	M.S	F	*p-value*
Treatment	5	5.9	1.18	1.6857	0.1764
Error	24	16.8	0.7		
Total	29	22.7			

Appendix C. ANOVA showing the effect of varied kinetin concentrations levels on number of leaves of vanilla accessions from Busia county

Source of Variation	D.F	S.S	M.S	F	*p-value*
Treatment	5	1.2	0.24	0.1871	0.9647
Error	24	30.8	1.283		
Total	29	32			

Appendix D. ANOVA showing the effect of varied kinetin concentrations levels on number of nodes of vanilla accessions from Busia county

Source of Variation	D.F	S.S	M.S	F	*p-value*
Treatment	5	5.77	1.154	1.832	0.1467
Error	24	15.2	0.63		
Total	29	20.97			

Appendix E. ANOVA showing the effect of varied kinetin concentrations levels on shoot length of vanilla accessions from Busia county

Source of Variation	D.F	S.S	M.S	F	*p-value*
Treatment	5	210.17	42.034	18.4699	<.0001
Error	24	54.62	2.2758		
Total	29	264.79			

Appendix F. ANOVA showing the effect of varied IAA concentrations levels on the number of plants with roots of vanilla accessions from Busia county

Source of Variation	D.F	S.S	M.S	F	*p-value*
Treatment	5	99.47	19.894	16.817	<.0001
Error	24	28.40	1.183		
Total	29	127.87			

Appendix G. ANOVA showing the effect of varied IAA concentrations levels on the number of roots per plant of vanilla accessions from Busia county

Source of Variation	D.F	S.S	M.S	F	*p-value*
Treatment	5	11.2	2.24	4.638	0.0042
Error	24	11.6	0.483		
Total	29	22.8			

Appendix H. ANOVA showing the effect of varied IAA concentrations levels on root length of vanilla accessions from Busia county

Source of Variation	D.F	S.S	M.S	F	*p-value*
Treatment	5	32.3	6.46	10.82	<.0001
Error	24	14.32	0.597		
Total	29	46.62			

Appendix I. ANOVA showing constraints of kinetin and IAA (Dead plants)

Source of Variation	D.F	S.S	M.S	F	*p-value*
Treatment	5	332.0	66.4	4.442	0.0053
Error	24	358.8	14.95		
Total	29	690.8			

Appendix J. ANOVA showing constraints of kinetin and IAA (Dormant plants)

Source of Variation	D.F	S.S	M.S	F	*p-value*
Treatment	5	210.8	42.16	17.44	<.0001
Error	24	58	2.417		
Total	29	268.8			

Appendix K. ANOVA showing constraints of kinetin and IAA (contaminated media)

Source of Variation	D.F	S.S	M.S	F	*p-value*
Treatment	5	360.4	72.08	28.49	<.0001
Error	24	60.8	2.53		
Total	29	421.2			

Appendix L. Data collection table for the effect of varied levels of kinetin on shoots of vanilla accessions from Busia county

Site	Treat	Rep	No. of plants with shoots	No. of shoots per plants	No. of leaves	No. of node	Shoot length (cm)
Busia	0	0	3	1	2	1	5.2
Busia		0	4	1	3	2	3.8
Busia		0	2	2	4	0	5.6
Busia		0	0	1	1	2	2.4

Busia		0	2	1	3	1	4
Busia	0.4	0.4	4	2	4	3	4.5
Busia		0.4	2	2	3	0	4.6
Busia		0.4	3	3	5	2	4.5
Busia		0.4	1	1	2	1	6
Busia		0.4	3	2	1	1	2.4
Busia	0.8	0.8	4	3	3	2	7.8
Busia		0.8	3	3	2	2	5.2
Busia		0.8	2	2	4	1	8.9
Busia		0.8	3	2	3	2	10
Busia		0.8	2	1	4	1	9.1
Busia	1.2	1.2	5	3	3	3	9.7
Busia		1.2	4	4	2	3	11
Busia		1.2	4	2	5	2	9.3
Busia		1.2	3	2	3	3	12.6
Busia		1.2	5	2	2	1	12.4
Busia	1.6	1.6	4	2	5	2	11.8
Busia		1.6	5	2	3	1	11.2
Busia		1.6	3	0	2	3	9.4
Busia		1.6	4	3	2	2	9.6
Busia		1.6	4	2	3	2	10
Busia	2.0	2.0	3	2	4	2	9.6
Busia		2.0	0	0	3	1	9.4
Busia		2.0	2	2	3	1	10.2
Busia		2.0	3	2	3	1	5.8
Busia		2.0	2	2	3	1	7

Appendix M. Data collection table for the effect of varied level of IAA on roots of vanilla accessions from Busia county

Site	Treat	Rep	No. of plants with roots	No. of roots per plant	Root length (cm)
Busia	0	0	1	2	2.4
Busia		0	3	2	2.6
Busia		0	3	3	1.8
Busia		0	4	2	2.2
Busia		0	1	3	1
Busia	0.4	0.4	5	3	1.9
Busia		0.4	3	2	3.1
Busia		0.4	4	3	2.4
Busia		0.4	2	3	0.6
Busia		0.4	3	2	1
Busia	0.8	0.8	4	3	3.8
Busia		0.8	3	2	2.2
Busia		0.8	4	3	2.9
Busia		0.8	4	3	3.1
Busia		0.8	3	4	1
Busia	1.2	1.2	7	3	4.3
Busia		1.2	5	4	3.7
Busia		1.2	6	4	4.6
Busia		1.2	8	3	4.4
Busia		1.2	8	4	3
Busia	1.6	1.6	5	5	3.6
Busia		1.6	7	4	3.4
Busia		1.6	6	3	3.1
Busia		1.6	5	4	3.9
Busia		1.6	6	5	4
Busia	2.0	2.0	1	4	5.2
Busia		2.0	2	3	3.8
Busia		2.0	3	2	4
Busia		2.0	0	4	5.3
Busia		2.0	2	4	4.7

Appendix N. Data collection table for micropropagation constraints, dead plants

Site	Treat	Rep	Kinetin (After 4 weeks)	Kinetin (After 4 weeks)	IAA (After 3 weeks)	IAA (After 5 weeks)	Total of KN & IAA
Busia	0	0	1	0	2	1	4
Busia		0	2	2	0	1	5
Busia		0	2	0	1	0	3
Busia		0	0	0	1	0	1
Busia		0	1	0	0	0	1
Busia	0.4	0.4	2	1	1	1	5
Busia		0.4	1	2	0	1	4
Busia		0.4	2	0	2	1	5
Busia		0.4	1	1	0	1	3
Busia		0.4	1	0	1	0	2
Busia	0.8	0.8	3	1	4	3	11
Busia		0.8	9	2	2	1	14
Busia		0.8	2	6	2	3	13
Busia		0.8	0	1	1	0	2
Busia		0.8	1	0	5	2	8
Busia	1.2	1.2	3	4	5	2	14
Busia		1.2	1	2	2	1	6
Busia		1.2	4	1	3	2	10
Busia		1.2	6	2	1	3	12
Busia		1.2	1	0	2	1	4
Busia	1.6	1.6	9	3	4	3	19
Busia		1.6	4	2	3	1	10
Busia		1.6	6	4	5	3	18
Busia		1.6	3	2	2	1	8
Busia		1.6	2	1	1	1	5
Busia	2.0	2.0	4	2	5	1	12
Busia		2.0	1	4	3	2	10
Busia		2.0	3	2	4	2	11
Busia		2.0	5	1	1	1	8
Busia		2.0	2	1	2	1	6

Appendix O. Data collection table for micropropagation constraints, dormant plants

Site	Treat	Rep	Kinetin (After 4 weeks)	Kinetin (After 4 weeks)	IAA (After 3 weeks)	IAA (After 5 weeks)	Total of KN & IAA
Busia	0	0	2	1	1	1	5
Busia		0	1	0	2	1	4
Busia		0	1	1	1	0	3
Busia		0	2	1	1	2	6
Busia		0	3	0	2	1	6
Busia	0.4	0.4	1	0	1	1	3
Busia		0.4	1	1	1	1	4
Busia		0.4	2	0	0	1	3
Busia		0.4	1	0	2	0	3
Busia		0.4	2	0	1	1	4
Busia	0.8	0.8	2	1	1	0	4
Busia		0.8	3	0	1	1	5
Busia		0.8	2	1	1	2	6
Busia		0.8	3	1	2	0	6
Busia		0.8	2	3	2	0	7
Busia	1.2	1.2	2	1	3	1	7
Busia		1.2	3	2	2	2	9
Busia		1.2	1	0	2	2	5
Busia		1.2	3	2	1	1	7
Busia		1.2	2	1	0	1	4
Busia	1.6	1.6	3	2	2	2	9
Busia		1.6	4	1	2	2	9
Busia		1.6	3	2	1	2	8
Busia		1.6	4	2	2	2	10
Busia		1.6	4	3	3	3	13
Busia	2.0	2.0	3	2	4	1	10
Busia		2.0	3	2	2	2	9
Busia		2.0	4	2	3	2	11
Busia		2.0	6	4	2	2	14
Busia		2.0	5	2	2	1	10

Appendix P. Data collection table for micropropagation constraints, contaminated media

Site	Treat	Rep	Kinetin (After 4 weeks)	Kinetin (After 4 weeks)	IAA (After 3 weeks)	IAA (After 5 weeks)	Total of KN & IAA
Busia	0	0	2	1	3	2	8
Busia		0	2	1	1	1	5
Busia		0	3	2	2	1	8
Busia		0	4	2	2	1	9
Busia		0	5	2	2	2	11
Busia	0.4	0.4	2	1	1	1	5
Busia		0.4	1	1	1	1	4
Busia		0.4	2	1	1	1	5
Busia		0.4	2	0	1	0	3
Busia		0.4	1	0	1	0	2
Busia	0.8	0.8	2	1	1	1	5
Busia		0.8	2	1	2	1	6
Busia		0.8	3	0	2	1	5
Busia		0.8	4	1	1	0	6
Busia		0.8	3	3	1	1	8
Busia	1.2	1.2	2	1	3	0	6
Busia		1.2	3	1	3	1	8
Busia		1.2	1	1	1	1	4
Busia		1.2	3	1	2	1	7
Busia		1.2	4	2	1	0	7
Busia	1.6	1.6	2	1	2	1	6
Busia		1.6	3	2	2	1	8
Busia		1.6	3	2	2	2	9
Busia		1.6	2	1	1	1	5
Busia		1.6	1	1	1	1	4
Busia	2.0	2.0	4	2	4	4	14
Busia		2.0	5	3	3	4	15
Busia		2.0	5	4	4	3	16
Busia		2.0	4	4	3	3	14
Busia		2.0	5	4	3	3	15

Appendix Q. Data collection table for qualitative traits of vanilla accessions from five counties of Kenya

QUALITATIVE DATA

NO.	OH	OLV	SC	SV	SS	LBTS	SSCS	PAR	LBC	LBB	PLBS	LBV	LBSA	LBS	LBCMV	FS	FST	FTSS
BUNGOMA COUNTY ACCESSIONS																		
1	2	2	2	2	2	2	2	2	2	1	2	2	2	3	2			
2	3	2	3	2	2	1	1	1	2	1	2	2	2	3	2			
3	2	2	2	2	2	2	2	3	2	1	2	2	2	3	2			
4	2	2	2	2	2	2	2	4	2	1	2	2	2	3	2			
5	3	2	3	2	2	1	1	1	2	1	2	2	2	3	2			
6	2	2	2	2	2	2	2	3	2	1	2	2	2	3	2			
7	2	2	2	2	2	2	2	3	2	1	2	2	2	3	2			
8	3	2	3	2	2	1	1	1	3	1	2	2	2	3	2			
BUSIA COUNTY ACCESSIONS																		
1	2	2	2	2	2	1	2	1	2	1	2	2	2	3	2			
2	2	2	2	2	2	1	2	1	2	1	2	2	2	3	2			
3	2	2	2	2	2	1	2	1	2	1	2	2	2	3	2	2	1	5
4	2	2	2	2	2	1	2	1	2	1	2	2	2	3	2			
5	2	2	2	2	2	1	2	1	2	1	2	2	2	3	2			
6	2	2	2	2	2	1	2	1	2	1	2	2	2	3	2			
7	2	2	2	2	2	1	2	1	2	1	2	2	2	3	2			
8	2	2	2	2	2	1	2	4	2	1	2	2	2	3	2			
9	2	2	2	2	2	1	2	1	2	1	2	2	2	3	2			
10	2	2	2	2	2	1	2	1	2	1	2	2	2	3	2			
11	2	2	2	2	2	1	2	2	2	1	2	2	2	3	2	2	1	5
12	2	2	2	2	2	1	2	2	2	1	2	2	2	3	2	2	1	5
13	2	2	2	2	2	1	2	1	2	1	2	2	2	3	2			
14	2	2	2	2	2	1	2	2	2	1	2	2	2	3	2			
15	2	2	2	2	2	1	2	1	2	1	2	2	2	3	2			
16	2	2	2	2	2	1	2	1	2	1	2	2	2	3	2			
17	2	2	2	2	2	1	2	1	2	1	2	2	2	3	2			
MOMBASA COUNTY ACCESSIONS																		
1	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			
2	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			
3	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			
4	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			
5	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			

6	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			
7	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			
8	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			
9	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			
10	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			
11	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			
12	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			
13	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			
14	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			
15	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			
16	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			
17	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			
18	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			
19	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			
20	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			
21	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			

KWALE COUNTY ACCESSIONS

1	3	2	2	1	2	1	1	1	2	2	2	1	2	1	1			
2	3	2	2	1	2	1	1	1	3	2	2	1	2	1	1			
3	3	2	3	1	2	1	1	1	3	2	2	1	2	1	1			
4	3	2	3	1	2	1	1	2	2	2	2	1	2	1	1			
5	3	2	3	1	2	1	1	1	2	2	2	1	2	1	1			
6	3	2	3	1	2	1	1	2	3	2	2	1	2	1	1			
7	3	2	2	1	2	1	1	1	3	2	2	1	2	1	1			
8	3	2	2	1	2	1	1	1	3	2	2	1	2	1	1			
9	3	2	2	1	2	1	1	1	3	2	2	1	2	1	1			
10	3	2	2	1	2	1	1	1	2	2	2	1	2	1	1	2	1	2
11	3	2	2	1	2	1	1	2	2	2	2	1	2	1	1			
12	3	2	2	1	2	1	1	1	3	2	2	1	2	1	1			
13	3	2	3	1	2	1	1	1	3	2	2	1	2	1	1			
14	3	2	2	1	2	1	1	1	3	2	2	1	2	1	1			
15	3	2	2	1	2	1	1	1	2	2	2	1	2	1	1			
16	3	2	3	1	2	1	1	1	3	2	2	1	2	1	1			
17	3	2	2	1	2	1	1	1	2	2	2	1	2	1	1			
18	3	2	2	1	2	1	1	1	3	2	2	1	2	1	1	2	1	2
19	3	2	2	1	2	1	1	1	3	2	2	1	2	1	1	2	1	2
20	3	2	2	1	2	1	1	1	2	2	2	1	2	1	1	2	1	2
21	3	2	2	1	2	1	1	1	2	2	2	1	2	1	1	2	1	2
22	3	2	2	1	2	1	1	1	2	2	2	1	2	1	1			
23	3	2	2	1	2	1	1	1	2	2	2	1	2	1	1			
24	3	2	2	1	2	1	1	1	2	2	2	1	2	1	1	2	1	2
25	3	2	2	1	2	1	1	1	2	2	2	1	2	1	1	2	1	2
26	3	2	2	1	2	1	1	1	2	2	2	1	2	1	1			

KILIFI COUNTY ACCESSIONS

1	2	1	2	1	2	1	1	1	2	2	2	1	2	1	1			
2	2	2	2	1	2	1	1	1	2	2	2	1	2	1	1			
3	3	2	2	1	2	1	1	2	2	2	2	1	2	1	1			
4	2	2	2	1	2	1	1	1	3	2	2	1	2	1	1			

Appendix R. Data collection table for quantitative traits of vanilla accessions from five counties of Kenya

QUANTITATIVE

BUNGOMA COUNTY ACCESSIONS									
NO.	**OSH (m)**	**AR (m)**	**NOF**	**LPL (cm)**	**SIL (cm)**	**LBW (cm)**	**LBL (cm)**	**SC (cm)**	**FL (cm)**
1	3.83	1.93		1.5	8.75	4.53	12	2.83	
2	3.8	2.9		1.5	11.38	4.33	13	2.8	
3	1.95	1.6		1.5	7.88	4.33	11.5	2.38	
4	2.68	1.3		1.5	8	3.88	9.75	2.03	
5	2.2	0.8		1.5	9.25	4.2	10.5	2.65	
6	2.4	1.5		1.5	10.63	4.88	11.38	2.23	
7	3.47	15.2		1.5	12.5	4.25	15.5	3.25	
8	2.19	15.9	3	1.5	11.25	5.12	11.95	3.08	
BUSIA COUNTY ACCESSIONS									
1	4.4	1.9		1.5	11.25	4.45	14.5	3.03	
2	3.3	1.9		1.5	13.25	4.6	15.5	3.1	
3	3.5	1.8		1.5	13.75	4.55	13.75	2.58	17
4	5	4	5	1.5	11	4.45	14.93	2.73	
5	5	2.6		1.5	10.13	4.2	12.58	2.85	
6	6	2.8		1.5	10.63	4.55	14	2.68	
7	4	2.4		1.5	11.13	4.6	12.88	2.05	
8	2.5	1.5		1.5	8	4.08	10.63	2.8	
9	5	2		1.5	11.75	3.83	11.38	2.5	
10	5	2.5		1.5	12.63	4.33	13.38	3.35	
11	6	4		1.5	11.67	4.48	15.13	2.73	20
12	3.5	1.6		1.5	10	5.03	15.13	2.45	21
13	1	2.5		1.5	11.38	4.7	13.13	2.8	
14	8	1.8		1.5	9.67	4.48	14.75	2.93	
15	7.5	3	3	1.5	10	4.88	12.08	2.38	
16	8	2.5	2	1.5	11.63	5.18	17.05	2.6	

17	7.2	3		1.5	10	4.53	14.05	2.95	

MOMBASA COUNTY ACCESSIONS

1	1	1		0.5	3.5	2.5	7.7	1.6	
2	0.92	6		0.5	5.8	1.7	7.5	1.85	
3	1.28	11		0.7	4.5	2.4	10.5	2.1	
4	1.25	6		0.5	9	2.5	9	3.25	
5	1.47	7.5		0.3	6	2	9	3.1	
6	1.82	7		0.5	6	2.2	8.7	3.05	
7	1.32	5		1	5.3	2.9	10	2.5	
8	1.09	4		0.6	4.5	2.1	9.3	2.93	
9	2.1	4.5		0.3	5.5	1.2	7.5	2.5	
10	0.89	2		0.5	3.5	2.7	6.2	1.38	
11	2.81	5.4		0.9	6.4	2	8.5	2.13	
12	2.08	3.5		0.8	3.7	2.1	6.6	2.35	
13	2.08	4.5		0.5	4.5	1.2	6.6	2.05	
14	1.08	2		0.3	5.5	2.4	8	2.7	
15	1.52	5		0.7	3.7	1.8	7.5	1.88	
16	5.2	2		0.3	1.3	2.9	6.3	1.5	
17	1.4	5		0.5	2.8	1.5	6	2.35	
18	1.8	6		1	3.6	1.4	6.5	2.13	
19	2.2	4.5		1.2	5	1.5	9	2.63	
20	1.4	2.6		1.6	3.5	2.2	8	2.7	
21	1.2	4		1	3.5	2.1	7.5	2.1	

KWALE COUNTY ACCESSIONS

1	18.2	14		0.5	2.5	2.2	5.5	2.68	
2	22,36	13.6		1.2	3.4	2.3	7	2.4	
3	17.55	10.9		1	5	3.5	10.8	2.13	
4	0.79	3		0.4	2.3	2.3	5	1.93	
5	2.09	4.8		1.2	5.6	3.3	10	2.38	
6	39.48	9.4		1.2	4.7	2.5	9	2.38	
7	21.24	8.9		1.1	3.6	2	7.6	2.13	
8	29.5	11.8		1	6.3	1.9	7.2	2.13	
9	30.4	91.5		1.2	6	2.7	9.7	2.53	
10	21.3	10.4		1.2	3.1	3.2	10	2.3	19.1
11	2.13	3.1		1	4	3.1	9.8	2.33	
12	7.5	6.3		1	6.9	3	10.8	2.9	
13	41.4	13.2		1	6.4	2.6	8.6	2.18	
14	0.94	11.5		0.7	1.2	2	4.4	2.25	
15	20.64	89.5		1	4	2	8	2.38	
16	16.8	10.2		1	6.2	2.2	11.1	2.4	

17	19.76	11		1.4	4.5	3.3	11.5	2.8	
18	32.55	12.1		1.2	5.4	3.5	11.3	2.58	14.5
19	21.2	6.8		1.5	4.8	2.5	7.5	2.6	18.4
20	25.6	10.4		1.5	4.5	3	11	2.5	19.2
21	59.2	15.2		1	6	1.9	11	2.1	17.3
22	22.1	13.2		1	3.5	2	8.2	2.48	
23	1.71	5.1		0.9	5	2	8.5	1.88	
24	44.88	4.8		0.8	4.6	1.4	6.8	2.13	16.4
25	64.8	12.2		0.5	4	3.4	9.5	2.78	18
26	8.1	6.8		2	7.5	2	10	2.3	

KILIFI COUNTY ACCESSIONS

1	7.67	1.67		0.5	4	2.5	4.3	2.13	
2	3	0.75		1	1.7	3	9.3	2.3	
3	1.6	3.5		0.8	1	2	7.8	2.48	
4	5.7	1.5		0.5	2	1.5	6.8	1.93	

Appendix S. DNA extraction protocol

- Genomic DNA were extracted from dried young plant leaf samples stored in silica gel using CTAB protocol according to Ibrahim (2011) with slight modification.

1. The day before DNA were extracted, polyvinylpyrrolidone (PVP) and β-mercaptoethanol were added to CTAB buffer and put in a water bath at 65^0c to dissolve PVP (antioxidant). (For 5ml CTAB buffer add 0.015g PVP and 15µl β-mercaptoethanol). CTAB buffer with PVP was used within 2-3 days stored capped.
2. Vanilla accessions samples were cleaned at initial stage to remove phytates using TAE buffer (Tris Acetate-EDTA).
3. Grinded about 0.02g of silica dried leaf samples in a 1.5ml eppendorf tubes using pelleted pestle and mortar over liquid nitrogen. Added small amount of sand using a small spatula, to aid the grinding.

4. Added 500µl (five hundred microliters) of the pre-warmed (65^0c) CTAB buffer onto the powder and grinded a bit more. Incubated for 30minutes at 65^0 c. Mixed by inverting every 5minutes as I put to water bath in 30minutes.
5. Centrifuged at 10000rpm for 10minutes. Following centrifugation, two layers were formed: top: aqueous phase, bottom: debris. Went on to the next step quickly so that the phase do not remix.
6. Using a pipette 1ml, carefully transferred the upper aqueous phase to new 1.5ml eppendorf tubes. (A void removing any material from the debris).
7. Added (400µl) an equal volume of Chloroform: Iso-amyl alcohol (24:1) (in the fume hood) and mixed well using a rotary shaker for 5 minutes or converting the tubes manually for 5 minute to obtain an emulsion. (Excessive shaking degrade the DNA).
8. Centrifuged at 10000rpm for 10minutes. Following centrifugation, three layers were formed: top: aqueous phase, middle: debris and proteins, bottom: chloroform. Went on the next step quickly so that the phase do not remix.
9. Using appetite (1ml), carefully transferred the upper aqueous phase to a new 1.5ml eppendorf tubes. (Avoiding to remove any material from the interface).
10. Repeated the Chloroform extraction (step 7-9) to increase purification of DNA for long storage term.
11. Added 200 µl of ice-cold Iso-propanol and mixed by inversion or swirling.
12. Then centrifuged at 14000rpm for 5 minutes. The solutions were allowed to precipitate for 18 hours (overnight) at -20 ^{0}c. (Longer time tend to yield more DNA).
13. At this point, whitish DNA pellets became visible. Poured or pipetted off the liquid carefully and DNA pellets left on the bottom of the tube.
14. Added 500 µl of cold 70% ethanol and mixed by vortexing. Left them to stand for 5 minutes.

15. Centrifuged the DNA solution at 14000rpm for 5 minutes to avoid pelleting carryover impurities. Poured or pipetted off the liquid alcohol carefully leaving the DNA pellets at the bottom of the centrifuged tubes.
16. Ethanol washing process was repeat to have more purified DNA.
17. Drying the DNA pellet: tubes with DNA pellets were inverted on sterile tissue paper for 10 minutes on lab-bench. (a void over-drying, pellets were suspend when damp).
18. Then DNA pellets were re-suspended (dissolved) in 50µl of TE buffer for each. (Tris – EDTA buffer helps to maintain constant pH between 6-8 in order to prevent hydrolysis at lower pH, and DNA denaturation at high pH).
19. The DNA extracted were ran on agarose gel 1% on electrophoresis to quantify the DNA and the bands compared to a DNA standard.

Appendix T. Preparation of 1% agarose gel electrophoresis for separating DNA by size

- ✓ **Equipment -** Casting tray, Well combs, Voltage source, Gel box, UV light source, Microwave.
- ✓ **Reagents -** TAE buffer, Agarose powder, Ethidium bromide (stock concentration of 10 mg/mL).

Procedure

1. Measure 1 g of agarose.
2. Mix 1gram of agarose powder in 100 ml TAE in a microwavable flask.

   *(**Note:** The same buffer was used in the gel box)*

3. Microwaved for 1-3 minutes until the agarose was completely dissolved (but do not overboil the solution, as it may cause the buffer to evaporate, thus altering the final percentage of agarose in the gel. Microwaving was done in pulses of 30 seconds, stop and swirl as the solution heats up).

4. Let the agarose solution cool down to about 50 °C (about when you can comfortably keep your hand on the flask) for about 5 minutes.

Appendix U. Table of Nanodrop

Sample ID	**Nucleic Acid Conc**	**Unit**	**260/280**	**Sample Type**
1	75	ng/µl	1.75	DNA
2	88.4	ng/µl	1.68	DNA
3	30.3	ng/µl	1.76	DNA
4	54.9	ng/µl	1.7	DNA
5	152.6	ng/µl	1.52	DNA
6	74.2	ng/µl	1.51	DNA
7	325.1	ng/µl	2.06	DNA
8	111.4	ng/µl	1.87	DNA
10	74.2	ng/µl	2.37	DNA
11	116.1	ng/µl	2.17	DNA
13	82.1	ng/µl	2.36	DNA
14	101.1	ng/µl	2.09	DNA
15	119.9	ng/µl	1.79	DNA
17	238.4	ng/µl	1.5	DNA
26	374.3	ng/µl	1.61	DNA
18	65.7	ng/µl	2.4	DNA
19	114.8	ng/µl	1.76	DNA
20	51.6	ng/µl	2.88	DNA
21	132.5	ng/µl	2.22	DNA
22	149.6	ng/µl	1.63	DNA
23	163.9	ng/µl	2.11	DNA
24	92.9	ng/µl	1.78	DNA
25	74	ng/µl	2.41	DNA
16	136.6	ng/µl	1.82	DNA
28	40.8	ng/µl	2.23	DNA
30	64.6	ng/µl	2.53	DNA
31	81.8	ng/µl	2.16	DNA
32	111.6	ng/µl	2.39	DNA
33	294.2	ng/µl	2.02	DNA
34	288	ng/µl	2.17	DNA
35	11.4	ng/µl	2.09	DNA
36	19.4	ng/µl	1.98	DNA
37	37.1	ng/µl	2.05	DNA
39	30.9	ng/µl	2.06	DNA
40	17.2	ng/µl	2.05	DNA
41	28.2	ng/µl	2.09	DNA
45	56.4	ng/µl	1.99	DNA
44	18.1	ng/µl	2.12	DNA
46	87	ng/µl	1.98	DNA
48	111.7	ng/µl	1.91	DNA
49	48.5	ng/µl	1.72	DNA

51	91.3	ng/µl	1.94	DNA
52	83.6	ng/µl	1.94	DNA
53	144.6	ng/µl	1.88	DNA
54	64.3	ng/µl	1.93	DNA
55	59.4	ng/µl	2.12	DNA
56	215.3	ng/µl	1.99	DNA
57	93.1	ng/µl	2.02	DNA
58	62.3	ng/µl	1.96	DNA
59	92.6	ng/µl	1.95	DNA
60	119.7	ng/µl	1.67	DNA
61	583.1	ng/µl	1.93	DNA
62	117.1	ng/µl	2	DNA
63	168.3	ng/µl	2.24	DNA
64	132.1	ng/µl	2.03	DNA
65	170.9	ng/µl	2.04	DNA
66	36.6	ng/µl	2.49	DNA
67	167.6	ng/µl	2.07	DNA
68	168	ng/µl	2.01	DNA
69	201.5	ng/µl	1.79	DNA
70	39.5	ng/µl	1.91	DNA
71	83.7	ng/µl	2.08	DNA
72	190.3	ng/µl	2.27	DNA
73	169.4	ng/µl	2.4	DNA
74	-1.7	ng/µl	0.86	DNA
75	-2.9	ng/µl	0.88	DNA
76	229.1	ng/µl	2.28	DNA

Appendix V. Table for DNA bands scores

Sample	M02	M03	M05	M10	M15	M16	M19	M22	M25	M26	M28	M31	M47	M50
BGM/1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BGM/2	0	0	0	0	0	0	0	0	0	0	1	0	0	0
BGM/3	0	0	0	0	0	0	0	0	0	0	1	0	0	0
BGM/4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BGM/5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BGM/6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BGM/7	0	0	0	0	0	0	1	1	0	0	1	0	0	0
BGM/8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BSA/1	0	0	0	0	0	0	1	1	0	0	1	0	0	0
BSA/2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BSA/3	0	0	0	0	0	0	0	0	0	0	1	0	0	0
BSA/4	0	0	0	0	0	0	1	1	0	0	1	0	0	0
BSA/5	0	0	0	0	0	0	1	1	0	0	1	0	0	0
BSA/6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BSA/7	0	0	0	0	0	0	1	1	1	0	0	0	0	0
BSA/8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BSA/9	0	0	0	0	0	0	1	1	0	0	0	0	0	0
BSA/10	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BSA/11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BSA/12	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BSA/13	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BSA/14	0	0	0	0	0	0	0	0	1	0	0	0	0	0
BSA/15	0	0	0	0	0	0	1	1	1	0	1	0	0	0
BSA/16	0	0	1	0	1	0	0	0	0	0	0	0	0	0
BSA/17	0	0	1	0	1	0	1	1	1	0	0	0	0	1
MSA/1	0	0	0	0	0	0	0	0	1	0	1	0	0	1
MSA/2	0	0	0	0	1	0	0	0	0	1	0	0	0	0
MSA/3	0	0	0	0	1	0	0	0	1	1	0	0	0	0
MSA/4	0	0	1	1	0	0	0	0	0	0	0	0	0	0
MSA/5	1	1	1	1	1	0	0	0	1	1	0	0	0	0

MSA/6	0	0	1	0	0	0	0	0	1	1	0	0	0	0
MSA/7	0	0	0	1	1	0	0	0	0	0	0	0	0	0
MSA/8	0	0	0	1	1	0	0	0	0	0	0	0	0	0
MSA/9	1	1	0	1	0	0	0	0	0	1	0	0	0	0
MSA/10	1	1	0	0	0	0	0	0	1	0	0	0	1	1
MSA/11	1	1	0	0	1	0	0	0	0	0	0	0	1	1
MSA/12	0	0	0	1	0	0	0	0	1	0	0	0	0	0
MSA/13	0	0	0	1	0	0	0	0	0	0	1	0	0	0
MSA/14	1	0	0	0	0	0	1	0	1	1	1	0	0	0
MSA/15	1	0	0	0	1	0	1	1	1	1	0	0	0	1
MSA/16	1	1	1	0	1	0	1	1	0	0	0	0	1	0
MSA/17	1	0	1	0	1	0	0	1	0	0	0	0	0	0
MSA/18	1	0	0	0	1	0	1	0	0	0	1	0	0	0
MSA/19	0	0	0	0	1	0	1	1	0	1	0	0	0	1
MSA/20	0	0	0	0	0	1	1	1	1	0	1	0	0	0
MSA/21	0	0	1	1	1	0	0	1	1	1	0	0	0	1
KWL/1	1	0	1	0	1	1	1	1	0	1	0	0	1	0
KWL/2	0	0	0	0	1	0	1	1	0	0	1	0	1	0
KWL/3	0	0	1	0	1	0	1	1	1	1	0	0	1	1
KWL/4	0	0	0	0	1	1	0	0	0	1	0	0	1	0
KWL/5	0	0	0	1	1	0	1	1	0	1	0	0	0	0
KWL/6	0	0	0	1	1	0	0	0	0	0	1	0	0	0
KWL/7	0	0	0	1	1	1	1	1	1	0	1	0	0	0
KWL/8	1	0	1	1	1	0	0	0	1	0	1	0	1	0
KWL/9	0	0	0	0	1	1	0	0	0	0	0	0	0	0
KWL/10	0	0	0	0	0	0	0	1	1	0	1	0	0	0
KWL/11	0	0	0	1	1	0	1	1	1	0	0	0	0	0
KWL/12	1	0	0	0	0	0	0	1	1	0	0	0	0	1
KWL/13	0	0	0	0	0	0	1	1	0	0	0	0	0	0
KWL/14	0	0	0	1	0	0	0	0	0	0	0	0	0	0
KWL/15	0	0	0	1	1	0	1	1	0	0	0	0	0	0
KWL/16	1	0	0	1	1	1	1	1	0	0	0	0	0	1

KWL/17	0	0	0	1	1	1	0	0	0	0	0	0	0	1
KWL/18	1	0	0	1	1	1	0	0	0	1	1	0	0	0
KWL/19	0	0	0	0	1	0	0	0	0	0	1	0	0	0
KWL/20	0	0	0	0	1	0	0	0	0	0	1	0	0	0
KWL/21	0	0	1	1	1	0	0	0	0	0	1	0	0	0
KWL/22	0	0	0	1	1	0	0	0	0	0	0	0	0	0
KWL/23	0	1	0	1	1	0	0	0	0	0	0	0	0	0
KWL/24	0	0	0	0	1	0	0	0	1	0	0	0	0	0
KWL/25	0	0	0	1	1	0	0	0	0	0	1	0	0	0
KWL/26	0	0	0	1	1	0	0	0	0	0	0	0	0	0
KLF/1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
KLF/2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
KLF/3	0	0	1	0	0	0	0	0	0	0	0	0	0	0
KLF/4	0	0	1	0	0	0	0	0	0	0	0	0	0	0

Appendix W. Research permit

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: 729996

Date of Issue: 12/August/2020

RESEARCH LICENSE

This is to Certify that Ms.. LEAH NASIMIYU SIMIYU of Egerton University, has been licensed to conduct research in Bungoma, Busia, Kilifi, Kwale, Mombasa, Nakuru on the topic: MICROPROPAGATION AND DIVERSITY ASSEESSMENT OF VANILLA (Vanilla spp.) FROMSELECTED COUNTIES OF KENYA USING MORPHOLOGICAL AND DNA MARKERS for the period ending : 12/August/2021.

License No: NACOSTI/P/20/5987

729996

Applicant Identification Number

Director General

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document, Scan the QR Code using QR scanner application.

THE SCIENCE, TECHNOLOGY AND INNOVATION ACT, 2013

The Grant of Research Licenses is Guided by the Science, Technology and Innovation (Research Licensing) Regulations, 2014

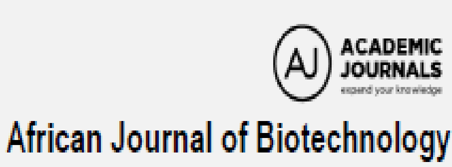
CONDITIONS

1. The License is valid for the proposed research, location and specified period
2. The License any rights thereunder are non-transferable
3. The Licensee shall inform the relevant County Director of Education, County Commissioner and County Governor before commencement of the research
4. Excavation, filming and collection of specimens are subject to further necessary clearence from relevant Government Agencies
5. The License does not give authority to tranfer research materials
6. NACOSTI may monitor and evaluate the licensed research project
7. The Licensee shall submit one hard copy and upload a soft copy of their final report (thesis) within one of completion of the research
8. NACOSTI reserves the right to modify the conditions of the License including cancellation without prior notice

National Commission for Science, Technology and Innovation
off Waiyaki Way, Upper Kabete,
P. O. Box 30623, 00100 Nairobi, KENYA
Land line: 020 4007000, 020 2241349, 020 3310571, 020 8001077
Mobile: 0713 788 787 / 0735 404 245
E-mail: dg@nacosti.go.ke / registry@nacosti.go.ke
Website: www.nacosti.go.ke

Appendix X. Research publication

Vol. 19(10), pp.736-746, October, 2020
DOI: 10.5897/AJB2020.17216
Article Number: A7A61E565167
ISSN: 1684-5315
Copyright ©2020
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJB



African Journal of Biotechnology

Full Length Research Paper

Diversity assessment of vanilla (*Vanilla* species) accessions in selected counties of Kenya using simple sequence repeats (SSRs) markers

Leah N. Simiyu*, Joseph N. Wolukau and Maurice E. Oyoo

Department of Crops, Horticulture and Soils, Faculty of Agriculture, Egerton University, P. O. Box 536-20115, Egerton, Kenya.

Received 1 August, 2020; Accepted 29 September, 2020

Diversity assessment of vanilla (*Vanilla* species) in Kenya is a key strategy for germplasm conservation and improvement. Production of vanilla crop in Kenya is limited due to inadequate knowledge on genetic diversity. This study was carried out to characterize 76 vanilla accessions from five counties of Kenya using 14 microsatellite DNA markers. POPGENE version 1.32 was used to compute variety factors. Amplicons ranged between 1 and 4. A total of 27 (96.43%) alleles were observed and their number ranged from 1.00 to 2.00 with a mean of 1.93. Effective allele values ranged from 1.00 to 1.99 with a mean of 1.63. Gene diversity ranged from 0 to 0.50 with a mean of 0.35, mean Shannon information index was 0.50 and Polymorphic information content values ranged from 0 to 0.38 with a mean of 0.35. Jaccard's similarity coefficient ranged from 0.08 to 1.00 with an average of 0.54. Unrooted phylogenetic tree was constructed in DARwin 6.0.8 using Unweighted Pair Group Method with Arithmetic Mean, clustering the samples into 3 main clusters (A 99.6%, B 98.96% and C 100%) and 6 sub-clusters (A1, A2, B1, B2, B3 and C1). Vanilla accessions grown in Kenya have a broad genetic background but low genetic diversity. Results inform the need to introduce other vanilla species as sources of genetic variation for breeding.

Key words: Genetic variation, DNA markers, breeding, Kenya.