PASSION FRUIT WOODINESS DISEASE IN COASTAL LOWLANDS OF KENYA AND GENETIC TRANSFORMATION SYSTEM OF FARMER PREFERRED PASSION FRUIT (*Passiflora edulis* SIMS)

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DECLARATION

I, Lydia Kwamboka Asande, declare that this thesis is my original work and has not been presented for a degree in any other University or for any other award.

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DEDICATION

I dedicate this thesis to my husband Mr. Thomas Bwaley and my daughter Libby Bwaley. I also dedicate it to my parents Mr. Samson Asande and Mrs. Rebecca Asande as well as my siblings.

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2, 4, 5- T	2, 4, 5-tricholorophenoxyacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
4-Cl-IAA	4-chloroindole-3-acetic acid
4-FA	4-flourophenoxyacetic acid
ABA	Abscisic acid
ACP	Antigen coated plate
ANOVA	Analysis of variance
A.s.l	Above sea level
AUDPC	Area under disease progress curve
B5	Gamborg medium
BAP	6-benzyl amino purine
Bp	Base pair
CABMV	Cowpea aphid borne mosaic virus
CaMV 35S	Cauliflower mosaic virus 35S protein
Conc.	Concentration
CP	Coat protein
CTAB	Cetyltrimethylammonium bromide
Dicamba	2-methoxy-3,6- dichlorobenzoic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EAPV	East Asian Passiflora Virus
EC	Embryogenic callus
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked Immunosorbent assay
GA_3	Gibberellic acid
g-DNA	Genomic DNA
GS	Globular stage
GUS	Beta-glucuronidase
HCDA	Horticultural Crops Development Authority
HS	Heart shaped
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KALRO	Kenya Agricultural and Livestock Research Organization
KB	Kilobase
KHCP	Kenya Horticulture Competitiveness Project
KIN	Kinetin
KPF	Kenya passion fruit

LB	Luria bertoni
LTB	Left T-DNA border sequence
MCPA	2-methyl-4-chlorophenoxyaceticacid
MCS	Multiple cloning site
Min	Minutes
Mm	Millimeters
mRNA	Messenger RNA
MS	Murashige and Skoog
MT	Megatonne
NAA	α-naphthalene acetic acid
NaCI	Sodium chloride
NaOH	Sodium hydroxide
NOA	2-naphthyloxyacetic acid
NOS	Nopaline synthase
nptII	Neomycin phosphotransferase II
OD	Optical density
PAA	2-phenylacetic acid
PaYMV	Patchouli Yellow Mosaic Virus
PCR	Polymerase chain reaction
PGRs	Plant growth regulators
Picloram	4-Amino-3, 5, 6-trichloro-2-pyridinecarboxylic acid
Poly A	Polyadenylation tail
PTGS	Post transcriptional gene silencing
PWD	Passion fruit woodiness disease
PWV	Passion fruit woodiness virus
RB	Right border
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
Rpm	Revolutions per minute
RT-PCR	Reverse-transcriptase polymerase chain reaction
SAPV	South African Passiflora Virus
SBM	Shoot bud initiation medium
SE	Standard error
SEM	Shoot elongation medium
SPD	Spermidine
SPM	Spermine
SRAP	Sequence related amplified polymorphism
SYN	Syntron
TAE	Tris-acetate-EDTA
T-DNA	Transfer DNA

Ti plasmid	Tumor inducing plasmid
TS	Torpedo stage
UPGMA	Unweighted pair group method with arithmetic mean
UPV	Ugandan Passiflora virus
UV	Ultraviolet
V/V	Volume/volume
Vir	Virulence
X-gluc	5-bromo-4-chloro-3-indolyl glucuronide
W/V	Weight/volume

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ABSTRACT

Passion fruit (*Passiflora edulis* [Sims]) is an important economic crop grown for both export and domestic market world-wide. In Kenya, passion fruit productivity is low due to both biotic and abiotic constraints. Passion fruit woodiness disease (PWD) complex is the most damaging viral disease causing yield losses of up to 100%. The most effective way to manage PWD is by using resistant cultivars. Conventional breeding of passion fruit for resistance against PWD is hampered by sexual barriers, narrow genetic variability, high ploidy levels and long generation cycles. Thus there is need to incorporate alternative approaches such as genetic transformation in passion fruit improvement. The objectives of this study were to determine the occurrence of passion fruit woodiness disease in selected Counties at the Coastal lowlands of Kenya and develop regeneration and transformation systems for farmer preferred passion fruit genotypes. A survey of PWD was undertaken in major passion fruit growing areas in Kwale and Kilifi Counties. Evaluation of five passion fruit genotypes for resistance to PWD was carried out under greenhouse and field conditions. For shoot induction and multiplication from leaf and nodal explants, respectively, Murashige and skoog (MS) medium supplemented with benzylamino purine (BAP) ranging from 1.0 - 3.0 mg L^{-1} as well as 2.0 mg L^{-1} BAP in combination with 0.5 mg L^{-1} kinetin were tested. The effect of putrescine on root induction was tested. The genetic fidelity of both *in vitro* regenerated and putrescine treated plants was established using sequence-related amplified polymorphism markers. Callus induction and somatic embryogenesis was carried out using leaf explants and immature seeds on MS supplemented with 0.5 – 16.0 mg L^{-1} 2, 4- dichlorophenoxyacetic acid (2, 4-D) and 1.0 mg L^{-1} thidiazuron (TDZ). Data on disease severity was used to compute area under disease progress curve (AUDPC). The data was subjected to analysis of variance and means were separated according to Tukey's Honest Significant Difference test at 5% confidence level. The highest disease incidence of 59.16% and 51.43% was observed at Kilifi and Kwale Counties, respectively. A significant difference ($p < 0.05$) in symptom severity was observed within the tested genotypes with purple and banana passion fruits presenting the highest and lowest AUDPC values, respectively, both under greenhouse and field conditions. Efficient induction and multiplication of shoots was achieved on MS supplemented with 2 mg L^{-1} and 3 mg L^{-1} BAP, respectively. The homogenous banding pattern based on SRAP analysis confirmed genetic uniformity of *in vitro* regenerated and macropropagated plants. Exogenous application of putrescine induced root formation on nodal explants. The conditions for optimum *gus* expression in the histochemical assays were optical bacterial density of 0.5, 30 min infection time, 200 µM acetosyringone and 3 days co-cultivation period. The regenerated putative transgenic lines showed presence of transgene by PCR. The *in vitro* regeneration system developed can be utilized for mass clonal propagation of passion fruit. The *Agrobacterium* mediated transformation system can be used to introduce genes which encode beneficial traits such as resistance to PWD and other agronomically important traits into KPF4 variety.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Passion fruit (*Passiflora edulis* Sims) is an economically important perennial fruit crop in many tropical and sub-tropical countries, mainly grown for its nutritional, ornamental and medicinal value (Zibadi and Watson, 2004; Borges *et al.*, 2005; Mwita *et al.*, 2016). It is ranked fourth amongst fruit exports in Kenya (HCDA, 2017). The crop is mainly grown by smallholder farmers who comprise of more than 80% of all growers, majority of whom are women (Kormelinck and Janssen, 2012; Karani *et al.,* 2015). Passion fruits are rich in iron, calcium, phosphorous and beta-carotene making it an important health food as source of vitamin A and C (Sema and Maiti, 2006; Cerqueira-Silva *et al.*, 2015).

Passion fruit is native to tropical America and widely grown in Brazil (Cerqueira-Silva *et al.*, 2015; Yang and Wang, 2020). In Kenya, the vine grows well in a vast range of altitudes of up to 1,800 m a.s.l in East of the Rift Valley and up to 2,000 m a. s. l. in West of Rift valley (Kormelinck and Janssen, 2012). Two distinct forms of *Passiflora edulis* Sims are predominantly grown in Kenya: the purple passion fruit which grows in cool environments at higher altitudes with an optimum temperature of 18 - 25 °C, and the yellow passion fruit, which grows in the tropical lowlands with an optimum temperature of 25- 30 °C. For both types of fruits, rainfall should be well-distributed, between 900 - 2,000 mm per year (NAFIS, 2008; Kormelinck and Janssen, 2012; Mwirigi *et al.*, 2016).

The world's total production of passion fruit is about 1.5 million tonnes. Brazil is the world's largest producer generating about 90% of the total production. This is followed by Peru, Venezuela and South Africa (Junqueira *et al.*, 2017; Altendorf, 2018). In Kenya, passion fruit is an important component of the horticulture industry which sustains millions of livelihoods through local and export markets (Gichimu *et al.*, 2013). Moreover, it has a commercial potential owing to the increasing demand for both fresh fruit and processed juice and also expanding export markets (Wangungu *et al.*, 2010). In 2017, passion fruit contributed Kshs 1.05 billion under an area of 2735 Ha and production of 21,983 MT (HCDA, 2017). However, although there is great potential in passion fruit, production has remained low at an average of 8 ton ha^{-1} compared to a potential of 24 ton ha^{-1} (Gichimu *et al.*, 2013; HCDA, 2017) mainly due to pests, diseases and inadequate disease-free planting materials (Wangungu *et al.*, 2010; HCDA, 2017). These constraints have also led to the reduction of the lifespan of this crop in Kenya from 7 years (Morton, 1987) to an average of 1 to 2 years (Gichimu *et al.*, 2013).

Among diseases limiting passion fruit production, is the passion fruit woodiness disease (PWD)-complex. This is mainly transmitted by aphids in all passion fruit production areas in Kenya (KALRO, 2001), affecting both the purple and the yellow forms (McCarthy, 1995). The disease has been reported to cause up to 100% loss in fruit yields in Kenya (Amata *et al*., 2009). The mode of propagation which entails use of seed and grafting has led to build-up especially of the woodiness virus complex resulting in total yield losses. The control of passion fruit woodiness disease is difficult and often impossible, mostly due to the non-circulative mode of virus transmission by several aphid vectors and to the fact that both *Cowpea Aphid Borne Mosaic Virus* (CABMV) and *Passion Fruit woodiness virus* (PWV) have several wild hosts that can serve as virus reservoirs (Bock and Conti, 1974). It is therefore necessary to control PWD in order to increase or at least maintain reasonable yields of passion fruit and protect susceptible germplasm from total loss. Woodiness virus-resistant varieties of passion fruit can be effective for virus control. A good genetic transformation system is vital for developing these varieties. This option is also important because host plant resistance has not yet been reported in cultivated species or close relatives with which they can be crossed, as a basis for conventional breeding and selection for the trait.

Nonetheless, the application of recombinant DNA technology for crop improvement calls for an efficient and reproduble genetic transformation system. Hence, there is need to establish an efficient and stable genetic transformation system for passion fruit which can be tailored to the precise needs of the changing environments in which passion fruit is grown across the world. This bids the potential to resolve yield gaps wherever they occur.

Micropropagation through stem nodal cuttings is the most popular and offers production of true-to-type plants in a short period of time and availability of superior individuals for large scale commercial plantation with quick productive gains (Kesari *et al.*, 2009; Pandey *et al.*, 2011). However, the difficulty in rooting of stem nodal cuttings is the main obstacle that should be overcome (Deshmukh *et al.*, 2017). Polyamines are growth regulators present in all plant tissues and are important for cell division, signal transduction and protein synthesis (Kusano *et al.*, 2008). They can speed up or slow down micro-shoot rooting, depending on their type and concentration. An innovative *ex vitro* rooting protocol that uses *in vitro* explants derived from short-term cultures is required to offer an alternative for mass propagation of healthy planting materials of passion fruit and at the same time reduce the cost of production of micropropagated plantlets.

Maintaining the genetic stability of *in vitro* regenerated plants is indispensable before adopting the protocol for large-scale clonal propagation and conservation (Jin *et al.*, 2008). Genetic variability can be induced during micropropagation of plants due to the environment and culture conditions and therefore it is fundamental to confirm the clonal identity to the mother plants using molecular markers (Gupta and Roy, 2002).

1.2 Statement of the problem

Woodiness viral disease is the most limiting factor for passion fruit production worldwide (Cerqueira-Silva *et al.*, 2014a; Gonçalves *et al.*, 2017). Unlike bacterial and fungal diseases, viral diseases including woodiness have no effective chemical control on infected plants, thus causing heavy yield losses in most vegetatively propagated plants (Lebot, 2009). In Kenya, CABMV and PWV have been identified as the causative agents of the woodiness disease (Ngeranwa, 2012; Kilalo *et al.*, 2013). The prevalence of the disease in addition to its relationship with the aphid vectors in the field contributes to shortage of clean (disease-free) planting materials (Kilalo *et al.*, 2013). This has further led to reduction in production and longevity of passion fruit, reducing the life-span of passion fruit production from 7 to 1.5 years (Trevisan *et al.*, 2006). While passion fruit breeding programs have developed cultivars with high yield and fruits with preferable characteristics, it has not been possible to develop a cultivar of *P. edulis f. flavicarpa* Deg. with high resistance to CABMV (Gonçalves *et al.*, 2017). Lack of passion fruit woodiness disease resistant varieties and clean health planting materials has forced farmers in Kenya to abandon passion fruit cultivation.

Since the first passion fruit transgenic plants were reported (Manders *et al.*, 1994), more transgenic plants have been recounted (Trevisan *et al.*, 2006; Correa *et al.*, 2015; Tuhaise *et al.*, 2019). However, these previous reports involved complicated procedures which took more than 5 months to generate transgenic seedlings. In addition, previous reports on transformation were based on model yellow passion fruit cultivars, which are not farmer-preferred due to low yield.

1.3 Justification of the study

Although passion fruit woodiness disease is a major challenge in passion fruit growing locations in Western and Eastern Kenya (Kilalo *et al.*, 2013), no survey has been reported in the coastal lowlands of Kenya to establish disease incidence, severity and prevalence. Moreover, there have been lamentations from farmers reporting incidences of pests and diseases that devastate passion fruit orchards (Kilalo *et al.*, 2013). The present survey was vital in the advancement of proper and sustainable disease management measures in the coastal lowlands of Kenya.

Furthermore, control of PWD is difficult hence the need to generate virus resistant cultivars if productivity has to be increased (Trevisan *et al.*, 2006; Cerqueira-Silva *et al.*, 2014a). Conventional breeding strategies for passion fruit against PWD are limited by narrow genetic variability and long generation times with the breeding cycle for passion fruit germplasm development requiring on average 6-20 years (Hortinews, 2014). Therefore, there is need to use alternative approaches such as genetic engineering to develop resistant genotypes.

The development of transgenic plants requires an efficient regeneration and genetic transformation system. There is no report on *Agrobacterium*-mediated transformation of KPF 4 and purple passion available in Kenya which is the preferred method for genetic engineering. *Agrobacterium* mediated transformation of passion fruit is cultivar dependent (Tuhaise *et al.*, 2019) and due to the long generation cycles of passion fruit, it is not possible to transform one of the cultivars, and use it as a donor plant for breeding and backcrossing the transgenes into other passion fruit cultivars that may be difficult to transform. Thus the development of cultivar independent passion fruit transformation protocols would complement the crop improvement efforts and enhance food security in the region. *Agrobacterium*-mediated transformation system offers several advantages over direct gene transfer methodologies such as possibility of higher efficiencies with low cost and the transfer of very large DNA fragments with minimal rearrangement (Gelvin, 2003; Komari *et al.*, 2004). It also generates transformants with low gene copy numbers (Ishida *et al.*, 1996). Therefore, this study was carried out with the purpose of ascertaining the incidence and severity of passion fruit woodiness disease in coastal lowlands of Kenya, determining resistant genotypes and development of regeneration and transformation systems for farmer preferred genotypes.

1.4 Hypotheses

i. Passion fruit woodiness disease is not widely spread in Coastal Kenya.
ii. Passion fruit varieties grown in Kenya are not resistant to woodiness disease.
iii. An efficient regeneration system for farmer preferred passion fruits can be developed.
iv. An efficient transformation protocol for farmer preferred varieties of passion fruit can be established.

1.5 Objectives

1.5.1 General objective

To determine the occurrence of passion fruit woodiness disease in selected Counties at the Coastal lowlands of Kenya and develop a regeneration and transformation system for farmer preferred passion fruit varieties for sustainable management of biotic stresses.

1.5.2 Specific objectives

i. To determine the prevalence, incidence and severity of passion fruit woodiness disease in the coastal lowlands of Kenya.
ii. To determine resistance of selected passion fruit genotypes to woodiness disease under greenhouse and field conditions.
iii. To determine the effect of different concentrations of auxins and cytokinnins on *in vitro* regeneration of selected farmer preferred passion fruits grown in Kenya.

iv. To determine factors affecting the development and optimization of an *Agrobacterium*-mediated transformation system for selected Kenyan passion fruit cultivars using *gus* reporter gene

1.6 Significance of the study

Findings on the occurrence of passion fruit woodiness disease in the Coastal lowlands of Kenya would impact positively in the innovation of mitigation measures to improve productivity of passion fruits. Screening for resistance to woodiness disease is correspondingly indispensable in the identification of promising genotypes for integration into future breeding program to mitigate the damage caused by this disease. Findings on the development of a regeneration and transformation system for selected farmer preferred passion fruit cultivars available in Kenya will enable future transfer of agronomic traits to improve the overall productivity of the passion fruits. This will enhance food security of Kenyan communities that rely on passion fruit as a staple crop and as a source of income.

CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy of passion fruit

Passion fruit is a vigorous perennial vine belonging to the family Passifloraceae Juss. order Violales, class Magnoliopsida and division Magnoliophyta (Cerqueira-Silva *et al.*, 2014b). The family Passifloraceae is estimated to have a number of species ranging from 520 (MacDougal and Feuillet, 2004) to 700 (Feuillet *et al.*, 2004) and about 18 genera (Feuillet *et al.*, 2004). Examples of species include *Passiflora aurantia*, *P. cinnabarina*, *P. laurifolia*, *P. herbertiana*, *P. cupiformis*, *P. henryi*, *P. jugorum*, *P. moluccana*, *P. siamica*, *P. edulis*, *P. incarnata*, *P. quadrangularis*, *P. maliformis*, *P. alata*, *P. caerulea*, *P. foetida* (Cerqueira-Silva *et al.*, 2014a).

In Kenya, five species of edible forms of passion fruit are cultivated. These are purple passion (*P. edulis f. edulis* (Sims), yellow passion (*P. edulis var flavicarpa*), sweet granadilla (*P. ligularis*), banana passion (*P. mollissima*) and giant granadilla (*P. quadrangularis*) (NAFIS, 2008; Ghosh *et al.*, 2017). Other varieties developed by Kenya Agricultural and Livestock Research Organization (KALRO) through crossing purple and yellow varieties of passion fruit include KPFs 4, 11 and 12 (Ssemwanga, 2007). The purple form is more preferred by farmers due to its sweetness, reduced acid content plus a richer aroma (Fushimi *et al.*, 2001; Prammanee *et al.*, 2011; Hortinews, 2014).

2.2 Passion fruit karyotype and chromosome morphology

The analysis of karyotypes allows the evaluation and comparison of chromosome variation between closely related taxa (Viana and Souza, 2012). This is an important tool for differentiation of similar species within the same subgenus or species that are taxonomically clustered. The chromosome number allows use of this information in taxonomy and in evolutionary study of the genus, although such cytological markers do not reveal structural chromosomal modifications (Melo and Guerra, 2003; Viana and Souza, 2012). The genus *Passiflora* has well established cytogenetics and exhibits karyotype diversity, as different ploidy levels exist between subgenera that are characterized by different basic chromosome number: x = 6 ($2n$ = 12, 24 and 36) in the subgenus *Decaloba*, x = 9 ($2n$ = 18, 36 and 72) and x = 10 ($2n$ = 20) in the subgenus *Passiflora*, and x = 12 ($2n$ = 24) in the subgenera *Astrophea*, *Deidamioides* and *Tetrapathea* (Hansen *et al.*, 2006). The genus *Passiflora* has been described as diploid but the chromosome number varies, for example *P. edulis* and *P. alata* have $2n$ = 18, while *P. foetida* has $2n$ = 20 (Melo *et al.*, 2003; Souza *et al.*, 2008). *Tetra-*, *hexa-*, or *octoploid* species are rare (Melo *et al.*, 2003). Although there is no precise estimation of the basic number of chromosomes across the genus, studies have suggested 6 or 12 as the precise number (Hansen *et al.*, 2006). Based on the karyotypic formula, the genus *Passiflora* has metacentric (m) and submetacentric (sm) chromsomes (Souza *et al.*, 2008).

The basic chromosome number (x) of the genus has been a matter of controversy, and $x = 3$, 6, 9 and 12 have been proposed (Hansen *et al.*, 2006; Melo and Guerra, 2003; Melo *et al.*, 2001). The lower number of rDNA sites in species with $2n = 12$ suggested an ancestral $x = 6$ for the genus, with increase of rDNA sites in polyploid lineages (Melo and Guerra, 2003). On the other hand, Hansen *et al.* (2006) employed a maximum parsimony (MP) approach (giving all transition types equal weights) to reconstruct ancestral chromosome numbers in the genus. The authors hypothesized $x = 12$ as the basic chromosome number of the genus. This placement implied descending dysploidy (from $n = 12$ to $n = 9$ and $n = 6$) without events of polyploidy.

2.3 Passion fruit propagation

Propagation of passion fruit can be achieved via seeds, stem cuttings or grafting (Alexandre *et al.*, 2009; Thokchom and Mandal, 2017). Propagation through stem cuttings is common all over the world in order to retain all vital superior traits of the genotype like pest and disease resistance, fruit size and fast maturity. However, the vegetative propagation method possesses the risk of carry-over of pathogens from mother plant to the next generation (Knight and Sauls, 1994). Therefore, commercial farmers of passion fruit worldwide prefer using seedlings in establishing plantations, in order to curb the spread of passion fruit pathogens like woodiness virus (Otoni *et al.*, 2013). Nevertheless, propagation through seeds has challenges of insufficient and seasonal supply (Isutsa, 2004).

2.4 Seed germination

In most species of plants the period of seed development and seed germination is separated by a season of dormancy characterized by failure of seeds to germinate due to factors associated with the seed coat or embryo (Montana *et al.*, 2014). Pre-germination experiments have been conducted using concentrated sulphuric acid for 5 minutes, soaking in tap water for 7 to 14 days and fermentation in 10% sucrose (Mabundza *et al.*, 2010). Enhancement of germination in passion fruit seeds using sodium chloride (salinity) has also been documented (Montana *et al.*, 2014). In spite of results from these experiments, there is need for more research, because variable germination rates are observed when available protocols are applied for seed germination (Gil *et al.*, 2015).

2.5 Economic and nutritional importance and distribution of passion fruit

In 2017, passion fruit was ranked third (1.75%) after avocado (80.62%) and mango (15.84%) in Kenya in terms of foreign exchange earnings (HCDA, 2017). Its major markets are both domestic and regional (HCDA, 2017). Kenya is the market leader of fruit juice exports in East Africa and also among the large scale producers of passion fruit in African countries (KHCP, 2011). In Kenya, passion fruit is an important high market value horticultural crop (Wangungu *et al.*, 2011; HCDA, 2017). Passion fruit is used as food, which has high content of iron and vitamin C. Rinds are a good source of fiber and pectin (Silva *et al.*, 2008) while leaves and pulp contain free amino acids, alkaloids and flavonoids, mainly *C*-glycosylflavones (Zeraik *et al.*, 2010). The plant is also used in the making of cosmetics and phytotherapeutic products (Rudnicki *et al.*, 2007). In Italy, passiflorine has been extracted from dry leaves of *Passiflora edulis.* The fruits also have important antioxidants found to deter the fast growth of malignant cells (Sridhar, 2011). Research shows that the consumption of purple passion fruit peel extract can reduce asthma symptoms (Watson *et al.*, 2008). The seeds produce 23% oil comparable to oils from sunflower and soybean which have several industrial uses. The oil from passion fruit seeds has antibiotic, antiulcer and spermicidal properties (Okwu, 2004).

In Kenya, *Passiflora* was first introduced in Kisii and Sotik areas in 1920s before spreading to all other regions that favor its production (HCDA, 2005). Among the Counties growing passion fruit in Kenya, Kwale County presents the highest tonnage (1,075) of yellow passion (Table 2.1) while Elgeyo Marakwet presents the highest tonnage (4,672) of purple passion (Table 2.2).

Table 2.1: Production of yellow passion fruits in selected counties in Kenya, 2016-2017

County	2016			2017			% of Total value
	AREA (HA)	VOLUME (Tons)	VALUE (KES)	AREA (HA)	VOLUME (Tons)	VALUE (KES)	
Kwale	63	706	21,680,000	115	1,075	30,450,000	23
Meru	102	848	41,755, 000	41	443	21,645,000	16
Bungoma	50	450	14,500,000	55	475	13,500,000	10
Embu	31	247	10,770,000	36	261	11,670,000	9
Kilifi	27	163	4,890,000	47	516	11,267,440	8
Kitui	12	44	1,300,000	50	400	10,000,000	7
Migori	205	920	19,134,000	98	395	9,100,000	7
Busia	21	239	6,147,166	20	172	4,613,332	3
Taita Taveta	35	146	3,474,170	46	177	4,094,170	3
Kisumu	51	94	3,803,159	53	106	3,808,159	3
Siaya	4	18	400,000	20	128	3,640,000	3
Kisii	39	457	16,610,000	15	98	3,440,000	3
Tharaka Nithi	15	60	2,600,000	15	60	2,600,000	2
Vihiga	4	22	1,150,000	9	51	2,400,000	2
Kakamega	16	52	3,040,000	18	53	1,338,000	1
Others	77	738	25,351,000	3	14	661,000	0
TOTAL	**751**	**5,203**	**176,604,495**	**639**	**4,422**	**134,222,101**	**100.0**

Source: HCDA validated report 2016 – 2017.

Table 2.2: Production of purple passion fruits in selected counties in Kenya, 2016-2017

County	2016			2017			% of Total value
	AREA (HA)	VOLUME (Tons)	VALUE (KES)	AREA (HA)	VOLUME (Tons)	VALUE (KES)	
Elgeyo Marakwet	438	4,809	205,760,000	381	4,672	206,600,000	22
Uasin Gishu	82	1,023	72,980,000	128	1,132	60,076,000	7
Kirinyaga	70	943	46,890,000	75	1,112	53,120,000	6
Embu	47	752	41, 400,000	60	934	52,700,000	6
Baringo	33	307	15,235,000	88	1,114	49,020,000	5
Kiambu	51	548	32, 964,000	55	743	43,212,000	5
Meru	87	540	25,050,000	100	806	42,890,000	5
Kitui	12	44	2,200,000	24	66	42,000,000	5
Machakos	112	607	41,766,000	108	620	41,950,000	5
Narok	84	870	38,880,005	92	848	41,130,000	4
Bomet	114	1,790	107,400,00	134	484	29,040,000	3

Nyamira	59	323	21,737,000	62	616	28,580,000	3
Trans Nzoia	43	442	26,890,000	41	380	22,690,000	2
Kericho	30	500	27,927,500	28	435	22,270,750	2
Siaya	34	143	9,572,800	52	392	21,792,000	2
Others	857	8,392	342,090,041	670	3,207	163,641,367	18
TOTAL	**2,151**	**22,031**	**1,058,742,346**	**2,096**	**17,561**	**920,712,317**	**100.0**

Source: HCDA validated report 2016 - 2017

Kwale and Kilifi Counties can be distributed over five Agro Ecological Zones (AEZ) (Figure 2.1) based on the mean annual precipitation, mean annual temperatures, topography, soil and vegetation that impact the potential of agricultural production (Jaetzold *et al.*, 2007).

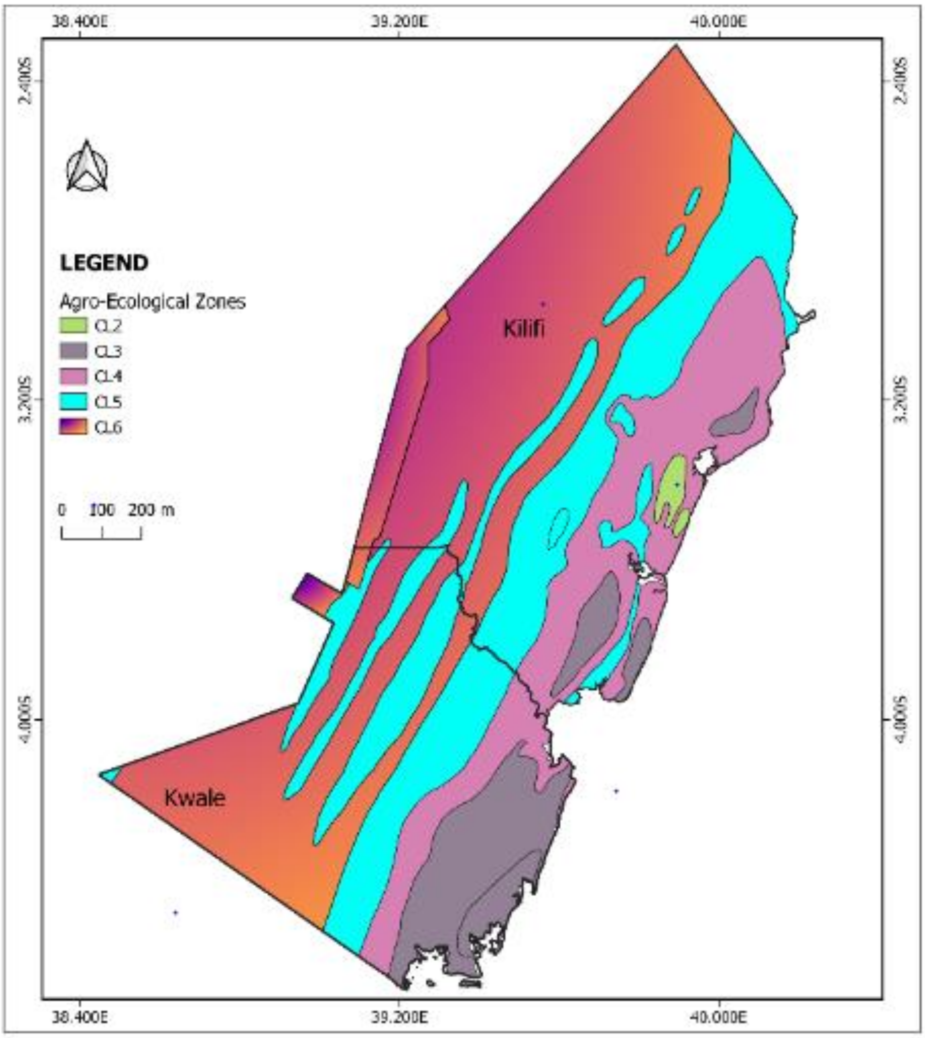


Figure 2.1: A map of Kwale and Kilifi Counties displaying Agro ecological zones (Map generated using shape files obtained from Kenya soil survey).

The AEZ (s) include Lowland Sugarcane Zone, (CL2) which only occurs in Kilifi County and is marked by a high yield potential (average 60–80 per cent of the optimum) for all vegetables, maize, sweet potatoes, simsim, sorghum, sunflower, cowpeas and soya beans. Cassava, avocadoes, bananas, bixa, coconuts, paw paws, mangoes and curcuma can be cultivated throughout the year. It is a rainy zone with an average annual rainfall of above 1200 mm per annum. CL2 covers approximately 23,500 ha and is appropriate for cassava and coconut production.

Coconut-Cassava Zone (CL3): This zone has a good yield potential for crop production in both Counties. It is the chief cropping zone of both Counties spreading along low-level coastal plains and the coastal uplands. It exhibits an average annual temperature of 24 °C and a mean annual precipitation of 1,300 mm per annum. This zone is suitable for tropical fruits such as passion fruit. Major tree crops include coconuts, mango, bixa, cassava, paw paw, avocadoes, pineapples, guavas, citrus and cashew nuts, vegetables such as okra, brinjals, cabbages and kales, food crops such as maize, sorghum, bananas, potatoes, beans, cowpeas, green grams, tomatoes and onions. Pasture and forage can also be grown in this zone.

Cashew nut - Cassava Zone (CL4): This area occurs expansively in both counties with a greater portion in Kilifi County. It spreads northwards along the coastal plain up to Sokoke forest. It displays an average annual precipitation of 900 mm and average annual temperature of 24 ºC. It is characterized by a small to good agricultural potential with similar crop types as in CL3 (Coconut-Cassava zone), however with less production. Pasture and forage can also be grown. However, the yield is less compared to the coconut-cassava zone.

Livestock-Millet Zone (CL5): The zone generally has a lower agriculture potential although few areas have good yield potential. It is marked by an annual mean precipitation of 700 – 900 mm. The zone is appropriate for dry land farming primarily drought tolerant crops. Millet, cassava, sorghum and green grams are suitable for this zone. Furthermore, small leaved bush land and livestock ranching are predominant in this region.

Lowland Ranching (CL6): This zone is characterized by poor soils and has the least potential for agricultural production. It has an average annual precipitation of 350-700 mm and mean annual temperature of 27 ºC. Its altitude ranges from 90 m to 300 m above sea level. The area is also marked with short grass diversified with small leaved bush land. Major activities in this area include livestock ranching and wildlife.

2.6 Constraints to passion fruit production

Passion fruit production in Kenya is limited by both biotic and abiotic factors. These include pests, diseases and drought. Pests and diseases lead to scarcity of clean planting materials (HCDA, 2013) and high production costs. Passion fruit diseases are caused by fungus, viruses and bacteria. Examples of passion fruit diseases causing major losses are *Fusarium* wilt, root rot, brown spot, septoria, crown rot, phytophthora blight, bacteriosis, woodiness disease (Gesimba, 2008; Mbora *et al.*, 2008; Amata *et al.*, 2009; Wangungu *et al.*, 2011).

In Kenya, passion fruit woodiness disease, die back, *Fusarium* wilt and bacteriosis, are among the main diseases that limit passion fruit production (Gesimba, 2008; Amata *et al.*, 2009).

2.6.1 Viral diseases

Passion fruit production can be affected by various viral diseases. These include leaf mottle disease, passion fruit mosaic disease, passion fruit vein clearing disease and passion fruit woodiness disease.

2.6.1.1 Leaf mottle disease

The disease is caused by *Passiflora leaf mottle virus*. Symptoms include: severe curling of leaves, deformation and mottling of leaves and fruits. The disease causes a reduction in yields and fruit quality (Novaes *et al.*, 2003). Infected passion fruit displays intense yellow mosaic of leaves and severe reduction in the leaf lamina (Novaes *et al.*, 2003). The virus is spread by *Bemisia tabaci* from infected passion fruit. Control of the disease can be achieved by using virus free seedlings for new plantings. Chemical control of viruses has been reported to be ineffective (Jones, 2003).

2.6.1.2 Passion fruit mosaic disease

The disease is caused by passion fruit yellow mosaic virus (Morales *et al.*, 2002). Symptoms include: bright yellow mosaic, yellow net and leaf crinkle (Fischer and Rezende, 2008). The virus is spread by *Diabrotics speciosa,* a vector commonly found in plantations of passion fruit.

2.6.1.3 Passion fruit vein clearing disease

The disease is caused by passion fruit vein clearing rhabdovirus family (Gardner, 1989; Yockteng *et al.*, 2011). The diseased plants display clearing of veins and small leaves and fruits. The disease can be contained using disease free seedlings and abolition of old plantations before planting new vines. There is no effective chemical control of passion fruit viruses (Pares *et al.,* 1983).

2.6.1.4 Passion fruit woodiness disease

Woodiness viral disease is the most limiting factor for passion fruit production worldwide as reported by Gonçalves *et al.* (2017). The viral pathogens designated as putative etiological agents of woodiness disease include

Passion Fruit Woodiness Virus (PWV) in Australia and Brazil (Iwai *et al.*, 2006), *East Asian Passiflora Virus* (EAPV) in Japan (Iwai *et al.*, 2006), *South African Passiflora Virus* (SAPV) in South Africa (Brand *et al.*, 1993), which was later found to be synonymous with CABMV (McKern *et al.*, 1994; Nascimento *et al.*, 2006) and Ugandan *Passiflora* virus (UPV) (Ochwo-Ssemakula *et al.*, 2012). In Kenya, *Cowpea Aphid Borne Mosaic Virus* (CABMV) has been reported as one of the causal agents of woodiness disease (Kilalo *et al.*, 2013; Munguti *et al.*, 2019). CABMV is also reported to be the main cause of woodiness disease in Brazil (Santana *et al.*, 1999; Nascimento *et al.*, 2006; Cerqueira-Silva *et al.*, 2008; Cerqueira-Silva *et al.*, 2014a), Uganda and Rwanda (Ochwo-Ssemakula *et al.*, 2012). *Cowpea Aphid Borne Mosaic Virus* (CABMV) belong to the genus *potyvirus*, family *potyviridae*; one of the largest groups of plant viruses (Berger *et al.*, 2005).

2.6.1.4.1 Symptoms, mode of spread and management of passion fruit woodiness disease

Cowpea Aphid Borne Mosaic Virus, causing woodiness disease in passion fruit, is known to be transmitted naturally by several aphid species including, *Aphis gossypii*, *A. fabae* and *Myzus persicae* in a non-persistent and non-circulative manner (Kilalo *et al.*, 2013). However, the major means by which the disease is spread is through vegetative propagation of infected plants, often contributing to multiple virus infections. If any of the mother plants are infected during vegetative propagation, it consequently increases the number of virus infected plants. Symptoms of CABMV on passion fruit leaves include severe mosaic, yellow spots, mottling, rugosity and distortion. Infected plants produce small, deformed fruits with a thick hard and woody rind, cracked fruits and small pulp cavity (Nascimento *et al*., 2006; Correa *et al*., 2015). The stems become shortened.

Currently, several cultural practices have been recommended to help prolong the life of orchards and minimize disease problem. These include, most importantly use of virus-free seedlings from certified nurseries, avoiding intercropping with leguminous plants which harbor the viruses and complete elimination of old and infected plants before new planting as they serve as new sources of infection (Gioria *et al*., 2006*)*. Pruning and thinning to prevent mechanical transmission of the viruses, systemic elimination of symptomatic plants within the first seven months after transplantation to the field is also a common practice (Cerqueira-Silva *et al*., 2014).

2.7 Genetic improvement of passion fruit through conventional breeding

Genetic improvement of passion fruit is fundamental for the enhancement of production in Kenya and the world. The main focus of genetic improvement of passion fruit is in the enhancement of fruit quality in order to meet the market demand, attainment of high yields and development of varieties resistant to pests and diseases (Viana *et al*., 2016). Santos *et al*. (2015) assessed resistance to Cowpea Aphid-Born Mosaic Virus in 178 genotypes of Passion fruit (*P. edulis* x *P. setacea*). The findings recommended that selected resistant hybrid plants can be recombined with *P. edulis* cultivars and reevaluated to verify their resistance to CABMV. Freitas *et al.* (2015) on the other hand, screened 369 genotypes of passion fruits for resistance to diseases to identify resistant cultivars in populations of *Passiflora* obtained through backcrossing. Their findings indicated that inheritance of disease resistance is polygenic and that selection is beneficial if large populations are used and more advanced breeding methods are employed such as back crossing. Molecular technology has also been employed in passion fruit breeding programs to reduce the long generation cycles and resources needed to retrieve genes of interest in the complex backcrossing cycles (Cerqueira-Silva *et al.*, 2015). Specifically, molecular markers have been utilized in characterization of genetic variability and construction of genetic maps (Ribeiro *et al.*, 2019).

2.8 *In vitro* culture and plant regeneration

2.8.1 Plant tissue culture

Tissue culture is the regeneration of entire plants from cells, tissues and organs as explants in nutrient-rich media under aseptic and controlled conditions. Plants cells and tissues under suitable environments have the ability to induce cell division, differentiation and development to mature plants. The ability of plants to regenerate *in vitro* under optimum conditions and express full genetic potential of the mother plant is referred to as totipotency (Shariatpanahi *et al.*, 2006).

The optimum *in vitro* conditions are genotype specific which include composition of culture medium and the external environment. The nutrient medium typically comprises of macro- and micro-nutrients necessary for growth and development, vitamins and amino acids (Murashige and Skoog, 1962). Other components are supplements which include carbon source, hormones (plant growth regulators), gelling agents, complex extracts (plant extracts), organic nitrogen compounds and organic acids (Singh, 2018; Oseni *et al.*, 2018).

Micropropagation is an essential tool for the retrieval, conservation of germplasm and embryo rescue. It facilitates genetic modification of plants via somatic hybridization which formerly was difficult to achieve, thus has helped to address the challenges of conventional breeding. It is also effective in maintaining disease free plants and avoiding genetic instability (Delgado-Sanchez *et al.*, 2006).

2.8.2 Factors affecting *in-vitro* plant regeneration

2.8.2.1 Plant growth regulators

Plant hormones are key media components, which determine the regeneration pathway the explant will take. Naturally, phytohormones are produced in minute quantities and they control particular physiological properties in plants (Waadt *et al.,* 2015). They also have an intense effect on the morphology of the tissue or organ formed from the explants. The hormones are categorized into gibberellins, abscisic acid, ethylene, auxins such as Indole-3-acetic acid, α- naphthalene acetic acid and cytokinins such as 6-benzyl amino purine and kinetin (Waadt *et al.*, 2015).

Gibberellins are key in promoting cell elongation and essential constituents influencing plant development. Among the different gibberellins, GA_3 is the most frequently used (Davies, 2004). It also has an auxin like activity of promoting callus growth although it inhibits growth of somatic embryos. It also enhances shoot formation once meristems are formed (Moshkov *et al.*, 2008). Ozarowski *et al.* (2012) reported an increased shoot regeneration rate plus a high bud forming capacity index from nodal explants of *P. caerulea* when the tissue culture media was supplemented with BA in combination with gibberellic acid (GA_3).

Abscisic acid constrains cell division and mostly promotes development of somatic embryos (Rai *et al.*, 2011). Some plant cells release ethylene which inhibits growth and development of explants (Kumar *et al.*, 1998*)*.

Auxins control cell division, growth of cells, initiation and development of roots (Finet and Jaillais, 2012). In combination with cytokinins they enhance cell division in callus cultures and deter root growth (Machakova *et al.*, 2008). At low concentrations, the auxins stimulate root induction (Machakova *et al.*, 2008).

Indole-3-acetic acid (IAA), 4-chloroindole-3-acetic acid (4-Cl-IAA) and 2-phenylacetic acid (PAA) are natural auxins synthesized in plants while 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-butyric acid (IBA), α-Naphthaleneacetic (α-NAA), 2,4,5-tricholorophenoxyacetic acid (2,4,5- T), 2-methoxy-3,6- dichlorobenzoic acid, 2-naphthylacetic acid (NOA), 4-amino-2,5,6-trichloropicolinic acid (picloram), 4-flourophenoxyacetic acid (4-FA) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) are classified as synthetic auxins (Korasick *et al.*, 2013).

In *in vitro* regeneration, endogenous auxins have restricted activity resulting from instability since they are easily altered by oxidation and increased light intensity (Dunlap and Robacker, 1988). Synthetic auxins are the most frequently used and are effective in tissue culture for they are not quickly metabolized by the plant as compared to natural auxins (Dunlap *et al.*, 1986). Since they are also not destroyed by oxidases they persist in plants (Salisbury and Ross, 1986). Synthetic auxins are broadly used in *in vitro* regeneration and transformation of passion fruit (Oggema *et al.*, 2007; Santa-Maria *et al.*, 2009).

Like auxins, cytokinins are also an essential regulator for different features of plant growth and development. Cytokinins promote cell division, initiation and growth of shoots (Murai, 2014; Dani *et al.*, 2016; Malinowski *et al.*, 2016). In *in vitro* regeneration, the ratio of auxin to cytokinin has an effect on callus differentiation, where high auxin: cytokinin promotes callus formation while low auxin: cytokinin promotes shoot initiation (Gaspar *et al.*, 2003).

Adenine-type and phenylurea-type are the two kinds of cytokinins documented. Adenine-types include kinetin, benzylaminopurine (BAP) and zeatin whereas phenylurea-types include diphenylurea and thidiazuron (TDZ) (Jabłońska-Trypuć *et al.*, 2016). Unlike zeatin, kinetin, BAP and thidiazuron are more commonly used in plant tissue culture medium due to their relative stability (Szucova, 2009). Kinetin is well-known to control cell division in presence of an auxin (Miller *et al.*, 1955; Murai, 2014).

2.8.2.2 Effect of explants used and genotype

Explants have a key role in many *in vitro* regeneration experiments. They determine the capacity to regenerate various cultivars or genotypes like passion fruit (Becerra *et al.*, 2004). Explant type, source and age affect the success of a tissue culture system. Examples of explants include hypocotyls, epicotyls, leaves with petioles, leaves, petioles, roots, stem cuttings, protoplasts, cotyledons, meristems, mature and immature zygotic embryos (Okada *et al.*, 2002*;* Shekhawat *et al.*, 2015)

In vitro regeneration of passion fruit has been done through organogenesis and somatic embryogenesis from several species, using many combinations of plant hormones and kinds of explants (Amugune *et al.*, 1993; Pinto *et al.*, 2011; Da Silva *et al.*, 2011; Rocha *et al.*, 2015; Shekhawat *et al.*, 2015; Ferreira *et al.*, 2015). Genotype also affects regeneration of plants in tissue culture. Same treatment given to different genotypes will generate different results. This has been documented in passion fruit among other plants such as rice and sweet potato (Yookongkaew *et al.*, 2007; Santa-Maria *et al.*, 2009; Marcin and Barbara, 2013).

2.8.2.3 Polyamines

Polyamines are organic compounds taking part in plant growth and development. They have important roles in controlling cell division (Zhao and Yang, 2008). The exogenous use of polyamines on stem cuttings for vegetative propagation has been successfully used in Desert ash (Tonon *et al.*, 2001), sweet orange (Mendes *et al.*, 2011) and Indian soybean (Arun *et al.*, 2014) to induce rooting. However, there is no report on the effect of polyamines on induction of roots on stem nodal cuttings of passion fruit.

2.8.3 Tissue culture techniques

2.8.3.1 Regeneration by organogenesis

Organogenesis refers to initiation of adventitious roots or shoots from explants or callus. Two kinds of organogenesis are documented; direct organogenesis occurs by direct initiation of plantlets from explants; while indirect organogenesis takes place when plantlets form via callus phase.

Plantlets regenerated through organogenesis arise from adventitious buds after initiation (Gahan and George, 2008). Organogenesis relies on plant plasticity and media composition such as the ratio of auxin to cytokinin (Gahan and George, 2008). Organogenesis in plants is also largely dependent on the levels of endogenous and exogenous hormones (Gaspar *et al.*, 2003). Hence it is vital to establish the optimum media composition required for organogenesis on the specific cultivar.

Organogenesis has been reported from root explants of wild passion fruit species (Cerqueira-Silva *et al.*, 2011), from leaf explants of yellow passion fruit in Kenya and Brazil (Amugune *et al.*, 1993; Trevisan *et al.*, 2005), nodal segments of commercial varieties in Uganda (Mukasa *et al.*, 2016) and cotyledon explants of an Australian passion fruit hybrid (Hall *et al.*, 2000).

2.8.3.2 Regeneration by somatic embryogenesis

Somatic embryogenesis is the induction of embryo like structures, analogous to zygotic embryos, from somatic cells, which can develop into whole plants. Two major steps in somatic embryogenesis are initiation and maturation (Jimenez, 2001). Embryogenesis can be direct or indirect. For direct embryogenesis, somatic embryos are formed directly from cells while in indirect somatic embryogenesis, somatic embryos are formed via a callus phase. Embryos develop through different stages ranging from globular to heart-shape to the torpedo shapes and lastly to cotyledonary stage forming the apical meristems (Von Arnold and Clapham, 2008).

Bunnag and Chamnanpon (2016) reported embryogenic callus induction from hypocotyls of passion fruit cultured on Murashige and Skoog solid medium supplemented with 0.5 mg L^{-1} 2, 4-dichlorophenoxy acetic acid and 1 mg L^{-1} 6-benzylaminopurine. Successful embryogenic callus induction has also been reported in different species of genus *Passiflora* using 2, 4-D (2, 4-dichlorophenoxyacetic acid) and KIN (Antognoni *et al.*, 2007). Pinto *et al.* (2011) reported successful induction of embryogenic callus of *P. edulis* with a combination of 2, 4-D and BAP (6-benzylaminopurine). Morphogenic callus induction has been reported from leaf discs cultured on MS medium supplemented with NAA, IAA and KIN (Amugune *et al.*, 1993). They also reported regeneration of shoots from leaf discs on BAP and KIN and induction of roots on 0.1- 1.0 mg L^{-1} NAA.

Green friable callus was induced efficiently from the leaf explants of *Passiflora foetida* L. (Passion fruit) on Murashige and Skoog (MS) medium supplemented with 2 mg L^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.5 mg L^{-1} kinetin (KIN) after 16 days (Rasool *et al.*, 2011). Combinations of picloram and kinetin have been reported to induce production of friable callus from leaf explants of *in vitro* germinated seedlings of native passion flower *Passiflora gibertii* (Artioli-Coelho *et al.*, 2015).

Somatic embryos have been reported from mature zygotic embryos of wild passion fruit (*Passiflora cincinnata*) cultured in Murashige and Skoog induction media supplemented with 2,4-dichlorophenoxyacetic acid and 6-benzyladenine (Rocha *et al.*, 2012). Pinto *et al.* (2011) reported induction of somatic embryos from zygotic embryos of FB-100, FB-200 and FB-300 passion fruits cultured in medium consisting of MS salts and B5 vitamins supplemented with 0.01 % (w/v) myoinositol, 3 % sucrose, 0.28 % (w/v) phytagel, 4.0 - 8.0 mg L^{-1} of 2, 4-D and 1 mg L^{-1} of BAP.

2.8.4 Genetic stability of *in vitro* regenerated plants

A major challenge associated with *in vitro* regeneration of plants is the occurence of genetic variability, a consequence of the environment and culture conditions. Therefore, it is vital to confirm the clonal identity to the mother plants using molecular markers (Gupta and Roy, 2002). Molecular techniques are potent and valuable tools used in genetic fidelity analysis of *in vitro* regenerated plants. Sequence-Related Amplified Polymorphism (SRAP) is a novel molecular marker technique based on two-primer amplification that preferentially amplifies open reading frames (ORFs) of genes (Li and Quiros, 2001). Due to their unique primer design, SRAP markers are more reproducible, more stable and highly simple in terms of operation in comparison to other molecular marker techniques (Li and Quiros, 2001). In addition, SRAP markers are more powerful than SSR, ISSR or RAPD markers in revealing genetic variation between different varieties within a species (Budak *et al.*, 2004) and are easier to assay than AFLPs (Li and Quiros, 2001). It has been proven that SRAP is more efficient for identifying epigenetic variations in *in vitro* cultured plants (El-Shahed *et al.*, 2017).

2.8.5 Plant genetic transformation

Genetic transformation is a technique of introducing foreign genes into a plant of interest and subsequent expression of the genes (Birch, 1997). Two methods of plant transformation have been documented namely, direct and indirect transformation. The direct method includes electroporation (Hassanein *et al.*, 2006) and particle gun bombardment (Okada *et al.*, 2002). Indirect methods include viral-mediated phage transfer (Wang *et al.*, 2005) and *Agrobacterium-mediated* transfer (Opabode, 2006). The methods have been utilized in different crop improvement programs like banana (Tripathi *et al.*, 2010), passion fruit (Trevisan *et al.*, 2006). Sweet potato has been transformed for resistance to sweet potato feathery mottle virus using microprojectile bombardment (Okada *et al.*, 2002) and for *gusA* gene using *Agrobacterium-mediated* transfer (Song and Sink, 2004).

2.8.5.1 Direct transformation methods

2.8.5.1.1 Electroporation

Electroporation involves use of an electrical pulse to improve cell membrane permeability by briefly opening the pores in the cell membrane hence improving the probability of gene transfer. Electroporation doesn't require specialized vectors and so well suited for a wide range of cells (Sambrook and Russell, 2001). However, this method results in low frequencies of stable transformants, very high DNA re-arrangements and in plants it can only be applied on protoplasts (Kandušer and Miklavčič, 2009).

2.8.5.1.2 Biolistics or micro-projectile

Biolistics require fast propelled tungsten microprojectiles coated with exogenous DNA fired onto cells or explants. This method doesn't require specialized vectors and can be applied on a wide range of plants since delivery of DNA is controlled by physical rather than biological factors. However, the probability of inserting the gene of interest within functional genes is high, resulting in undesired gene silencing (Zawleski *et al.*, 2012).

2.8.5.2 Indirect transformation methods

2.8.5.2.1 Virus-mediated gene transfer

Viral-mediated transfer is commonly applied in animal sciences and it involves use of viruses like retro-viruses. This method is highly efficient though not practical in producing stable introgression of foreign genes in plants (Akuta *et al.*, 2002).

2.8.5.2.2 *Agrobacterium*-mediated gene transfer

Agrobacterium is a gram negative soil bacterium known to cause crown gall disease in many dicot plants. It achieves this by infecting wounds and subsequently transferring its transfer DNA into the host. Virulence is conferred by a tumor-inducing plasmid (Ti plasmid) carrying genes encoding phytohormones and enzymes that catalyze the production of opines. The phytohormones are responsible for the decontrolled cell division causing crown gall growth, while opines are synthesized by the plant cells and utilized by the *Agrobacterium* for food. These genes are located on the T- DNA (transfer DNA) region of the Ti plasmid. The transfer of T-DNA into the plant genome is controlled by vir (virulence) genes on the Ti plasmid.

Scientists have taken advantage of the ability of *Agrobacterium tumefaciens* to transfer T-DNA into plant genome. In this case, the oncogenes in the T-DNA region have been substituted with beneficial genes (genes of interest) to facilitate artificial transfer into plant cells (Riva *et al.*, 1998). Transfer of T-DNA is instigated when *Agrobacterium* senses the presence of phenolics, which induce virulence (*vir*) genes (Gelvin, 2003).

Agrobacterium-based transformation is the best method for plant transformation owing to its simple operation, transfer of pieces of DNA with defined ends and minimal rearrangements, ability to insert relatively huge segments of foreign DNA into the host plant genome, low copy numbers, relatively low experimental cost and it is highly reproducible (Li *et al.*, 2017). *Agrobacterium-mediated* transformation has been utilized in soybean (Li *et al.*, 2017), passion fruit (Trevisan *et al.*, 2006), rice (Cao *et al.*, 2005) and sweet potato (Zang *et al.*, 2009) in production of transgenic plants.

2.8.5.3 Requirements for transgene expression

The necessities for transgene expression comprise of a gene construct with various elements to allow transfer of genes into the host plant genome. The elements include, gene of interest like disease resistance genes, or reporter genes e.g. beta-glucuronidase (*gus*) gene (Li *et al.*, 2017). Promoters are also key to the gene transfer process. They are used to control or drive genes. They can be classified as constitutive like cauliflower mosaic virus (CaMV) 35S

(Shimanda *et al.*, 2017) or inducible like sporamin (Chen *et al.*, 2016) and lastly polyadenylation signal sequence (Mayr, 2016*)*. Poly (A) sites may be at any location during gene transcription and efficiently regulate protein length or effect mRNA stability, translation and transport (Wu *et al.*, 2011).

2.8.5.4 Selectable markers

Many foreign genes incorporated into plant genomes usually do not confer a phenotype that can be appropriately used for identification of transformed cells. Therefore, a selectable marker gene is incorporated into the gene construct at the same time as the foreign DNA. This enables the transformed cells to stay alive in the presence of a selective agent, toxic to non-transformed cells. An example of a selectable agent is kanamycin (Acanda, 2017; Rana, 2017).

2.8.6 Improvement of passion fruit through genetic engineering

Reproducible *in vitro* regeneration and transformation protocols are essential pre- requisites for genetic improvement of any crop (Nyaboga *et al.*, 2013). Most of the transformation protocols for citrus fruits rely on friable embryogenic callus as the starting material for generation of transgenic plants. These embryogenic callus leads to development of uniformly transformed plants without chimeras because of their unicellular nature. Nonetheless, generation of friable embryogenic callus is tedious, prolonged and cultivar dependent. Besides, some passion fruit cultivars are recalcitrant to establishment of friable embryogenic callus. Hence the need to develop alternative regeneration and transformation protocols to generate transgenic plant within a short period and from a variety of cultivars.

One of the alternative approaches is the use of leaf explants for transformation of a variety of passion fruit cultivars. *Agrobacterium* mediated transformation of passion fruit using leaf explants has been reported (Quoirin *et al.*, 2002). However the utilization of leaf explants for genetic transformation yields low efficiency and establishment of chimeras. Dilution and elimination of chimeras can however be achieved via *in vitro* regeneration manipulations and optimized selection procedures to obtain uniformly transformed plants (Tripathi *et al.*, 2008).

Chimerism has been documented in *Agrobacterium tumefaciens* mediated transformation of many citrus fruits including Carrizo citrange and Mexican lime (Domínguez *et al.*, 2004), strawberries (Mathews *et al.*, 1995) and apple (Flachowsky *et al.*, 2008). Numerous mechanisms have been suggested to explain the production of chimeras, including the probability of an organ emanating from a blend of transformed and non-transformed cells (Zhu *et al.*, 2007) and transformation effects in cells that don’t divide or divide to daughter cells making only a section in a shoot. Chimerism can be associated with challenges of cross protection whereby the non transformed cells are shielded via effective detoxification of the antibiotic by transformed cells or the selective agents in varieties with endogenous tolerance may not be effective (Zhu *et al.*, 2007). Transient expression of the selectable marker gene during initial stages of regeneration or *Agrobacterium* cells recurrence in infected cells could be attributed to formation of chimeras and escapes.

Genetically modified passion fruit plants by *Agrobacterium*-mediated transformation were first reported about twenty five years ago (Manders *et al.*, 1994). Since then, limited progress has been achieved in the development of genetic transformation technologies and subsequent gene-function assessments have been done in only three passion fruit germplasms. Trevisan *et al.* (2006) reported transformation of two Brazilian yellow passion fruit (*Passiflora edulis* f. *flavicarpa*) cultivars IAC-275 and IAC-277 using a gene construct for resistance to *Cowpea aphid borne mosaic virus* (CABMV), which resulted in transformation efficiencies of 0.11 and 0.21%, respectively. Transformation of *Passiflora alata* for resistance to CABMV has been reported with 0.89% transformation efficiency (Correa *et al.*, 2015). Recently, Tuhaise *et al.* (2019) documented a transformation efficiency of 0.456% following genetic transformation of Uganda’s yellow passion fruit (*Passiflora edulis* f. *flavicarpa*). These reports show that *Agrobacterium*-mediated transformation of passion fruit is restricted to few species and/or cultivars and is species- and/or cultivar-dependent.

The success of an *Agrobacterium*-mediated transformation system is influenced by many variable parameters including the type of explants, duration of pre-culture of explant, *Agrobacterium* strain, bacterial cell density, infection time, co-cultivation duration, concentration of acetosyringone, cultivars and antibiotic selection (Madhulatha *et al*., 2007). Therefore, there is an urgent need to optimize genetic transformation procedure for each passion fruit species and/or cultivar in order to evaluate the technology in passion fruit cultivars adapted to different environments.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Assessment of prevalence, incidence and severity of passion fruit woodiness disease (PWD) in Kwale and Kilifi Counties

3.1.1 Description of study sites

The study sites were located in Kwale (CL3) and Kilifi (CL4) Counties (Figure 3.1) in the coastal region of Kenya. Kwale County borders Indian Ocean to the East, Tanzania to the South West, Taita Taveta to the West, Kilifi to the North and Mombasa to the North East. The County lies within a longitude of 38° 27' E and 39° 40' E, latitude of 3° 30’ S and 4° 40' S and an altitude of 0 to 462 m above sea level. The County receives rainfall in the range of 900 mm and 1500 mm per annum with a bimodal distribution pattern and temperatures in the range of 22 °C to 34 °C (Mutuku *et al.*, 2013). The area is also characterized by loamy, clay and sandy soils (Nawiri, 2018).

Kilifi County on the other hand, borders Indian Ocean to the East, Kwale County to the South West, Taita Taveta County to the West, Mombasa County to the South and Tana River County to the North. It lies between a latitude of 2° 20' S and 4° 0' S, longitude of 39^{o} 05' E and 40^{o} 14' E and altitude ranges from 0 to 450 m above sea level. The County receives rainfall in the range of 400 mm to 1,300 mm with a bimodal distribution pattern and a mean annual temperature of about 27 °C. Additionally, Kilifi County is characterized by well drained, fine sandy loam to fine sandy clay loam soils (Jaetzold *et al.*, 2007).

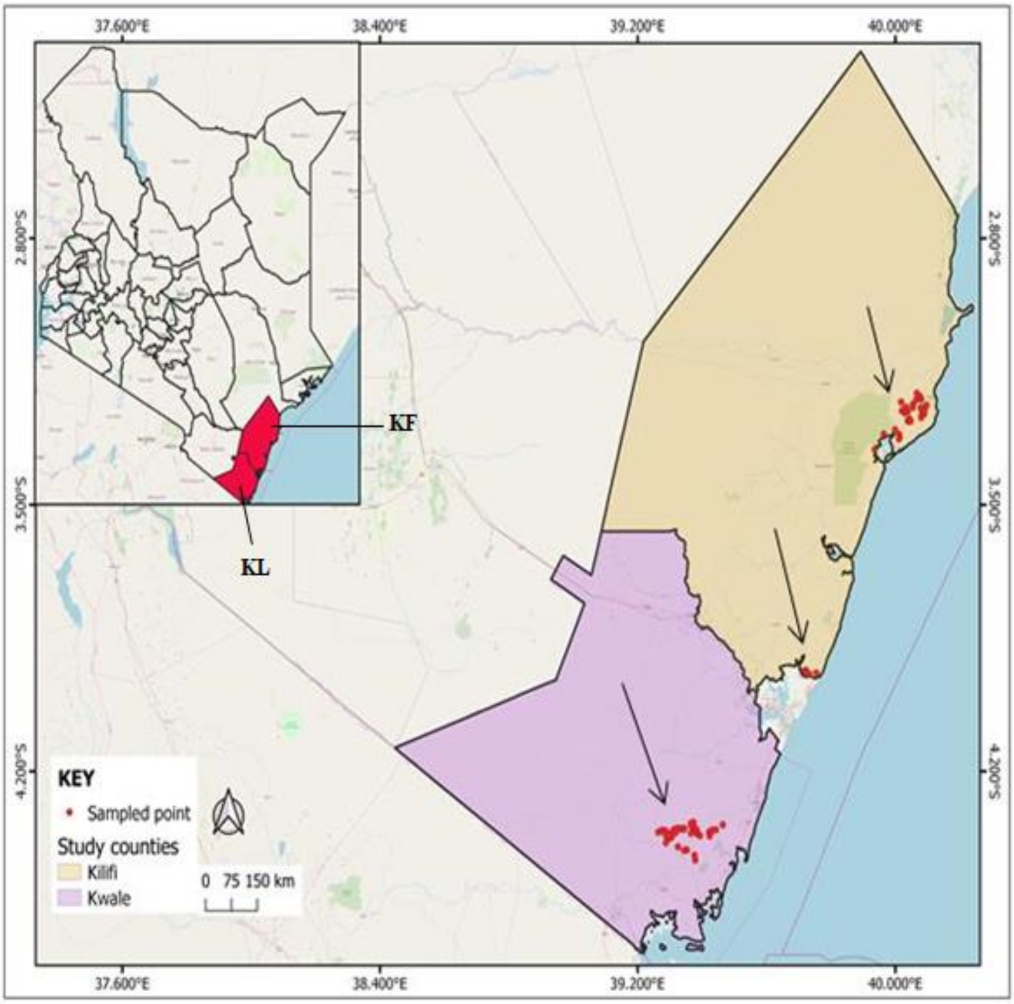


Figure 3.1: A map of Kenya displaying Kilifi (KF) and Kwale (KL) counties where the survey was undertaken. Red points indicate sampled orchards.

3.1.2 Selection of survey study sites

A survey study was carried out in passion fruit orchards in major growing areas in Kwale and Kilifi Counties. The specific locations of the survey were chosen in consultation with Kenya Agricultural and Livestock Research

Organization (KALRO), located in Matuga, Kwale County and Msabaha, Kilifi County. The survey area encompassed administrative locations where groups of orchards were found.

3.1.3 Sampling procedure in farmers fields

Selected farms were at least 1 km apart and each farm contained a minimum of 100 passion fruit plants aged between 6 months and 3 years. A sample size of 40 passion fruit orchards was determined in accordance with the formula by Mugenda and Mugenda (1999):

$$nf = \frac{n}{1+\frac{n}{N}}$$

Where nf = the desired sample size, n= 384 (a constant) and N= 45 farmers.

Sampling was conducted using a semi-structured questionnaire and disease assessment was carried out.

3.1.3.1 Semi-structured interviews

A semi-structured questionnaire was administered to passion fruit farmers to capture background information, their knowledge and experience with passion fruit woodiness disease (PWD) and document how they address the problem (Appendix 1). Information captured in the questionnaire included farm size, passion fruit varieties planted, preferred varieties, source of passion fruit seedlings, farm management practices, types of pests and other diseases affecting passion fruit. Prior to the formal data collection, the questionnaire was pre-tested on a small group of farmers and adjustments were made to ensure validity and clarity of the content.

3.1.3.2 Disease assessments and data collection in the field

Passion fruit woodiness disease assessments were conducted between September and November 2019. Fields were randomly sampled at 1 km intervals on the main and rural accessible roads. On a 50 × 50 m area, an examination of the farm was carried out diagonally at random and diseased plants were counted along the two diagonals according to Kilalo *et al.* (2013). Disease incidence was obtained by calculating the ratio of the diseased plants with PWD symptoms to the total number of plants assessed expressed as a percentage (Gashaw *et al.*, 2014). Disease severity was also determined in all orchards surveyed. Plants were evaluated for woodiness disease symptoms using a five category scale where, 1 = absence of infection, 2 = mild infection, leaf deformation; 3 = moderate infection, leaf deformation and stunting; 4 = severe infection and stunting; 5 = very severe infection, severe stunting and plant death (Kilalo *et al.*, 2013). Disease prevalence was obtained by calculating the ratio of the fields with disease symptoms to the total number of fields assessed expressed as a percentage (James, 1974). Fungal diseases were identified according to Amata *et al.* (2009). The pests were identified with the assistance of an entomologist from the National Museums of Kenya.

3.2 Evaluation of selected passion fruit genotypes for resistance to passion fruit woodiness disease

3.2.1 Description of experimental sites

Greenhouse and field experiments to evaluate resistance of passion fruit genotypes to woodiness disease were carried out between September and December, 2019 at the Department of Plant Sciences, Main campus, Kenyatta University (Figure 3.2). The University is located between Nairobi and Thika at approximately 20 km by road from Nairobi city at an altitude of 1608 m above sea level and longitude of 36° 55' 0E and latitude of 1° 10' 60S. The area receives rainfall range of between 1000 - 1100 mm with a bimodal distribution pattern while temperatures range between 12 °C and 24.6 °C. The site is in upper midland agro-ecological zone 1 (UM3). The area is characterized by dark reddish brown to dark brown loam soils.

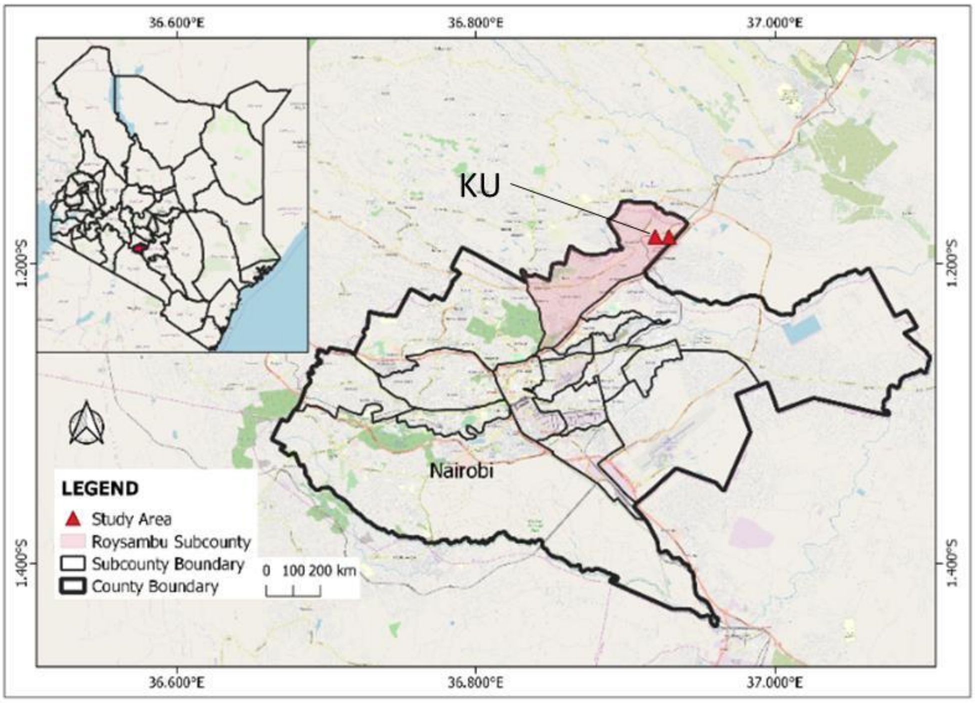


Figure 3.2. A map showing study sites at Main campus, Kenyatta University (© QGIS 2019 software version 3.4).

3.2.2 Sources and types of passion fruit genotypes used for assessment of resistance to woodiness disease

Five different passion fruit genotypes namely purple passion, yellow passion, sweet granadilla passion, Kenya Passion Fruit 4 (KPF 4) and banana passion (Plate 3.1) were acquired from Jomo Kenyatta University of Agriculture and Technology (JKUAT), KALRO (Thika) and KALRO (Mtwapa) (Table 3.1). The fruit genotypes were selected based on the species of passion fruit cultivated in Kenya and also breeders' lines. The purple passion fruit was used as a susceptible control. There was no resistant passion fruit genotypes used in this study since there is no information available on the level of resstance of Kenyan passion fruit germplasm. The passion fruit genotypes were screened under field and greenhouse conditions at Kenyatta University, Kenya.

Plate 3.1: Passion fruit varieties screened for resistance against woodiness disease. A, Purple passion (*Passiflora edulis* f. *edulis*) vine (Bar = 10 cm); B, Purple passion (*Passiflora edulis* f. *edulis*) fruits (Bar = 2 cm); C, Yellow variety (*Passiflora edulis* f. *flavicarpa*) vine (Bar = 10 cm); D, Yellow variety (*Passiflora edulis* f. *flavicarpa*) fruits (Bar = 2 cm); E, Sweet granadilla (*Passiflora ligularis*) vine (Bar = 10 cm); F, Sweet granadilla (*Passiflora ligularis*) fruits (Bar = 2 cm); G, KPF 4 vine (Bar = 10 cm), a cross between *Passiflora edulis* f. *edulis* and *Passiflora edulis* f. *flavicarpa* (Ssemwanga, 2007); H, KPF 4 fruits (Bar = 2 cm); I, Banana passion (*Passiflora mollissima*) vine (Bar = 10 cm); J, Banana passion (*Passiflora mollissima*) fruits (Bar = 2 cm).

Table 3.1: Sources and types of passion fruit genotypes used for assessment of resistance to woodiness disease

Source	Genotype	Species	Characteristics
KALRO (Thika)	Purple passion Fruit	*Passiflora edulis* f. *edulis*	Round-shaped fruits with a purple rind, pulp is rich in aroma and flavor, less acidic compared to yellow passion fruit, susceptible to PWD
KALRO (Mtwapa)	Yellow passion Fruit	*Passiflora edulis* f. *flavicarpa*	Resistant to *Fusarium* wilt and nematodes, fruits have yellow rind with an acidic flavor, response to PWD inoculation not known
JKUAT	Sweet granadilla	*Passiflora ligularis*	Orange to yellow fruit colour with lesser light markings, round-shaped fruits with a tip that ends in the stem, response to PWD inoculation not known
JKUAT	KPF 4	Hybrid (*Passiflora edulis* f. *edulis* × *Passiflora edulis* f. *flavicarpa*)	Drought tolerant, yellow and sweet fruit with high juice content, farmer-preferred variety, response to PWD inoculation not known
KALRO (Thika)	Banana passion	*Passiflora mollissima*	Yellow and oblong–shaped fruit, sweet fruits with a characteristic flavor, response to PWD inoculation not known

Source: NAFIS (2008)

3.2.3 Source and preparation of inoculum

Symptomatic fruits and leaf tissues (Plate 3.2) obtained from diseased plants were used for screening passion fruit genotypes against PWD. The diseased leaves and fruits were ground in the presence of 0.05 M potassium phosphate (1 g in 5 ml) buffer pH 7.0 and the extracts were filtered through cheese cloth. A small quantity (1.0 g) of carborundum (600 mesh) was added to the plant extracts having the virus (Nascimento *et al.*, 2006). The extracts were then used to inoculate healthy plants both under greenhouse and field conditions at Kenyatta University, Kenya.

Plate 3.2: Symptomatic passion fruits infected with passion fruit woodiness disease. A, Infected purple passion fruits (Bar = 2 cm); B, Infected yellow passion fruits (Bar = 2 cm); C, Infected plant with symptomatic leaves (Bar =10 cm).

3.2.4 Greenhouse based evaluation

Seedlings of different passion fruit genotypes were planted in 5-litre plastic pots comprising of top soil well melded with farmyard manure (3:1). At planting, 10 g of Diammonium phosphate (DAP) was also applied per individual seedling. A completely randomized design was used to lay out the experiments. Each treatment had 20 plants (one plant per pot) and each treatment was replicated 3 times.

3.2.4.1 Plant inoculation with passion fruit woodiness virus complex

Prior to inoculation, the plants were screened for potyviruses through indirect ACP ELISA to ensure they were free from infection. The first two leaves of healthy plants (8 to 12 weeks old) were mechanically inoculated through conventional leaf rub method (Gonçalves *et al.*, 2018). The control was not inoculated with the virus. Instead, 20 plants per genotype were inoculated with the extraction buffer alone as negative controls.

Inoculations were repeated twice at a seven-day interval and the plant responses were observed for three months. Watering (500 ml per plant) was carried out once a day, in the morning. The plants were protected from pests using ESCORTE® (Emamectin benzoate 19 g L^{-1} w/v) and THIOVIT JET® 80WG (Sulpher 500 g L^{-1} w/v) to prevent spread of the disease to other plants. THIOVIT JET® 80WG was also effective in controlling fungal diseases. Reaction of different genotypes to the potyviruses was monitored and scored on a weekly basis. Symptom severity on passion fruit plants was scored beginning 1 week after inoculation on a category scale of 1 - 5 by visual examination of the disease symptoms on specific plants where; 1= absence of infection, 2 = mild infection, leaf deformation, 3 = moderate infection, leaf deformation and stunting; severity 4 = severe infection and stunting and 5 = very severe infection, severe stunting and plant death (Kilalo *et al.*, 2013) (Plate 3.3).

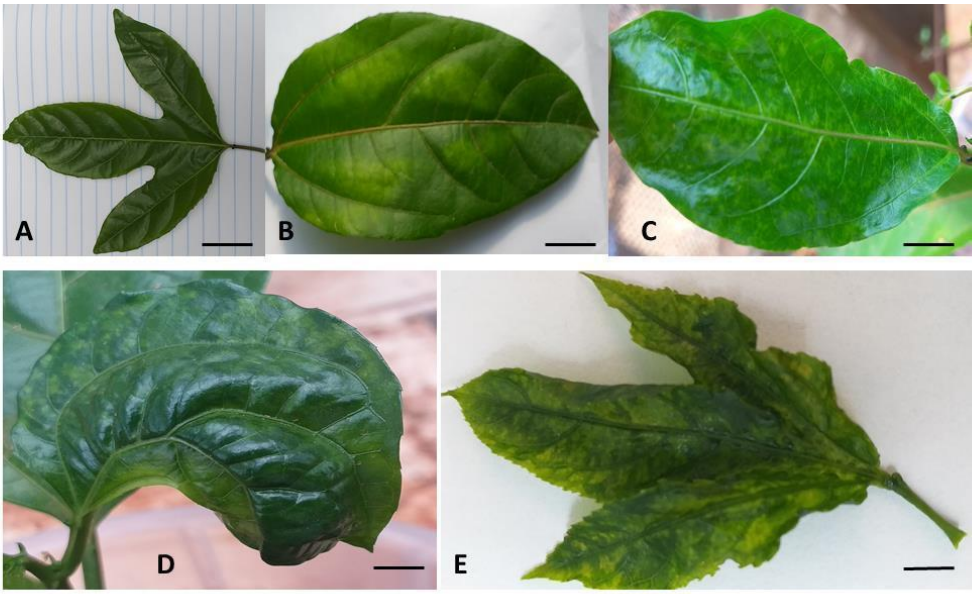


Plate: 3.3: Disease severity category scale (Bars = 1 cm). A, 1 = absence of infection; B, 2 = mild infection; C, 3 = moderate infection; D, 4 = Severe infection; E, 5 = very severe, severe mottling, deformation.

At the end of the experiment, data obtained using the disease severity scale was used to compute the Area Under the Disease Progress Curve (AUDPC) (Campbell and Madden, 1990) for every single genotype evaluated.

3.2.5 Field based evaluation

3.2.5.1 Field preparation and experimental design

Field plots established at Main campus, Kenyatta University were cleared and ploughed to obtain a fine tilth (Plate 3.4). A randomized complete block design was used to lay out the experiment. Five different passion fruit genotypes were planted in holes of 45 cm × 45 cm × 45 cm. Each plot had 4 rows with 5 plants per row. A spacing of 1 m between rows and 1.5 m within rows was maintained. The plots were 1 m apart. Each experimental plot had a single genotype and was replicated three times. There was an additional plot per variety without treatment (control).

Each planting hole was filled with topsoil well mixed with 1 kg of farmyard manure. At planting, 10 g of Diammonium phosphate (D.A.P, 18-46-0) was also applied per individual seedling.

Plate 3.4 Field plots for screening (of passion fruit) experiments at Kenyatta University (Bar = 2 cm).

3.2.5.2 Inoculation of healthy passion fruit plants with woodiness virus complex

Prior to inoculation, the plants were screened for potyviruses through indirect ACP ELISA to ensure they were free from infection. The first two fully expanded leaves of healthy plants (8 to 12 weeks old) were mechanically inoculated as described in section 3.2.4.1. The control plants were inoculated with the extraction buffer (Appendix II). Inoculations were repeated as described in section 3.2.4.1. The plants were maintained in the field as described in section 3.2.4.1

Assessment of disease resistance was carried out, based on visual symptoms of the disease and by comparing the rate of symptom development which includes leaf mosaic, distortion and reduction in size. Disease severity based on symptoms was assessed per variety using the five category scale described in section 3.2.3.1 above. At the end of the experiment, data obtained by the severity scale and plant height was recorded as described in section 3.2.4.1.

3.2.6 Enzyme-linked Immunosorbent Assay

The presense of PWD in passion fruit plants inoculated with PWD was ascertained through indirect ACP- Enzyme Linked Immuno-sorbent Assay (Agdia Inc., Elkhart, USA). Three leaves of each of the inoculated and control plants of passion fruit genotypes were collected 12 weeks after mechanical inoculation with potyviruses. The samples were assayed based on monoclonal antibodies (PTY 1) through ACP - Enzyme Linked Immuno-sorbent Assay as per Agdia's Potyvirus Group test. Crude leaf extracts were prepared from leaves of potyvirus inoculated and mock inoculated plants by grinding in an indirect sample extraction buffer (1 g in 100 ml) using a clean mortar and pestle.

A 100 µl of each sample extract was dispensed into the sample wells in an empty microtitre plate. A 100 µl of the positive control was also dispensed in two empty wells. Similarly, a 100 µl of the sample extraction buffer (IEB) was loaded into two empty wells. The plate was then set in a humid box and incubated for 1 hour at room temperature (18-30 ºC). All the enzymes conjugates and antibodies were prepared as per the manufactures instructions. When the incubation period was complete, the microtitre plate was cautiously washed using the wash buffer (1× PBST) and tapped firmly on paper towels. This procedure was repeated 8 times. Subsequently, 100 µl of prepared detection antibody (dissolved in 1× ECL buffer) was dispensed to each well. The plate was set in a humid box and incubated overnight at 4 °C. After incubation, the plate was again washed 8 times with 1× PBST (wash buffer). The plate was tapped firmly to remove excess buffer and bubbles. A 100 µl of alkaline phosphatase enzyme conjugate (dissolved in 1× ECL buffer) was added per well. The plate was again incubated for 1 hour at room temperature. After incubation, the plate was again washed thoroughly 8 times with 1×

PBST. The plate was tapped firmly on paper towels to remove excess buffer and air bubbles.

Lastly, 100 µl PNP substrate was added into each well and the plates were incubated for 60 minutes. The plates were covered with aluminium foil to protect them from direct and intense light. The wells were visually examined and optical density values read using a plate reader at 405 nm. Samples with optical density values greater than twice the average of negative controls, at 405 nm, were deemed positive. The positive controls were used to confirm that the experiment worked.

3.3 Development of a regeneration system for farmer preferred passion fruit cultivars

3.3.1 Source of explants

Two farmer-preferred passion fruit genotypes namely purple and KPF 4 were acquired as ripe fruits as well as potted seedlings from Kenya Agricultural and Livestock Research Organization (KALRO) and Jomo Kenyatta University of Agriculture and Technology (JKUAT), respectively.

3.3.2 Explant preparation

Mature seeds extracted from ripe fruits were rinsed with tap water before drying them in the sun for 3 days. The seeds were surface-sterilized with 70% (v/v) ethanol for 5 min, followed by 2.5% sodium hypochlorite for 20 min and then rinsed four times in sterile distilled water. Approximately 2 mm cut was carefully made on the lateral sides of each seed before germinating them aseptically in culture vessels containing MS with vitamins [(Murashige and Skoog, 1962), sucrose (2%), and gelrite (0.24%) pH 5.8]. They were then incubated at 26± 2 ^{o} C (Plate 3.5). Leaves from twenty one day old seedlings were excised and used as explants for organogenesis and somatic embryogenesis.

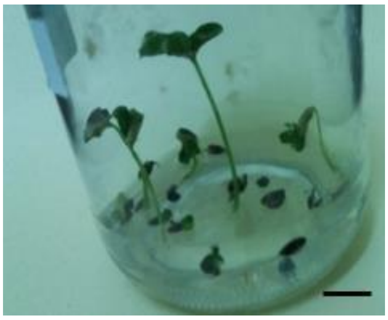


Plate 3.5: Seven day old passion fruit seedlings germinated on hormone free MS medium (Bar = 1 cm).

Immature fruits were also surface-sterilized with 70% (v/v) ethanol for 2 minutes, followed by 2.5% sodium hypochlorite for 10 minutes and then rinsed four times in sterile distilled water. The fruits were aseptically split open and immature seeds excised for induction of somatic embryos.

3.3.3 Organogenesis

3.3.3.1 Shoots regeneration from leaf disc explants

Leaf disc segments (6 mm^2) of both varieties were excised from the first two leaves of 21-day-old seedlings and used for *in vitro* regeneration of shoots.

The excised leaf segments were placed in honey jars containing shoot proliferation/induction medium (SIM; MS medium supplemented with 3% sucrose and 2.4% gelrite; pH adjusted to 5.8). The effect of different concentrations of 6-Benzylamino purine (BAP; 1.0 - 3.0 mg L^{-1}) and also a combination of 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} Kinetin (KIN) were tested on induction of shoots. Cultures were incubated at 26±2 °C with 16 hour photoperiod for 8 weeks. Micro-shoots were transferred into MS medium supplemented with 0.1 mg L^{-1} BAP, 3% sucrose and 2.4% gelrite (pH adjusted to 5.8) for 2 weeks for further proliferation before transfer to root initiation and development medium.

Experiments were set in a completely randomized design as described by Compton (1994). Each treatment had 6 replicates with 5 explants per replicate. The experiment was repeated three times. Data was recorded on the number of shoots per explant and the number of explants with clusters of shoots.

3.3.3.2 Rooting of *in vitro* regenerated plantlets

The elongated shoots of KPF 4 variety were transferred to root induction medium consisting of MS augmented with 0.1 mg L^{-1} naphthalene acetic acid (NAA) at 26±2 °C with 16 hour photoperiod for 4 weeks.

3.3.3.3 Acclimatization of regenerated plantlets

In vitro regenerated shoots with roots were taken out of the culture medium and the roots were washed thoroughly, but with care, under running tap water to remove the gelling agent. The plantlets were then transplanted onto small pots containing autoclaved garden soil and sand (1:1). All pots were covered with clear transparent polyethylene bags to allow in light to be received by the plants and to maintain humidity. The potted plantlets were kept under greenhouse conditions. The polythene bags were opened gradually. After two weeks the polyethylene bags were removed completely and plantlets were kept in the greenhouse. The plantlets were watered at regular intervals. Subsequently the plantlets were transferred to larger plastic pots (14 × 10.6 cm size) containing the same potting substrates and kept under greenhouse conditions.

3.3.3.4 *In vitro* micro propagation of KPF 4 plants using stem nodal explants

Nodal explants were surface-sterilized with 70% (v/v) ethanol for 2 minutes followed by 2.5% sodium hypochlorite for 10 minutes and rinsed four times with sterile distilled water. Sterile nodal explants (one explant per culture bottle) about 3 cm long with 2 nodes were inoculated vertically into MS medium supplemented with different concentrations of BAP (0, 1, 2 and 3 mg L^{-1}), KIN (0, 1, 2 and 3 mg L^{-1}) and a combination of 2 mg L^{-1} BAP and 0.5 mg L^{-1} KIN for multiple shoot induction. The cultures were incubated at 26±2

°C with 16 hour photoperiod for 8 weeks. Experiments were set up in a completely randomized design as described by Compton (1994). There were 10 replicates for each treatment and the experiments were repeated three times. The number of shoots, leaves and shoot length were recorded after a period of 4 weeks and 8 weeks. Elongated shoots were transferred to root induction medium consisting of MS augmented with 0.1 mg L^{-1} NAA for root initiation. The plantlets were transplanted onto small pots containing autoclaved garden soil and sand (1:1). All pots were covered with clear transparent polyethylene bags and maintained in the greenhouse as described in section 3.3.3.3.

3.3.4 Effect of putrescine on root induction of nodal explants under greenhouse

To determine the effect of polyamines (putrescine) in induction of roots from purple and KPF 4 variety, the nodal cuttings (2 nodes per explants; approximately 3 to 5 cm long) were treated with two concentrations (0.5 and 2%) of putrescine solutions for 4 hours. The nodal explants were immersed in distilled water for 4 hours as control experiments. The treated nodal explants were transferred to plastic pots (10 × 7 cm size), one explant in each pot, containing a sterilized mixture of soil and manure (3:1). The soil was moistened with tap water and maintained under greenhouse conditions for root induction. High humidity was maintained by covering each pot with a polyethylene bag throughout the experimental period. Each treatment had 10 nodal segments and was replicated five times. After 6 weeks the plants were carefully removed from the pots, soil attached to the plants was removed, and parameters including the percentage response in terms of root induction, number of primary roots per plant, root length and number of new leaves per plant were recorded.

3.3.5 Somatic embryogenesis from leaf disc and immature seed explants

Explants from both varieties (Purple and KPF 4) consisting of 6 mm^2 leaf discs were excised from the first two leaves of 60-day-old seedlings and used for induction of callus and somatic embryos. The excised explants were placed in petridishes (5 explants per petridish) containing MS medium supplemented with 3% sucrose, 0.8% agar and supplemented with 0.5- 16.0 mg L^{-1} 2, 4-D for callus induction. Cultures were incubated at 26±2 °C with 16 hour photoperiod for 4 weeks. Experiments were set up in a completely randomized design. There were 6 replicates for each treatment and the experiments were repeated three times. The embryogenic calli and early stages of somatic embryos were transferred to different maturation treatments: MS with 6% sucrose, MS with 1% activated charcoal, MS with 0.1 or 0.2 mg L^{-1} ABA for 4 weeks. Maturated somatic embryos were transferred into different media containing different concentrations of growth regulators; MS without hormones and MS with BAP (0.5-3.0 mg L^{-1}) and kinetin (0.5-3.0 mg L^{-1}) for shoot induction.

Seeds obtained from immature fruits (sterilized as described in section 3.3.2) were placed in culture bottles containing MS basal medium and B5 vitamins. The medium was supplemented with 3% sucrose, 2.4% gelrite, 200 mg L^{-1} glutamine and 500 mg L^{-1} casein hydrolysate. The medium was also augmented with 4.0 - 32.0 mg L^{-1} 2, 4 D and 1.0 mg L^{-1} TDZ. After 6 weeks, the embryogenic callus was transferred to MS basal salt and B5 vitamins containing 1% (w/v) activated charcoal or 0.1 mg L^{-1} ABA for maturation for 4 weeks. The somatic embryos were transferred into different media containing different concentrations of growth regulators; MS without hormones and MS with BAP (0.5-3.0 mg L^{-1}) and kinetin (0.5-3.0 mg L^{-1}) for plant regeneration.

3.3.6 Assessment of genetic fidelity using SRAP markers

Five *in vitro* regenerated KPF 4 plants (via organogenesis from nodal cuttings) were randomly selected from concentrations of PGRs (Kinetin and BAP), that resulted to the highest number of shoots and also from the donor mother plants for DNA isolation. Leaves of putrescine-treated plants were also collected for DNA isolation for subsequent genetic fidelity assessment.

3.3.6.1 Genomic DNA extraction from leaves

Genomic DNA was extracted from young fresh leaves (200 mg) of *in vitro* regenerated plants (via organogenesis from nodal cuttings), putrescine treated plants and the donor mother plants using cetyltrimethylammonium bromide (CTAB) protocol as described by Saghai-Maroof *et al.* (1984). To remove RNA, 2 µL of RNase A (10 mg/ml) was added to 20 µl of nucleic acid-TE buffer pH 8.0 and incubated at 37 °C for 30 minutes followed by heating at 65 °C for 15 minutes to inactivate the RNase A. The genomic DNA was stored at -4 °C in a fridge for subsequent molecular analysis.

3.3.6.2 Gel electrophoresis

Agarose gel (1%; w/v) electrophoresis was used to confirm the quality of genomic DNA. Five µl of the genomic DNA was loaded into the gel. Ethidium bromide (0.5 µg L^{-1}) was used for staining the DNA. The electrophoresis was run at 80 V for 45 minutes in Pharmacia biotech GNA horizontal tank and observed under ultraviolet (UV) transilluminator and photographed using a gel documentation system (Bio Rad).

3.3.6.3 Sequence-related amplified polymorphism (SRAP)-PCR amplification and agarose gel electrophresis

Twenty different combinations of SRAP markers were initially screened for their ability to amplify passion fruit genomic DNA. Out of the twenty, seven markers (Table 3.2) were selected based on production of clear and scorable bands and used for genetic fidelity analysis following amplification of DNA from *in vitro* regenerated (from nodal explants), putrescine treated plants and donor mother plants.

Table 3.2: Details of SRAP primer combinations used for assessment of genetic fidelity *in vitro* regenerated plants (via organogenesis), putrescine treated plants and donor mother plants

Primer code	Forward sequence (5' to 3')	Reverse sequence (5' to 3')
me1 – em 9	TGAGTCCAACCGGATA	GACTGCGTACGAATTCAG
me 5 - em 7	TGAGTCCAAACCGGAAG	GACTGCGTACGAATTCAA
me2 - em 10	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTCAG
me 1- em 12	TGAGTCCAACCGGATA	GACTGCGTACGAATTCTC
me11- em11	TGAGTCCAAACCGGTCC	GACTGCGTACGAATTCCA
me1 - em 15	TGAGTCCAACCGGATA	GACTGCGTACGAATTGTC
me2 - em 12	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTCTC

Amplifications were carried out in a total volume of 25 µL reactions. The amplification reaction contained 12.5 µL of premix (One*Taq*® Quick-Load® 2X Master Mix with Standard Buffer), 9.5 µL of nuclease -free PCR water, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 1 µL of genomic DNA. The PCR cycling conditions; were initial denaturation at 94 °C for 5 minutes followed by 5 cycles of 1 minute denaturation at 94 °C, 1 minute annealing at 35 °C and 1 minute extension at 72 °C. This was followed by 30 cycles with 1 minute denaturation at 94 °C, 1 minute annealing at 50 °C and 1 minute extension at 72 °C. This was followed by final extension at 72 °C for 7 minutes (Li and Quiros, 2001). Three independent amplification reactions were performed using DNA from the same plant to assess the consistency and accuracy of the reproducible bands. The amplified DNA fragments were run in 1.5% (w/v) agarose (Duchefa Biochemie, Netherlands) gel containing 1× Tris-Acetate EDTA (TAE) buffer. Ethidium bromide (0.5 µg L^{-1}) was used for staining of the DNA. Gels visualization was carried out using a UV transilluminator and photographed by a gel documentation system (Bio Rad). The amplicon sizes were estimated through comparison with a 1 kb ladder (Generuler, Thermo scientific). Only clear and distinct amplified PCR fragments were scored. The bands were scored based on their presence (1) or absence (0) in agarose gels.

3.4 Optimization of *Agrobacterium* – mediated transformation system for passion fruit varieties using *gus* reporter gene

3.4.1 *Agrobacterium* strain and binary vector used for transformation of passion fruit

Agrobacterium tumefaciens strain LBA4404 containing binary vector pCAMBIA 1301 was used in this study. The pCAMBIA 1301 contains hygromycin phosphotransferase (*hpt*) gene in the T-DNA region as selection marker and *gus* gene with a castor bean catalase intron as a reporter gene driven by a CaMV35S promoter (Figure 3.3).

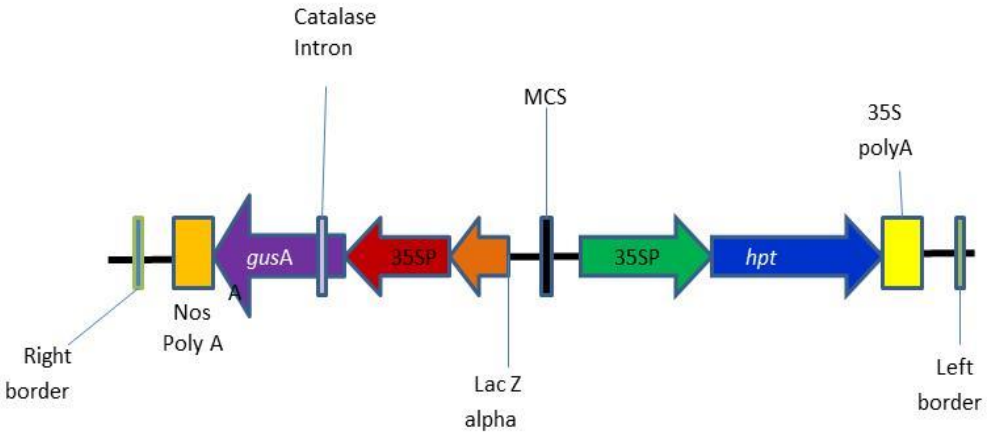


Figure 3.3: Schematic representation of T-DNA region of binary plasmid, pCAMBIA 1301 used for genetic transformation. NOS Poly A; Nopaline synthase polyadenylation signal (terminator), *gus*A; β-glucuronidase reporter gene, 35SP; CaMV 35S promoter, MCS; multiple cloning site, *hpt*; hygromycin phosphotransferase gene, 35S Poly A terminator.

3.4.2 Preparation of LBA 4404 inoculum harboring pCAMBIA 1301

Agrobacterium strain LBA 4404 harboring pCAMBIA 1301 containing *gus* gene was grown on Luria bertani (LB) agar plates supplemented with kanamycin (50 mg L^{-1}), rifampicin (50 mg L^{-1}) at 28 °C for 2 days. Single colonies from the LB agar plates were used to inoculate 2 ml LB medium starter cultures. After 48 hours of shaking at 150 rpm at 28 °C, the bacterium suspension was inoculated into a 20 ml LB medium supplemented with the same concentrations of kanamycin and rifampicin and incubated overnight with shaking at 150 rpm to attain an optical density of 1.0. The *Agrobacterium* culture was centrifuged for 15 minutes at 3500 rpm and a pellet was re-suspended in liquid SBM medium augmented with 200 µM acetosyringone (Sigma Chemical Co.) and incubated further for 2-3 hours at 25 °C on a shaking platform at 100 rpm. The optical density (OD_{600}) of culture was confirmed and adjusted to 0.5. The bacterium suspension was used for subsequent transformation experiments.

3.4.3 Evaluation of factors influencing *Agrobacterium*-mediated transformation efficiency

The influence of various parameters comprising of optical density of *Agrobacterium tumefaciens*, LBA 4404 (0.1, 0.25, 0.5, 0.75**)**, duration of infection (10, 20, 30, 40 and 50 minutes), co-cultivation period (1, 2, 3, 4 and 5 days) and the concentration of acetosyringone (0, 50, 100, 150, 200, 250 µM) on transfer of T-DNA by *Agrobacterium* to the explants and the efficiency of transformation was compared using *gus* positive explants. Thirty explants were used in each treatment and each treatment was repeated three times. Data collected was based on the number of *gus* staining explants after co-cultivation. Leaf explants from passion fruit variety KPF 4 were transformed using the optimum conditions to assess their influence on the transformation efficiency.

3.4.4 Inoculation of leaf disks from KPF 4 variety with *A. tumefaciens*, co-cultivation, resting, selection and regeneration of putative transgenic plants

3.4.4.1 Infection

Leaf disk explants (6 mm^2) were injured several times using sterile needles and immersed in *Agrobacterium* suspension (O.D_{600} 0.5) supplemented with 200 µM acetosyringone followed by gentle shaking at 25 rpm for 30 minutes at room temperature.

3.4.4.2 Cocultivation

After inoculation, the leaf explants were blotted dry on sterile paper towels and co-cultured on co-cultivation medium (MS with 2 mg L^{-1} BAP) for 3 days under dark condition at 21 - 23 °C, in petri dishes. The experimental design was completely randomized. Each treatment had 10 explants, replicated thrice and each experiment was repeated three times.

3.4.4.3 Resting

After co-cultivation, the leaf explants were rinsed three to four times with liquid co-cultivation medium (MS medium supplemented with 2 mg L^{-1} BAP) augmented with 450 mg L^{-1} cefotaxime and blotted dry on sterile paper towels and transferred onto resting medium augmented with 450 mg L^{-1}cefotaxime for 4 days at 28 ºC 16/8 hour photoperiod.

3.4.4.4 Selection and plant regeneration

After 4 days of incubation on resting medium supplemented with 450 mg L^{-1} cefotaxime, putatively transformed explants were transferred onto fresh SBM containing 450 mg L^{-1} cefotaxime and 7.5 mg L^{-1} hygromycin and incubated for 2 weeks at 28 ºC 16/8 hour photoperiod. The cultures were maintained in the selective medium for 2 months with subculturing after every 2 weeks into the same fresh medium. All dead explants were disposed off during subcultures. The putively transformed shoots were transferred onto root initiation medium (Appendix VIII) augmented with 0.1 mg L^{-1} NAA.

3.4.4.5 Acclimatization of putative transformants

Putative transgenic plantlets were taken out of the culture medium. The roots were washed carefully under running tap water and acclimatized as described in section 3.3.3.3.

3.4.4.6 *Gus* histochemical assays

The putative transgenic plants were subjected to *gus* histochemical assays as described by Jefferson (1987). Expression of the *gus* reporter gene was investigated in leaves excised from putative transformants regenerated on selection medium. Non-transformed leaf discs of the control plants were also assessed.

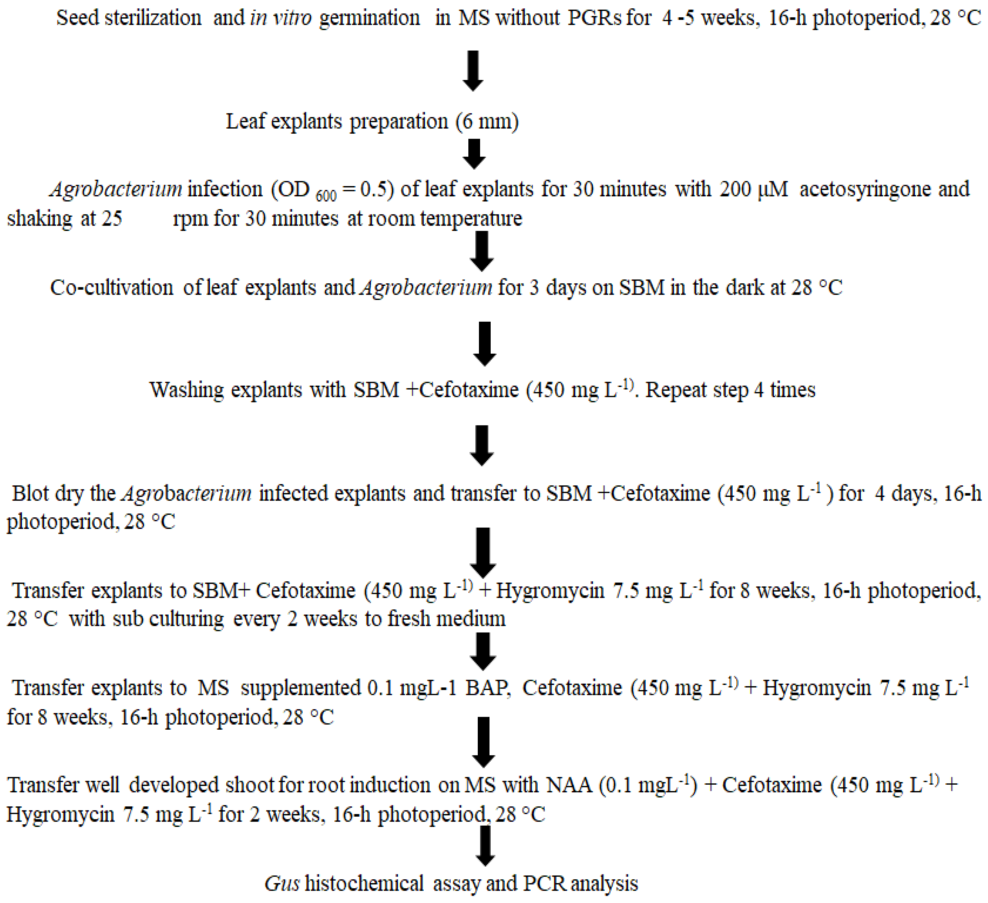
3.4.4.7 Isolation of total genomic DNA and polymerase chain reaction of putative transgenic lines

Total genomic DNA was obtained from *in vitro* regenerated shoots (200 mg) via the cetyltrimethylammonium bromide (CTAB) method (Soni and Murray, 1994). For PCR analysis, the *gusA* gene was amplified to confirm the presence of the transgene. The primers used had the following sequences: forward primer 5′-TTTAACTATGCCGGGATCCATCGC-3′, reverse primer 5′-CCAGTCGAGCATCTCTTCAGCGTA-3′ meant to amplify 500 bp *gus*A gene.

The amplification reaction mixture was 25 µl which contained 12.5 µl of premix (One*Taq*® Quick-Load® 2X Master Mix with Standard Buffer), 9.5 µl of nuclease -free PCR water, 1 µl of 10 uM forward primer, 1 µl of 10 µM reverse primer, 1 µL of genomic DNA. The PCR amplification was carried out in the following conditions; initial denaturation at 94 °C for 10 minutes followed by 35 cycles (15 s denaturation at 94 °C, 40 s annealing at 62 °C and 50s extension at 72 °C). This was followed by final extension at 72 °C for 7 minutes and holding at 4 °C (Li and Quiros, 2001).

3.4.4.8 Gel electrophoresis

The amplicons were separated in 0.8% agarose gel (Duchefa Biochemie, Netherlands) gel containing 1× Tris-Acetate EDTA buffer. Ethidium bromide (0.5 µg/mL) was used for staining of the DNA. The PCR product (4 µl) was loaded into each well. The electrophoresis was run at 70 V for 45 minutes in Pharmacia biotech horizontal tank. Gels visualization was carried out under UV transilluminator and photographed by a gel documentation System (Bio Rad). The amplicon sizes were estimated using a 100 bp DNA Ladder (New England Biolabs). A generalized scheme for genetic transformation system for KPF 4 variety is presented in Figure 3.4.



Seed sterilization and *in vitro* germination in MS without PGRs for 4 -5 weeks, 16-h photoperiod, 28 °C

Leaf explants preparation (6 mm)

Agrobacterium infection (OD $_{600}$ = 0.5) of leaf explants for 30 minutes with 200 µM acetosyringone and shaking at 25 rpm for 30 minutes at room temperature

Co-cultivation of leaf explants and *Agrobacterium* for 3 days on SBM in the dark at 28 °C

Washing explants with SBM +Cefotaxime (450 mg $L^{-1)}$. Repeat step 4 times

Blot dry the *Agrobacterium* infected explants and transfer to SBM +Cefotaxime (450 mg L^{-1}) for 4 days, 16-h photoperiod, 28 °C

Transfer explants to SBM+ Cefotaxime (450 mg $L^{-1)}$ + Hygromycin 7.5 mg L^{-1} for 8 weeks, 16-h photoperiod, 28 °C with sub culturing every 2 weeks to fresh medium

Transfer explants to MS supplemented 0.1 mgL-1 BAP, Cefotaxime (450 mg $L^{-1)}$ + Hygromycin 7.5 mg L^{-1} for 8 weeks, 16-h photoperiod, 28 °C

Transfer well developed shoot for root induction on MS with NAA (0.1 mgL^{-1}) + Cefotaxime (450 mg $L^{-1)}$ + Hygromycin 7.5 mg L^{-1} for 2 weeks, 16-h photoperiod, 28 °C

Gus histochemical assay and PCR analysis

Figure 3.4: A flow diagram displaying the protocol used for *Agrobacterium*-mediated transformation of passion fruit using leaf explants.

3.5 Data analysis

Pearson correlation, Pearson Chi-square and cross tabulation in SPSS statistics 20 were used to analyze data from the questionnaire. A logarithmic transformation ($\mathrm{Log}_{10} \times$, where $\times$ = severity score) was applied to the data on severity and then subjected to Analysis of Variance (ANOVA) to assess significant differences and separation of means carried out using Tukey's honest significant difference test at 5% level. The data was analyzed using Genstat 15th Edition statistical program.

Data obtained from the five category grading scale was used to compute Area under Disease Progress Curve (AUDPC) (Campbell and Madden, 1990) in all genotypes assessed, according to the expression;

$$AUDPC = \sum_{i=1}^{n-1} \left(\frac{y_i + y_{i+1}}{2} \right) (t_{i+1} - t_i)$$

y_i = disease level at time t_i.

$t_{i+1} - t_i$ =Time (days) between two disease scores

Data on *in vitro* regeration and *Agrobacterium*- mediated transformation parameters were transformed where necessary before subjection to ANOVA using Genstat 15th Edition and MS Excel programs. Transformation efficiency was determined using the following formula; Genetic transformation efficiency % = [(Number of PCR positive plants/ Number of leaf explants inoculated) × 100].

On genetic fidelity analysis, DendroUPGMA server was used to compute distance matrices between *in vitro* regenerants and their mother plants, based on binary data generated from SRAP profiles. The distance matrices were based on Jaccard's similarity coefficient. Similarity matrices were subjected to UPGMA (unweighted pair group method with arithmetic mean) cluster analysis and Fig tree software (Version 1.4.2) used to create the dendrograms.

CHAPTER FOUR

RESULTS

4.1 Prevalence, incidence and severity of passion fruit woodiness disease in Kilifi and Kwale Counties

4.1.1 Prevalence, incidence and disease severity of passion fruit woodiness disease

All surveyed farms in Kwale County displayed virus disease symptoms (disease prevalence of 100%). Virus disease incidence ranged from 32.50% to 51.43%, with an overall mean of 43.27% (Table 4.1). The highest disease incidence was recorded in Mivumoni (51.43%) while the lowest was recorded in Mangawani at 32.50%. Mean disease severity ranged from 2.40 to 2.77 across the six locations, with an overall mean of 2.66. The highest PWD severity (2.77) was scored in farmers' fields in Lukore location while the lowest was in Shimba hills location with a score of 2.40 (Table 4.1). There was a significant difference ($P \leq 0.05$) in mean disease severity between orchards surveyed in Shimba hills, and those in Mivumoni and Lukore.

Table 4.1: Prevalence, incidence and severity of passion fruit woodiness disease (PWD) in Kwale County, Kenya

Location	Sample size	Prevalence of PWD (%)	PWD incidence (%)	Severity of PWD [X] (Mean ± S.E)
Lukore	7	100	47.14 ± 4.48^{ab}	2.77 ± 0.07^{a}
Mwaluvanga	5	100	35.00 ± 3.53^{b}	2.59 ± 0.09^{ab}
Mivumoni	7	100	51.43 ± 4.46^{a}	2.75 ± 0.07^{a}
Shimba hills	5	100	47.00 ± 6.63^{ab}	2.40 ± 0.08^{b}
Mangawani	8	100	32.50 ± 2.83^{b}	2.64 ± 0.08^{ab}
Manyatta	8	100	48.12 ± 3.65^{ab}	2.65 ± 0.07^{ab}
Mean total		100	43.27	2.66

[X] Values represent mean ± standard error of 20 replicates per orchard. Means having the same letter within a column are not significantly different at P > 0.05 using Tukey's Honest significant difference test.

All surveyed farms in Kilifi County displayed virus symptoms (disease prevalence 100%). Virus disease incidence ranged from 33.75% to 59.16% (Table 4.2) with overall mean of 46.00% across the 8 locations. Disease severity ranged between from 2.65 to 3.18. Ganda recorded the highest disease incidence (59.16%) and disease severity (3.18%). There was a significant difference (P ≤ 0.05) in mean disease severity between orchards surveyed in Ganda and Mbaraka Chembe. Ganda had both KPF 4 and yellow variety of passion fruits varieties. Kilifi County displayed a higher mean disease incidence (46.00) and severity (2.96) compared to Kwale County (43.27 and 2.66, respectively).

Table 4.2: Prevalence, incidence and severity of passion fruit woodiness disease (PWD) in Kilifi County, Kenya

Location	Sample size	Prevalence of PWD (%)	PWD Incidence (%)	Severity of PWD^{X} (Mean± S.E)
Mbaraka Chembe	4	100	42.50 ± 3.22^{ab}	2.65 ± 0.11^{b}
Mkenge	8	100	44.37 ± 2.39^{ab}	2.97 ± 0.07^{ab}
Ganda	6	100	59.16 ± 8.28^{a}	3.18 ± 0.08^{a}
Kijiwetanga	7	100	45.71 ± 2.77^{ab}	2.91 ± 0.08^{ab}
Mida	4	100	33.75 ± 3.15^{b}	2.73 ± 0.14^{ab}
Dabaso	3	100	48.57 ± 8.82^{ab}	2.89 ± 0.12^{ab}
Goshi	3	100	36.66 ± 4.41^{ab}	2.82 ± 0.14^{ab}
Mtwapa	5	100	50.00 ± 5.70^{ab}	3.08 ± 0.09^{ab}
Mean total		100	46.00	2.96

^{X}Values represent mean ± standard error of 20 replicates per orchard. Means having the same letter within a column are not significantly different at $P > 0.05$ using Tukey's Honest significant difference test.

4.1.2 Disease symptoms observed on plants and fruits in the field

Virus disease symptoms were recorded in all the surveyed farms in both Kwale and Kilifi Counties. The plants exhibited characteristic symptoms of woodiness disease such as yellow leaf mosaic, leaf mottling, leaf puckering, distortion coupled with reduction in size and leaf curl (Plate 4.1A, B and C). The fruits were deformed, with unusually corky rinds and reduced in size (Plate 4.1D and E). In severe cases, affected plants displayed stunted growth (Plate 4.1F).

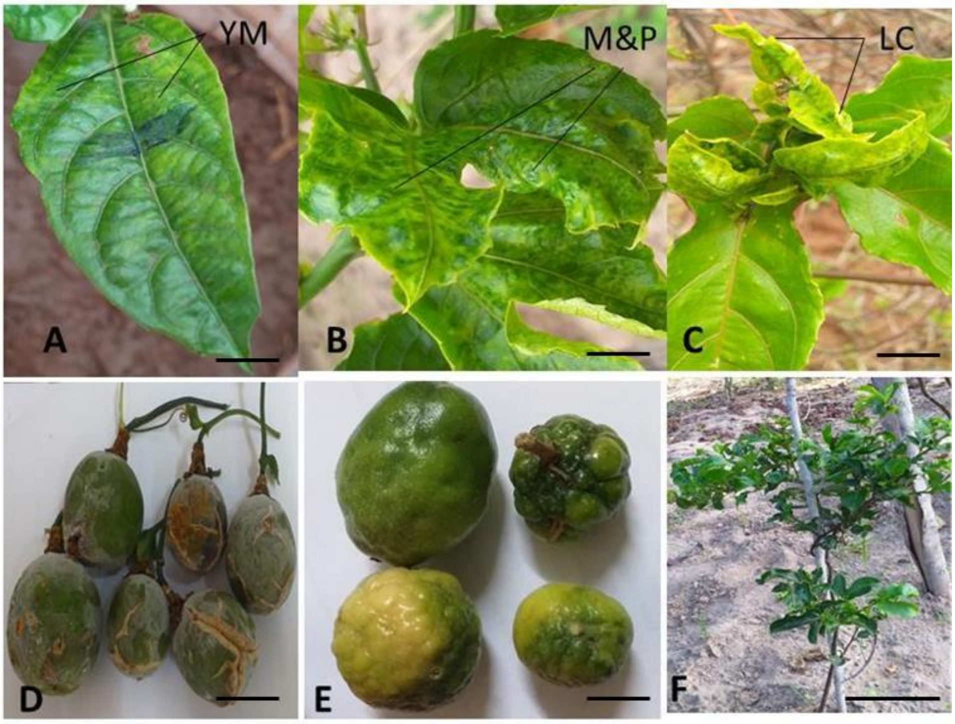


Plate 4.1: Symptoms of passion fruit woodiness disease observed in the field. A, Yellow leaf mosaic (YM) (Bar = 2 cm); B, Leaf mottling with puckering (M&P) (Bar = 2 cm), C, Leaf curl (LC) (Bar = 2 cm); D, Purple passion misshapen fruits with a corky rind (Bar = 2 cm); E, Yellow passion misshapen fruits with a corky rind (Bar = 2 cm), F, Three year old stunted plant (Bar = 10 cm).

4.1.3 Passion fruit woodiness disease management practices

A total of 45% and 55% farmers in Kwale and Kilifi Counties respectively were able to identify woodiness disease symptoms. However, there was no statistical association between management practices and the different locations within Kwale and Kilifi Counties (Table 4.3).

Table 4.3: Test of association between woodiness disease management practices and locations in Kwale and Kilifi Counties

Kwale County		Kilifi County	
Location	Pearson Chi-square	Location	Pearson Chi-square
Lukore	χ^2 =2, P = 0.849	Mbaraka Chembe	χ^2 =3.778, P = 0.707
Mwaluvanga	χ^2 =1, P = 0.963	Mkenge	χ^2 =4, P = 0.677
Mivumoni	χ^2 =8.5, P = 0.131	Ganda	χ^2 =3.852, P = 0.697
Shimba hills	χ^2 =2.556, P = 0.635	Kijiwetanga	χ^2 =6.966, P = 0.324
Mangawani	χ^2 =5.194, P = 0.393	Mida	χ^2 =1.263, P = 0.974
Manyatta	χ^2 =9.077, P = 0.106	Dabaso	χ^2 =2.267, P = 0.894
		Goshi	χ^2 =2.73, P = 0.965
		Mtwapa	χ^2 =6, P = 0.423

Regarding pruning, the highest percentage of farmers in both Counties (35.3% and 38.9% in Kilifi and Kwale respectively) did not sterilize their pruning tools (Figure 4.1). The highest proportion of farmers in Kilifi County used both methylated spirit (17.6%) and jik (17.6%). On the other hand, the highest proportion (22.2%) of farmers in Kwale County used methylated spirit. However, there was no significant difference (χ^2 = 4.181, P > 0.05) in the sterilization methods used in the two Counties (Figure 4.1)

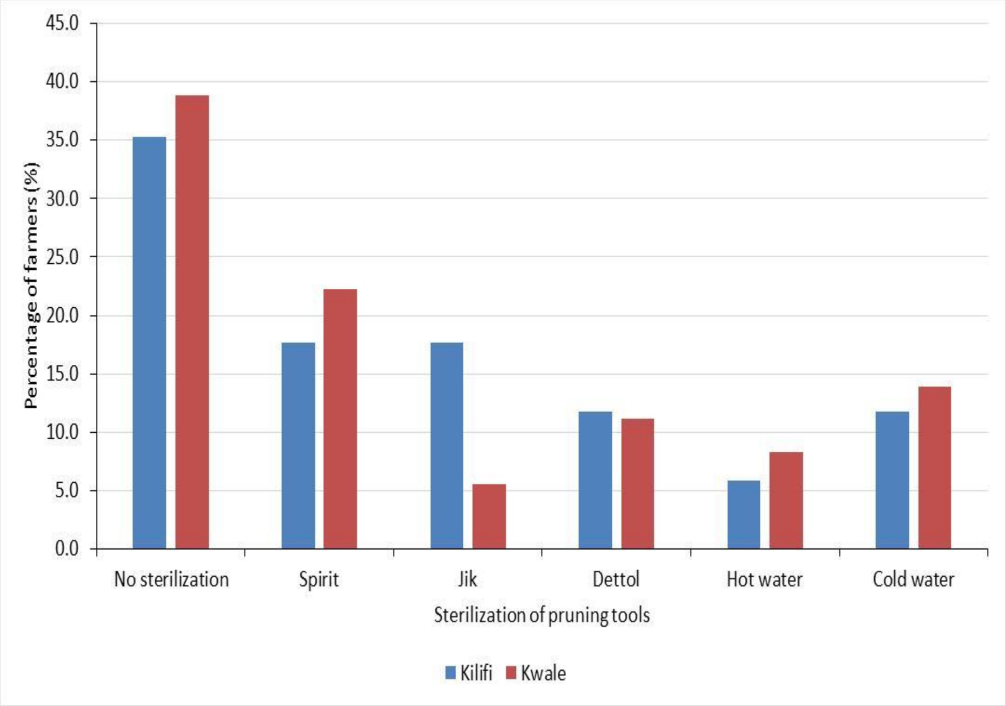


Figure 4.1: Percentage of farmers using different methods of sterilization of pruning tools in Kwale and Kilifi Counties.

The highest proportion of farmers in Kilifi (30%) and Kwale (32.5%) Counties did not practice intercropping (Figure 4.2). However, crops grown before or intercropped with passion fruit differed significantly between the two Counties ($\chi^2 = 20.696, p \leq 0.05$).

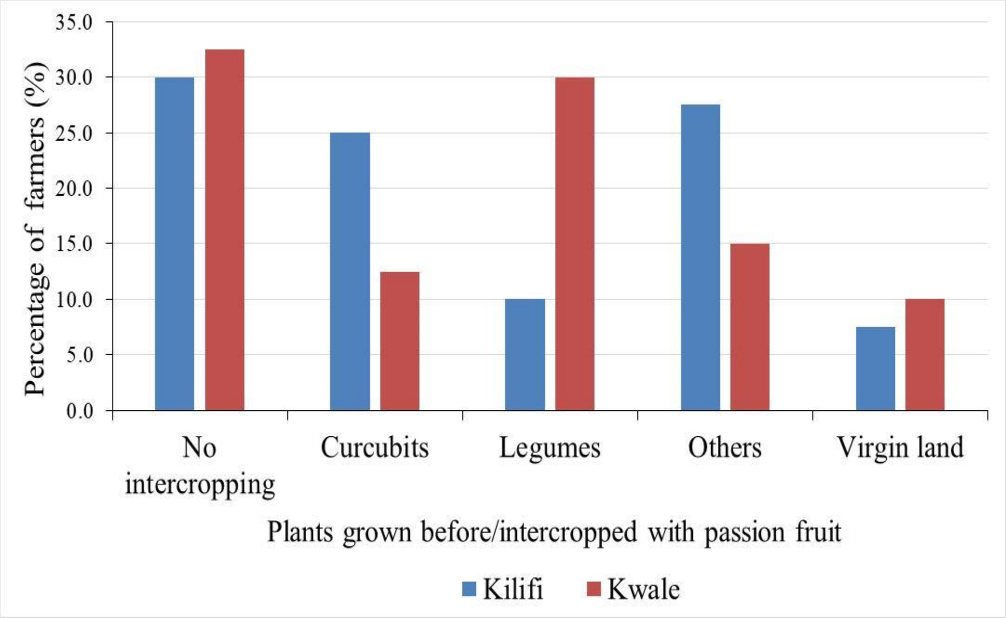


Figure 4.2: Percentage of farmers growing different crops before planting passion fruit or intercropping with passion fruits in Kwale and Kilifi Counties.

4.1.3.1 Correlation analysis of management practices and PWD incidence and severity in the different locations of Kwale and Kilifi Counties

In Kwale County, there was a positive, significant correlation between pruning and disease incidence (0.394). A negative, significant correlation was noted between foliar feeds/ fertilizers and disease incidence (-0.469). Additionally, a positive, significant correlation between pruning and disease severity was recorded in Kwale County (0.136).

In Kilifi County, there was a positive significant correlation between pruning and disease incidence (0.423). There was also a positive significant correlation between intercropping and disease incidence (0.312). Table 4.4 shows a positive significant correlation between disease severity and pruning (0.126), crop rotation (0.161) and intercropping (0.534).

Table 4.4: Correlation analysis of woodiness disease management practices and locations in Kwale and Kilifi Counties

County	Management practice							
	Response variable	Pruning	Weeding	Pesticides	Irrigation	Foliar feeds	Crop rotation	Intercropping
Kwale	Disease incidence	0.394*	-0.286	-0.227	-0.065	-0.469*	-0.042	0.225
	Disease severity	0.136*	0.079	0.0768	0.054	0.0721	0.0911	0.089
Kilifi	Disease incidence	0.423*	0.159	0.227	0.116	-0.25	0.282	0.312*
	Disease severity	0.126*	0.099	0.057	0.024	-0.057	0.161*	0.534**

**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).

4.1.4 Preferred genotypes

There was a significant difference (χ^2 = 7.671, P < 0.05) in the preferred genotypes of passion fruit grown in Kwale and Kilifi Counties. In Kwale County, all farmers (100%) preferred yellow passion fruit. In Kilifi County, the highest proportion of farmers (82.5%) showed a preference for yellow genotype of passion fruit while the lowest proportion (7.5%) showed a preference for KPF 4 genotype of passion fruit. A correlation analysis of the preferred genotype and different locations in Kilifi County revealed no statistical association (χ^2 =22.46, P > 0.05) between the different genotypes and the locations in the County (Table 4.5).

Table 4.5: Proportions of farmers showing preference for different genotypes of passion fruit grown in different locations in Kilifi County

Location	Varieties			
	Yellow	KPF 4	Yellow & KPF 4	Total (%)
Dabaso	9.09	0.00	0.00	7.50
Ganda	6.06	33.33	75.00	15.00
Goshi	6.06	33.33	0.00	7.50
Kijiwetanga	21.21	0.00	0.00	17.50
Mbaraka Chembe	12.12	0.00	0.00	10.00
Mida	12.12	0.00	0.00	10.00
Mkenge	24.24	0.00	0.00	20.00
Mtwapa	9.09	33.33	25.00	12.50

χ^2 =22.46, P = 0.070

4.1.5 Correlations between surveyed locations and preferred genotypes

All farmers in Kwale County showed a preference for yellow passion fruit. In Kilifi County, there was a negative correlation between disease incidence and number of farmers growing yellow passion fruit (-1.53). On the other hand, there was a positive correlation between disease incidence and farmers who showed preference for KPF 4 (0.302) in Kilifi. A positive significant correlation (0.188) was also noted between disease severity and farmers showing preference for both yellow passion and KPF 4 genotypes in Kilifi (Table 4.6).

Table 4.6 Correlations between surveyed locations and preferred genotypes

County	Responsive variable	Preferred genotypes		
	Disease	Yellow	KPF 4	Yellow and KPF4
Kwale	Disease incidence	-0.130	A	A
	Disease severity	0.072	A	A
Kilifi	Disease Incidence	-0.153	0.302	0.476
	Disease severity	-0.061	0.159	0.188**

**. Correlation is significant at the 0.01 level (2-tailed). A = Correlation coefficient could not be computed because at least one of the variables is constant.

Farmers in Kwale and Kilifi Counties obtained their seedlings from different sources which include KALRO, local nurseries, their own nurseries or a combination of sources (Figure 4.3). There was a significant difference ($\chi 2 = 51.536$, $P \leq 0.05$) in the source of seedlings in the two Counties.

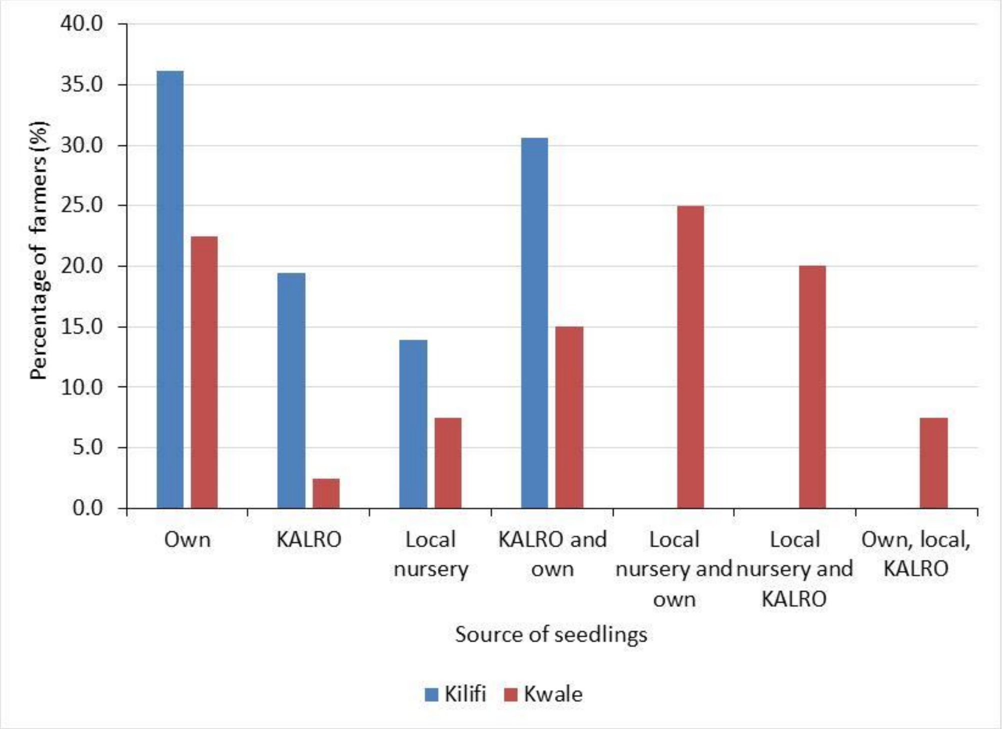


Figure 4.3: Sources of passion fruit seedlings grown by farmers in Kwale and Kilifi Counties.

4.1.6 Other constraints to passion fruit production highlighted by the farmers and observed in the farmers' fields

Symptoms of fungal diseases were noted in passion fruit orchards in Kwale and Kilifi Counties. These included brown spot, dieback, anthracnose, scab, septoria blotch and *Fusarium* wilt although to a lesser extent (Plate 4.2).

Plate 4.2: Symptoms of passion fruit fungal diseases observed in Kwale and Kilifi Counties (Bars = 1 cm). A, Anthracnose symptoms on a vine and fruit; B, Brown spot symptoms; C, Scab symptoms on passion fruits; D, Dieback symptom on passion fruit vines.

Insect pests were observed in passion fruit orchards among them black vine weevils (*Systates* spp.), passion vine bugs (*Leptoglossus gonagra*), brown marmorated stink bugs (*Halyomorpha halys*), mealy bugs (*Planococcus* spp.), horned coreid bug (*Cletus* spp.) (Plate 4.3), leaf miners (*Liriomyza* spp.), spider mites (*Tetranychus* spp.), nematodes (*Meloidogyne*) termites, fruit flies and snails defoliating and damaging different parts of the fruit vines (Plate 4.4).

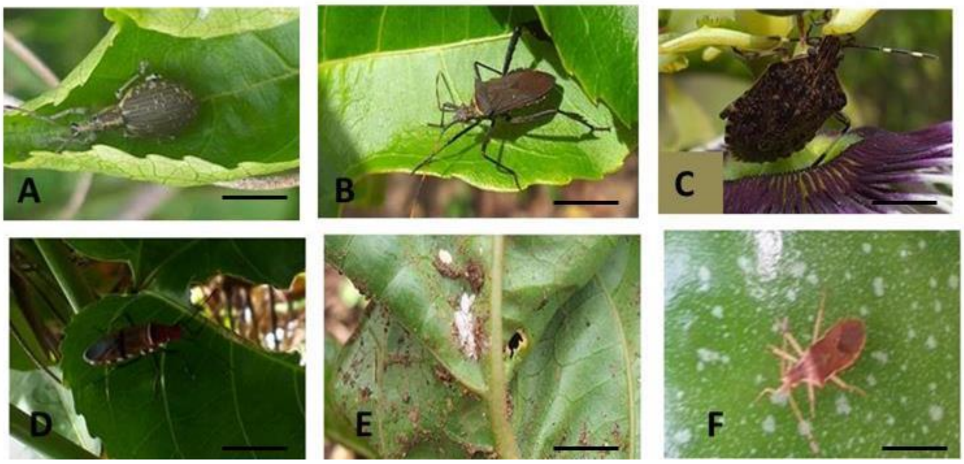


Plate 4.3: Some of the passion fruit pests observed in orchards located in Kwale and Kilifi Counties (Bars = 5 mm). A, *Systates sp*; B, *Leptoglossus gonagra*; C, *Halyomorpha halys*; D, *Dysdercus sp*; E, *Planococcus sp*; F, *Cletus sp*.

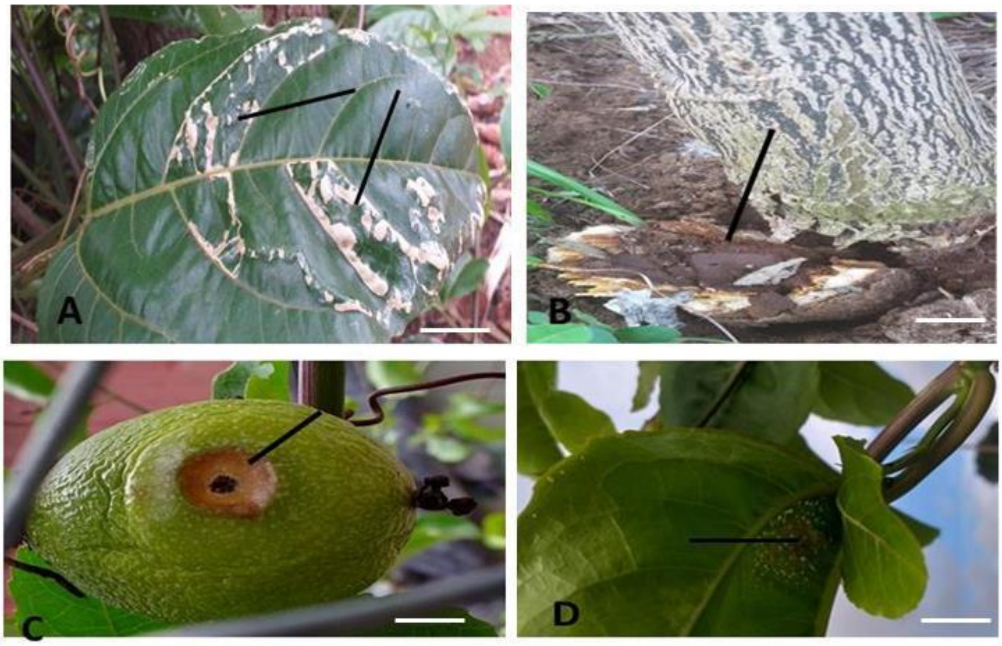


Plate 4.4: Damage caused by pests in passion fruit orchards observed in Kwale and Kilifi Counties (Bars = 2 cm). A, Leaf miner damage; B, Termites damage; C, Leaf sucking bags damage; D, Spider mites damage.

Other challenges of passion fruit production noted by the respondents in both counties were prolonged droughts, lack of water for irrigation leadig to flower abortion, lack of posts and wires for trellising, theft, poor fruit prices especially in Kwale County and poor roads. It was also observed that farmers preferred training their vines on growing trees (locally known as "bush system of farming") such as neem tree, cashew nuts and vanilla as opposed to the trellis system of stout posts and wires (locally known as pure stand system).

4.2 Greenhouse and field screening of passion fruit against woodiness disease complex

4.2.1 Symptomatology in inoculated plants in the greenhouse and field conditions

Leaves of uninoculated plants both under greenhouse and fields conditions, did not display any symptoms of woodiness disease throughout the evaluation period. Symptoms associated with woodiness disease viruses were observed in inoculated test plants at three weeks post-inoculation. The PWD symptoms observed in all genotypes in both the greenhouse and field were yellow mosaic, leaf mottling, leaf deformation, blister like symptoms and stunting (Plate 4.5).

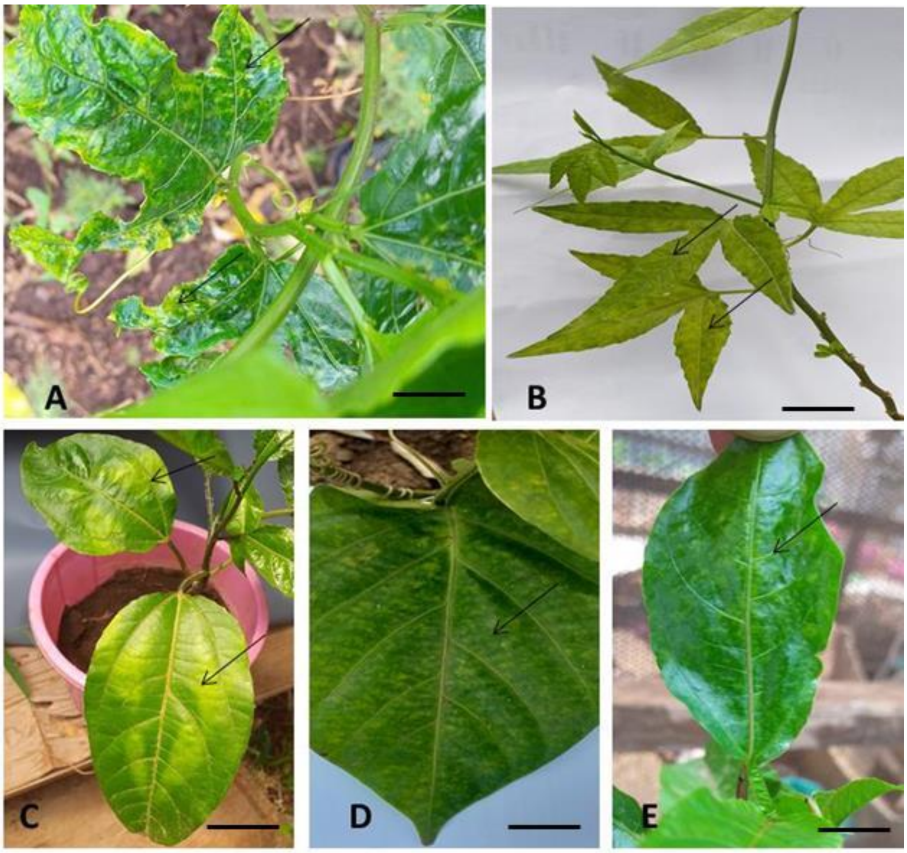


Plate 4.5: Symptoms of woodiness disease in passion fruit genotypes screened for resistance (Bars = 2 cm). A, yellow mosaic, leaf deformation and stunting in purple passion; B, leaf mottling in banana passion; C, yellow mosaic in KPF 4 ; D, leaf mottling in sweet granadilla; E, leaf mottling in yellow passion fruit.

4.2.2 Disease progression under greenhouse conditions

The inoculated plants of all tested varieties did not present any characteristic symptom of passion fruit woodiness disease at two weeks post-inoculation. The first PWD symptoms on inoculated plants were observed at three weeks after inoculation (Figure 4.4). The highest disease progression (1.81 to 3.87) was observed on purple passion fruit and the lowest on banana passion fruit (0.5 to 2.52) throughout the evaluation period. There was no significant difference ($p > 0.05$) in disease progression between purple passion fruit and

KPF 4 passion fruit. Yellow passion fruit displayed higher AUDPC values (0.9 to 3.18) compared to banana passion (0.5 to 2.52) which were significantly different ($p \leq 0.05$) on the 28th day and between 56 and 63 days post-inoculation. There was a significant difference ($p \leq 0.05$) in disease progression between genotypes KPF 4, purple passion fruit and genotypes yellow passion fruit, banana passion fruit and sweet granadilla.

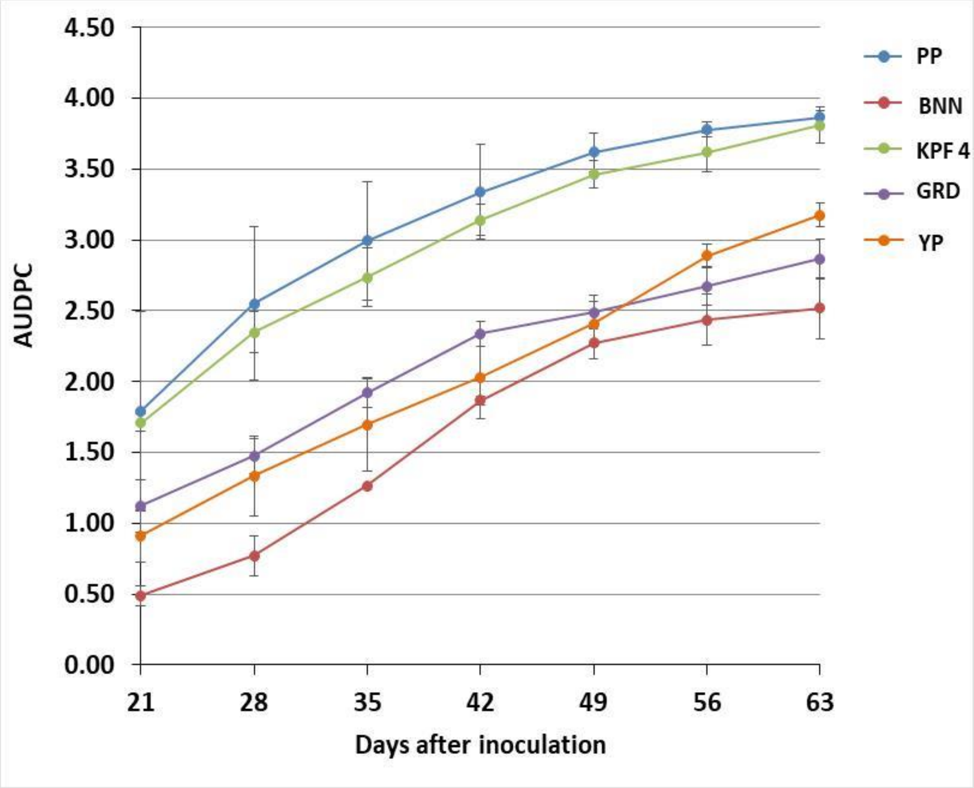


Figure 4.4: Area under disease progress curve (AUDPC) in passion fruit plants infected with passion fruit woodiness disease virus complexes at different days after inoculation in the greenhouse. PP, purple passion fruit; BNN, banana passion fruit; KPF4, KPF 4 passion fruit; GRD, sweet granadilla passion fruit; YP, yellow passion fruit.

Based on symptom development, banana passion, sweet granadilla and yellow passion fruits were classified into moderately tolerant while KPF 4 and purple passion fruits genotypes were susceptible (Table 4.7).

Table 4.7: Grouping of passion fruit genotypes based on symptom development under greenhouse conditions

Genotype	Disease severity range	Classification
Banana passion fruit	1.20 - 2.33	Moderately tolerant
Sweet granadilla passion fruit	1.40 - 2.73	Moderately tolerant
Yellow passion fruit	1.33 - 2.87	Moderately tolerant
KPF 4 passion fruit	1.67- 3.6	Susceptible
Purple passion fruit	1.66-3.66	Susceptible

4.2.2.1 Effects of PWD on plant growth under greenhouse conditions

There was a significant reduction ($p \leq 0.05$) in plant height of purple passion fruit at 28 days after inoculation after which no significant change in height was recorded through the evaluation period (Figure 4.5). There was a significant reduction ($p \leq 0.05$) in height of infected banana passion between 42 days and 63 days after inoculation after which a significant increase ($p \leq 0.05$) was recorded between 70 and 77 days. However, there was no significant difference ($p > 0.05$) in height between non-inoculated and infected banana passion between 42 days and 77 days post inoculation. There was a significant reduction ($p \leq 0.05$) in plant growth of KPF 4 between 35 days and 42 days post inoculation which was significantly different ($p \leq 0.05$) from the non-inoculated plants. No significant increase ($p > 0.05$) in height of infected sweet granadilla between 21 days and 49 days post inoculation was recorded, but a significant increase ($p \leq 0.05$) was recorded between 56 days and 77 days after inoculation. No significant difference was recorded in yellow passion fruit throughout the evaluation period.

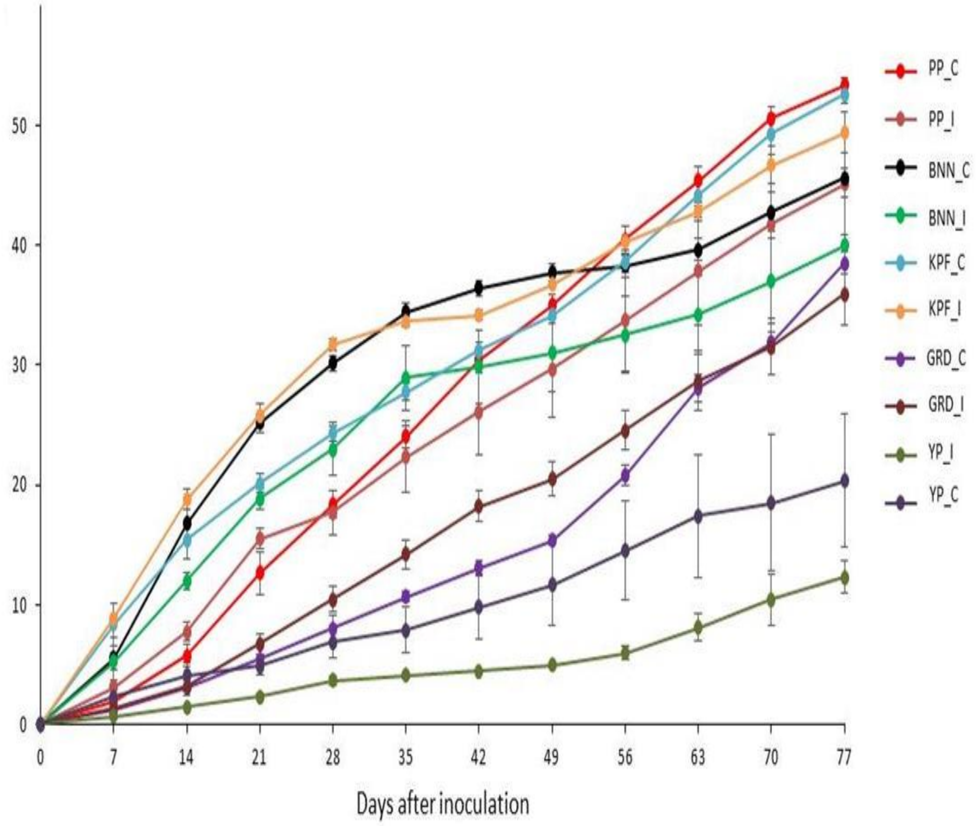


Figure 4.5: Growth of passion fruit genotypes not inoculated (control) and those mechanically inoculated with passion fruit virus complexes under greenhouse conditions. PP_C (control) PP_I (Infected), purple passion fruit; BNN_C (control) BNN_I (infected), banana passion fruit; KPF_C (control), KPF_I (infected) KPF 4 passion fruit, GRD_C (control) GRD_I (infected), sweet granadilla passion fruit; YP_C (control) YP_I (infected) yellow passion fruit.

4.2.2.2 Confirmation of passion fruit woodiness disease infection post mechanical sap transmission

All leaf samples from PWD-inoculated passion fruit plants tested positive for potyviruses (Table 4.8). Potyvirues were not detected in leaf samples from non inoculated plants. The highest absorbance was recorded in purple passion fruit (0.392) and the lowest absorbance in yellow passion fruit (0.244). Absorbance in yellow passion fruit was significantly ($p \leq 0.05$) different from all other genotypes except sweet granadilla genotype. There was no significant difference ($p > 0.05$) in absorbance recorded in non-inoculated plants of different genotypes (Table 4.8).

Table 4.8: Detection of potyviruses in leaf samples collected from PWD-inoculated plants under greenhouse conditions

Genotype		Absorbance (Abs_{405nm})	ELISA reaction[y]
PP	Non-inoculated	0.090 ± 0.00^{d}	-
PP	Inoculated	0.392 ± 0.03^{a}	+
KPF4	Non- inoculated	0.093 ± 0.00^{d}	-
KPF4	Inoculated	0.332 ± 0.02^{ab}	+
BNN	Non- inoculated	0.089 ± 0.00^{d}	-
BNN	Inoculated	0.377 ± 0.00^{ab}	+
YP	Non- inoculated	0.095 ± 0.00^{d}	-
YP	Inoculated	0.244 ± 0.02^{c}	+
GRD	Non- inoculated	0.086 ± 0.00^{d}	-
GRD	Inoculated	0.302 ± 0.03^{bc}	+
Negative control		0.083	-
Positive control (kit)		0.176	+
Positive control (source of inoculum)		0.173	+

[y] (+) = positive reaction to the presence of potyviruses; (−) = negative reaction to the presence of potyviruses. PP, purple passion fruit; BNN, banana passion fruit; KPF4, Kenya Passion Fruit 4; GRD, sweet granadilla passion fruit; YP, yellow passion fruit.

4.2.3 Disease progression under field conditions

The first PWD symptoms on inoculated plants were observed at three weeks after inoculation (Figure 4.6). The highest AUDPC values (2.34 to 3.22) were observed with KPF4 passion fruit between 21 days and 42 days post - inoculation. Purple passion fruit displayed the highest AUDPC values (3.46 to 3.60) between 49 days and 63 days post inoculation. However, there was no significant difference ($p > 0.05$) in disease progression between purple passion fruit and KPF 4 passion fruit. There was a significant difference in disease progression between yellow passion fruit and genotypes KPF4 and purple passion fruit between 21 to 35 days post-inoculation. There was a significant difference ($p \leq 0.05$) between banana passion and rest of the genotypes namely KPF 4, purple passion, yellow passion and sweet granadilla throughout the evaluation period.

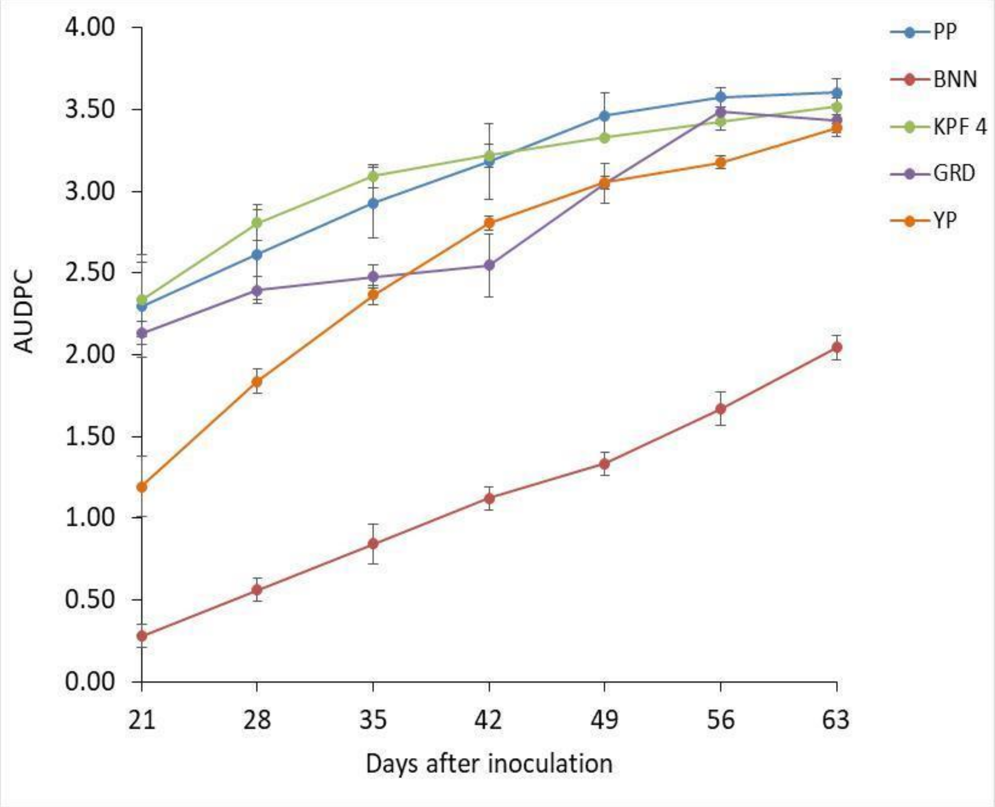


Figure 4.6: Area Under Disease progress curve (AUDPC) in passion fruit plants infected with passion fruit woodiness disease virus complexes at different days after inoculation under field conditions. PP, purple passion fruit; BNN, banana passion fruit; KPF 4, KPF 4 passion fruit; GRD, sweet granadilla passion fruit); YP, Yellow passion fruit.

Based on symptom development, banana passion fruit genotype was classified into moderately tolerant while sweet granadilla, yellow passion, KPF 4 and purple passion fruit genotypes were susceptible (Table 4.9).

Table 4.9: Grouping of passion fruit genotypes under field conditions based on symptom development

Genotype	Severity range	Classification
Banana passion fruit	1.07 - 2.13	Moderately tolerant
Yellow passion fruit	1.93 - 3.13	Susceptible
Sweet granadilla passion fruit	1.4-3.2	Susceptible
KPF 4 passion fruit	2.07 - 3.27	Susceptible
Purple passion fruit	2.12 - 3.33	Susceptible

4.2.3.1 Effect of PWD on plant growth under field conditions

The increase in plant height in infected banana passion was significantly lower ($p \leq 0.05$) compared to the non-inoculated plants between 35 to 77 days (Figure 4.7). A significant reduction ($p \leq 0.05$) in plant height was observed between 28 days after inoculation to 49 days post-inoculation. Between 56 and 77 days post- inoculation, a significant ($p \leq 0.05$) increase in plant growth was recorded. A significant increase ($p \leq 0.05$) in height was recorded in KPF 4 infected plants. The increase in height in the infected plants of KPF 4, purple passion and banana passion genotypes between 49 and 77 days was significantly ($p \leq 0.05$) lower compared to the non-inoculated plants.

No significant increase ($p > 0.05$) in plant height was recorded in purple passion between 35 and 49 days post inoculation. A significantly lower ($p \leq 0.05$) growth was recorded in infected purple passion compared to the non inoculated plants between 56 to 77 days post inoculation. No significant difference ($p > 0.05$) in increase in height of infected yellow passion fruit plants compared to the non-inoculated plants throughout the growth period.

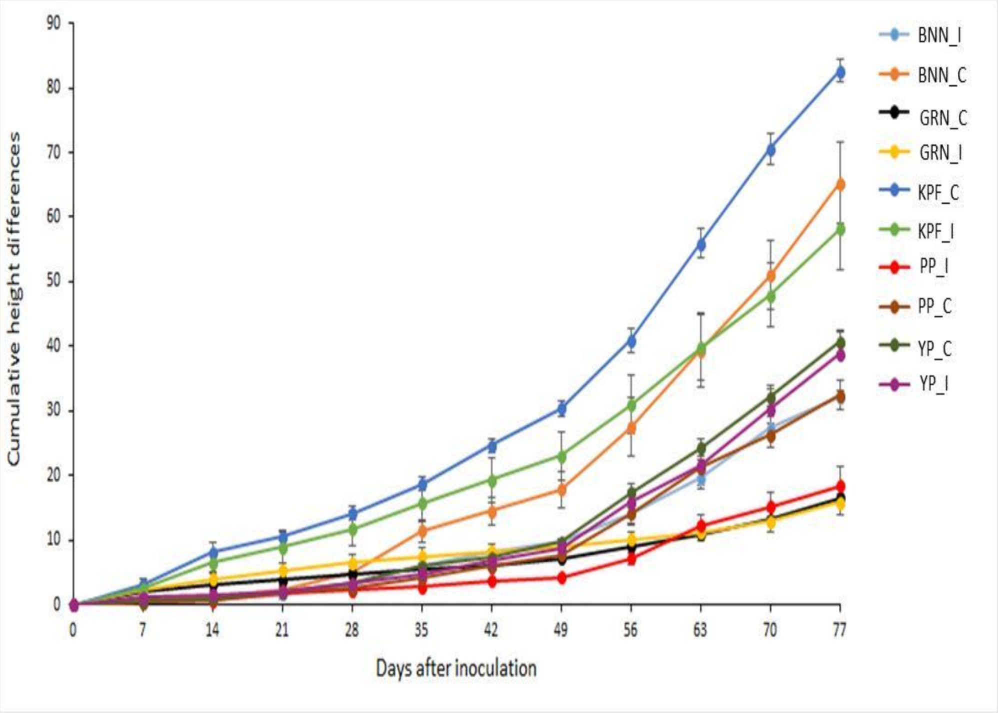


Figure 4.7: Growth of passion fruit genotypes mechanically inoculated with passion fruit virus complex under field conditions. BNN_C (control) BNN_I (infected), banana passion fruit; GRD_C (control) GRD_I (infected), sweet granadilla passion fruit; KPF_C (control) KPF_I (infected) KPF 4 passion fruit; PP_C (control) PP_I (Infected), purple passion fruit; YP_C (control) YP_I (infected) yellow passion fruit.

There was a significant negative correlation between disease severity and plant growth in banana passion (-0.787). A negative correlation was also recorded between disease severity and plant growth in KPF 4 (-0.366), purple passion (-0.189), yellow passion (-0.012) and sweet granadilla passion fruit (-0.204) (Table 4.10).

Table 4.10: Correlation analysis of plant growth and disease severity under field conditions

Experimental site	Responsive variable	Genotypes				
		BNN	KPF 4	PP	YP	SG
Kenyatta University (Main campus)	Plant height	-0.787**	-0.366	-0.189	-0.012	-0.204

**Correlation is significant at the 0.01 level (2 tailed). BNN, Banana passion; KPF 4, KPF 4 passion; PP, Purple passion; YP, Yellow passion; SG, Sweet granadilla passion

4.2.3.2 Enzyme Linked Immunosorbent Assay

All leaf samples from PWD-inoculated passion fruit plants tested positive for potyviruses (Table 4.11). Potyviruses were not detected in leaf samples from non-inoculated plants. The highest absorbance was recorded in purple passion fruit (0.371) and the lowest absorbance in sweet granadilla passion fruit (0.229). There was no significant difference ($p > 0.05$) in absorbance of inoculated plants in all genotypes tested. There was no significant difference ($p > 0.05$) in absorbance recorded in non-inoculated plants of the different genotypes (Table 4.11).

Table 4.11: Detection of potyviruses in passion fruit leaf samples collected from plants maintained under field conditions

Genotype		Absorbance (Abs_{405nm})	Potyvirus status^{y}
PP	Non- inoculated	0.087 ± 0.00^{b}	-
PP	Inoculated	0.371 ± 0.03^{a}	+
KPF4	Non- inoculated	0.092 ± 0.00^{b}	-
KPF4	Inoculated	0.304 ± 0.03^{a}	+
YP	Non- inoculated	0.091 ± 0.00^{b}	-
YP	Inoculated	0.259 ± 0.10^{ab}	+
BNN	Non- inoculated	0.090 ± 0.00^{b}	-
BNN	Inoculated	0.277 ± 0.02^{a}	+
GRD	Non- inoculated	0.089 ± 0.00^{b}	-
GRD	Inoculated	0.229 ± 0.00^{ab}	+
Negative control		0.083	-
Positive control (kit)		0.176	+
Positive control (source of inoculum)		0.173	+

^{y} (+) =positive reaction to the presence of potyviruses; (−) =negative reaction to the presence of potyviruses. PP, purple passion fruit; BNN, banana passion fruit; KPF4, Kenya Passion Fruit 4; GRD, sweet granadilla passion fruit; YP, yellow passion fruit.

4.3 Effect of different concentrations of auxins and cytokinins on *in vitro* regeneration of KPF 4 and purple passion fruit genotypes

4.3.1 Effect of BAP and KIN on shoot organogenesis from leaf disc explants of KPF 4 and purple genotype of passion fruit

Shoots were not formed on leaf explants of KPF4 genotypes cultured on MS medium without growth regulators (control) as indicated in Table 4.12. Instead, callus was formed from the cut surfaces. Morphogenetic responses of leaf disc explants were observed on MS medium augmented with BAP irrespective of the concentration of BAP tested. The first morphogenetic responses were visible after 21 days of culture, when shoot buds were observed from the leaf explants (Plate 4.6 A and B). The highest mean number of induced shoots (11.44 per leaf disc explant) was observed on MS supplemented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} kinetin, which was not significantly ($p > 0.05$) different from the average number of induced shoots observed on MS augmented with 2.0 mg L^{-1} BAP. Similarly, the highest mean number of explants with clusters of shoots (1.4 per leaf disc explant) was observed on MS augmented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} kinetin. However, there was no significant difference ($p > 0.05$) in the mean number of explants with clusters of shoots in this media augmented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} KIN compared with that augmented with 1.0 and 2.0 mg L^{-1} BAP (Table 4.12).

Table 4.12: Effect of BAP and KIN on *in vitro* shoot induction from leaf disc explants of KPF 4 passion fruit genotype after 8 weeks of culture

PGR	Conc. (mg L^{-1})	Percentage no. of explants with shoots	Mean no. of shoots per explant^{X}	Mean number of explants with clusters of shoots^{X}
BAP	0.0	0.0	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
	1.0	58.0	6.06 ± 0.7^{b}	1.22 ± 0.1^{ab}
	2.0	86.6	10.72 ± 0.9^{a}	1.38 ± 0.2^{a}
	3.0	50.0	4.5 ± 0.8^{b}	0.5 ± 0.1^{bc}
BAP+ KIN	2.0 + 0.5	88.8	11.44 ± 0.9^{a}	1.44 ± 0.2^{a}

^{X}Values represent mean ± standard error for 30 explants per treatment in three repeated experiments. Means having the same letter within a column are not significantly different at $P > 0.05$ according to Tukey's Honest significant difference.

Leaf disc explants of KPF 4 cultured on MS medium augmented with 3 mgL^{-1} BAP had the lowest mean number of induced shoots and lowest average number explants with clusters of shoots (Table 4.12). Micro-shoots from MS medium supplemented with 1.0 to 3.0 mg L^{-1} BAP or a combination of BAP and kinetin (2.0 mg L^{-1} BAP + 0.5 mg L^{-1} kinetin) developed further when they were transferred into MS medium supplemented with 0.1 mg L^{-1} BAP (Plate 4.6 C and D).

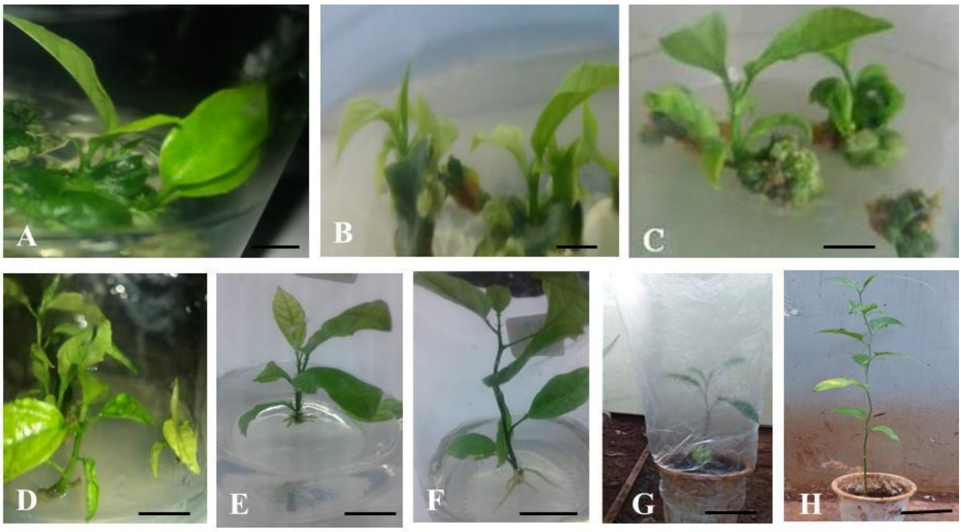


Plate 4.6: *In vitro* regeneration of plantlets from leaf disc explants of passion fruit genotype KPF 4 cultured on MS medium and acclimatization and growth in the greenhouse. A and B, Initiation of shoots from leaf disc explants cultured on MS + 2 mg L^{-1} BAP on the 3rd and 4th week after culture, respectively (Bars = 1 cm); C and D, further development of shoots on MS + 0.1 mg L^{-1} BAP on the 2nd and 4th week after culture, respectively (Bars = 1 cm); E and F, Rooted plants on MS + 0.1 mg L^{-1} NAA on the 1st and 2nd week after culture, respectively (Bars = 1 cm); G, acclimatization stage (2 week- old plant in a plastic pot) (Bar = 2 cm) H, one month-old plant growing in a plastic pot (Bar = 2 cm).

All of the elongated shoots cultured in MS medium augmented with 0.1 mg L^{-1} naphthalene acetic acid (NAA), roots were initiated after 1 week (Plate 4.6

E) and formed well-rooted plantlets within 2 weeks (Plate 4.6 F). Fully developed rooted plantlets were weaned and hardened for 4 weeks in small pots (Plate 4.6 G) before they were transferred onto larger pots (Plate 4.6 H) with a survival rate of 42%.

Plants regenerated through leaf discs in MS augmented with 1.0 - 2.0 mg L^{-1} BAP and a combination of 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} kinetin, were normal and no morphological abnormalities were noted for early stages of development in the potted plants. Shoots formed on MS medium supplemented with 3 mg L^{-1} BAP were abnormal with short or no stems and large malformed leaves (Plate 4.7A).

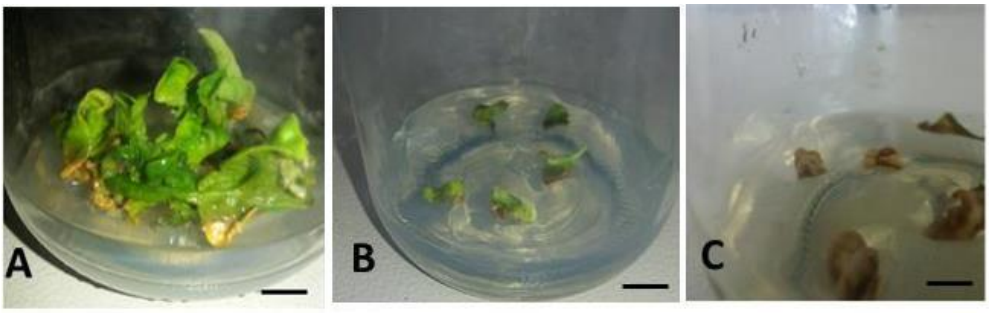


Plate 4.7. Effect of BAP on leaf explants of purple and KPF 4 genotypes of passion fruit (Bars = 1 cm). A, abnormal shoots from KPF 4 leaf explants after 4 weeks of culture on MS + 3 mg L^{-1} BAP; B and C, Non responsive leaf disc explants from purple genotype cultured on MS + 2 mg L^{-1} BAP on the 3rd and 8th week after culture respectively.

No shoots were initiated from leaf disc explants of purple genotype placed on hormone free MS medium. All of the leaf disc explants of purple variety cultured on MS augmented with different concentrations of BAP or a combination of BAP and KIN turned brown, with no further development into shoot buds (Plate 4.7 B and C).

4.3.2 *In vitro* micropropagation and multiplication of plants using stem nodal explants of KPF 4

Shoot multiplication of KPF 4 genotype of passion fruit occurred on MS medium augmented with different concentrations of BAP or KIN (Plate 4.8). On MS medium without PGRs (control), an average of 0.44 and 0.61 shoots per explant were recorded on 4th and 8th weeks of culture, respectively (Table 4.13). MS supplemented with 3 mg L^{-1} BAP had the highest mean number of shoots per explant (3.83) on the 8th week of culture, which was significantly ($p \leq 0.05$) different from other concentrations of either BAP or kinetin. The highest number of leaves per explant were 3.44 and 5.0 after the 4th and 8th week of culture, respectively, when the explants were cultured on MS augmented with 2 mg L^{-1} BAP which was significantly different from other concentrations of BAP, kinetin or a combination of BAP and kinetin **(**2.0 mg L^{-1} BAP + 0.5 mg L^{-1} kinetin) as indicated in Table 4.13. The highest average shoot height was 1.51 obtained on the 8th week of culture in MS augmented with 2 mg L^{-1} BAP which was significantly ($p \leq 0.05$) different from other concentrations of BAP or Kinetin.

The MS media supplemented with BAP (at 2 mg L^{-1}) in combination with KIN (at 0.5 mg L^{-1}) had the highest average number of shoots per explant (4.44 and 6.39 on the 4th and 8th week of culture, respectively) which was significantly ($p \leq 0.05$) different from the various concentrations of either BAP or KIN alone at both 4 weeks and 8 weeks of culture (Table 4.13).

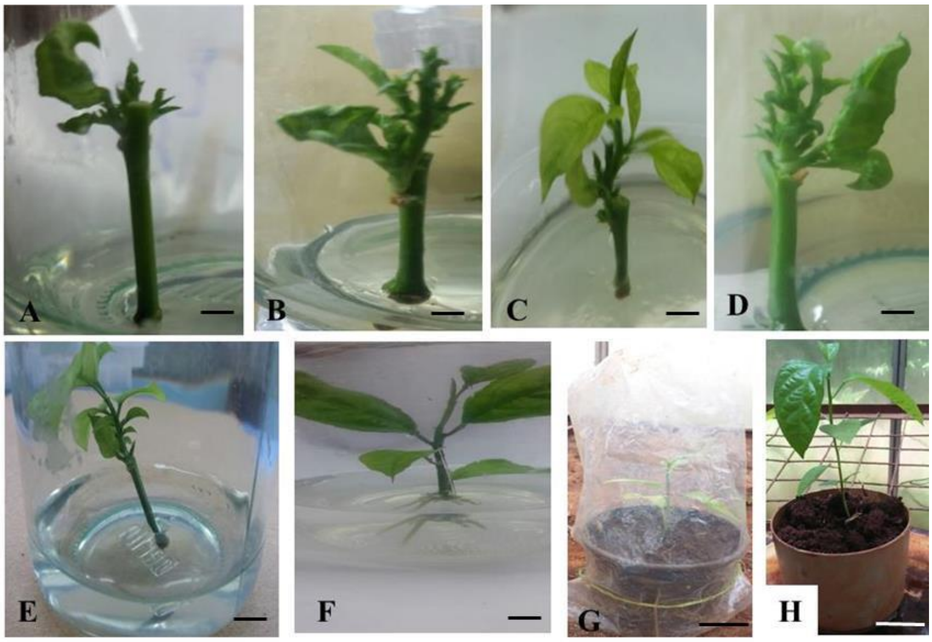


Plate 4.8: *In vitro* regeneration of plantlets from stem nodal explants of passion fruit genotype KPF 4 cultured on MS medium and acclimatization and growth in the greenhouse (Bars = 1 cm). A and B, Initiation of shoots from nodal explants cultured on MS + 3 mg L^{-1} BAP on the 4th and 8th week after culture, respectively; C and D, Initiation of shoots from nodal explants cultured on MS + 2 mg L^{-1} BAP+ 0.5 mg L^{-1} kinetin on the 4th and 8th week after culture, respectively; E, Initiation of shoots from nodal explants cultured on MS + 3 mg L^{-1} KIN on the 8th week after culture. F; Rooted plant (Individual shoot separated from multiple shoots on the nodal explant) on MS + 0.1 mg L^{-1} NAA on the 2nd week after culture; G, acclimatization stage (2 week- old plant in a plastic pot); H, one month-old plant growing in a plastic pot.

Table 4.13: Effect of BAP and KIN on shoot multiplication of nodal explants of KPF 4 passion fruit on 4th and 8th weeks of culture

		4 weeks				8 weeks			
PGR	**Conc. (mg L^{-1})**	**Percentage No. of explant with shoots (%)**	**Mean No. of shoots (per explant)^{X}**	**Mean No. of leaves^{X}**	**Shoot height (cm)^{X}**	**Percentage No. of explants with shoots (%)**	**Mean No. of shoots (per explant)^{X}**	**Mean No. of leaves^{X}**	**Mean shoot height (cm)^{X}**
BAP	0.0	44.0	0.44 ± 0.1^{c}	0.28 ± 0.1^{c}	0.04 ± 0.01^{d}	55.0	0.61 ± 0.12^{c}	0.72 ± 0.18^{d}	0.07 ± 0.02^{d}
	1.0	94.4	0.95 ± 0.1^{bc}	1.95 ± 0.3^{b}	0.16 ± 0.02^{cd}	100.0	1.0 ± 0.0^{c}	3.57 ± 0.31^{b}	0.85 ± 0.16^{b}
	2.0	100.0	1.0 ± 0.0^{bc}	3.44 ± 0.2^{a}	0.68 ± 0.07^{a}	100.0	1.06 ± 0.05^{c}	5.0 ± 0.23^{a}	1.51 ± 0.11^{a}
	3.0	94.4	2.06 ± 0.3^{b}	1.83 ± 0.3^{b}	0.22 ± 0.04^{cd}	100.0	3.83 ± 0.44^{b}	3.33 ± 0.37^{b}	0.83 ± 0.11^{b}
KIN	1.0	94.4	0.94 ± 0.1^{bc}	1.06 ± 0.2^{bc}	0.16 ± 0.03^{cd}	94.0	0.94 ± 0.06^{c}	1.72 ± 0.34^{cd}	0.36 ± 0.08^{cd}
	2.0	100.0	1.0 ± 0.0^{bc}	1.22 ± 0.2^{bc}	0.23 ± 0.04^{bcd}	100.0	1.0 ± 0.0^{c}	2.28 ± 0.36^{bc}	0.57 ± 0.09^{bc}
	3.0	100.0	1.0 ± 0.0^{bc}	2.11 ± 0.3^{b}	0.46 ± 0.08^{ab}	100.0	1.0 ± 0.0^{c}	3.61 ± 0.35^{b}	0.85 ± 0.10^{b}
BAP +KIN	2.0+ 0.5	94.0	4.44 ± 0.8^{a}	1.89 ± 0.2^{b}	0.36 ± 0.09^{bc}	100.0	6.39 ± 0.86^{a}	3.06 ± 0.30^{bc}	0.53 ± 0.10^{bcd}

^{X}Values represent mean ± standard error of 10 replicates per treatment in three repeated experiments. Means having the same letter within a column are not significantly different at $P > 0.05$ according to Tukey's Honest significant difference.

Roots were initiated after 7 days in all elongated shoots cultured on MS medium supplemented with 0.1 mg L^{-1} naphthalene acetic acid (NAA), forming well-rooted plantlets in a period of 2 weeks. Out of fifty rooted plantlets that were weaned and hardened for 4 weeks in small pots, 44 plants survived giving a survival rate of 88%.

4.3.3 Effect of putrescine on vegetative propagation of passion fruit nodal explants

All the concentrations of putrescine tested, induced rooting on nodal cuttings from the two passion fruit cultivars (Table 4.14). The purple variety of passion fruit had 100% rooting response when nodal cuttings were treated with putrescine. However, variation in putrescine concentration had no significant effect ($p > 0.05$) on the mean number of roots, mean root length and mean number of new leaves formed in purple passion fruit (Table 4.14). The KPF 4 variety had the highest rooting response (100%) when nodal cuttings were treated with 0.5% putrescine. Unlike purple variety, there was no induction of roots on nodal cuttings of variety KPF 4 which were not treated with putrescine (Plate 4.9). There was a significant ($p \leq 0.05$) difference between purple and KPF 4 varieties in regard to mean number of new leaves whereby KPF 4 had the lowest mean number of new leaves at 2% putrescine. On the other hand, there was no significant difference between the two varieties of passion fruit in regard to the mean number of roots and mean root length.

Table 4.14: Effect of putrescine on rooting of nodal explants of KPF 4 and purple passion fruit genotypes

Passion fruit variety	Conc. of Putrescine	Percentage rooting response (%)	Mean No. of roots^{X}	Mean root length^{X}	Mean No. of new leaves^{X}
Purple	0.0	66.7	3.33±1.18^{c}	1.2±0.60^{c}	0.0^{b}
	0.5	100	10.6 ± 1.01^{ab}	5.8±0.71^{a}	3.8± 0.25^{a}
	2.0	100	11.2± 0.81^{ab}	5.5±0.79^{ab}	3.8±0.32^{a}
	0.0	0	0.0^{c}	0.0^{c}	0.0^{b}
KPF4	0.5	100	11.7±2.23^{a}	5.5±0.84^{ab}	3.7±0.42^{a}
	2.0	80	7.8 ±3.61^{b}	3.4±0.27^{b}	1.0±0.71^{b}

^{X}Values represent mean ± standard error of 10 replicates per treatment in three repeated experiments. Means with the same letter within a column are not significantly different (P > 0.05) according to Tukey's Honest significant difference test at 5% level.

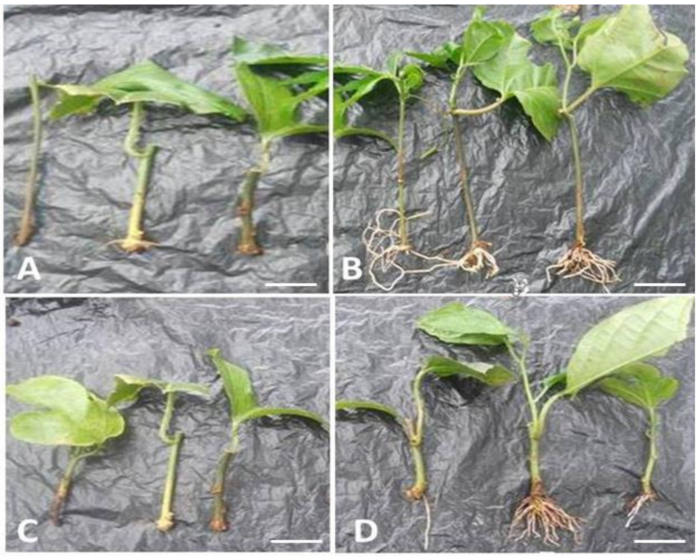


Plate 4.9: Root initiation from passion fruit stem nodal explants treated with different concentrations of putrescine (Bars = 1 cm): A, Purple skinned passion fruit nodal explants not treated with putrescine; B, purple skinned passion fruit nodal explants treated with 2% putrescine treatment; C, KPF 4 nodal stem explants not treated with putrescine; D, KPF 4 nodal stem explants treated with 2% putrescine treatment.

4.3.4 Effect of 2, 4-D on callus induction and somatic embryogenesis from leaf explants of two genotypes of passion fruit

In hormone free medium (control), callus was recorded on leaf explants from KPF 4 genotype. The leaves displayed some chlorosis as from the fourth week of culture on callus initiation medium and then turned brown. On the other hand, leaf explants from purple variety of passion fruit did not form callus on hormone free medium.

Explants from purple genotype of passion fruit generated loose non-embryogenic callus on different concentrations of 2, 4-D (Plate 4.10A). Embryos were not formed from purple genotype of passion fruit. For KPF 4, leaf explants inoculated on MS medium supplemented with 0.5 to 16 mg L^{-1} 2, 4-D, callus initiation was visible from the cut edges as from the fourth day of culture. Friable callus from KPF 4 leaf explants began to show some level of organization into defined structures which appeared like the globular stages of somatic embryos in media augmented with 4 mg L^{-1} 2, 4-D (Plate 4.10 B, C and D).

At lower concentrations of 2,4-D (< 4.0 mg L^{-1}) no somatic embryos were recorded in KPF 4 genotype. The number of KPF 4 leaf explants with somatic embryos increased with increase in concentration of 2,4-D up to 8 mg L^{-1} 2,4-D which generated the highest number of explants with somatic embryos which was significantly ($p \leq 0.05$) different from all other treatments except treatment with 10 mg L^{-1} (Table 4.15).

An increase in PGR concentration from 0.5 to 6 mg L^{-1} 2, 4-D resulted in an increase in the frequencies of callus induction in purple genotype after which a decline was recorded. In addition, an increase in PGR concentration resulted in an increase in the frequencies of explants with embrogenic callus and somatic embryos of KPF 4 genotype attaining the highest mean of 100 and 91.1, respectively at 8 mg L^{-1} 2, 4-D (Table 4.14).

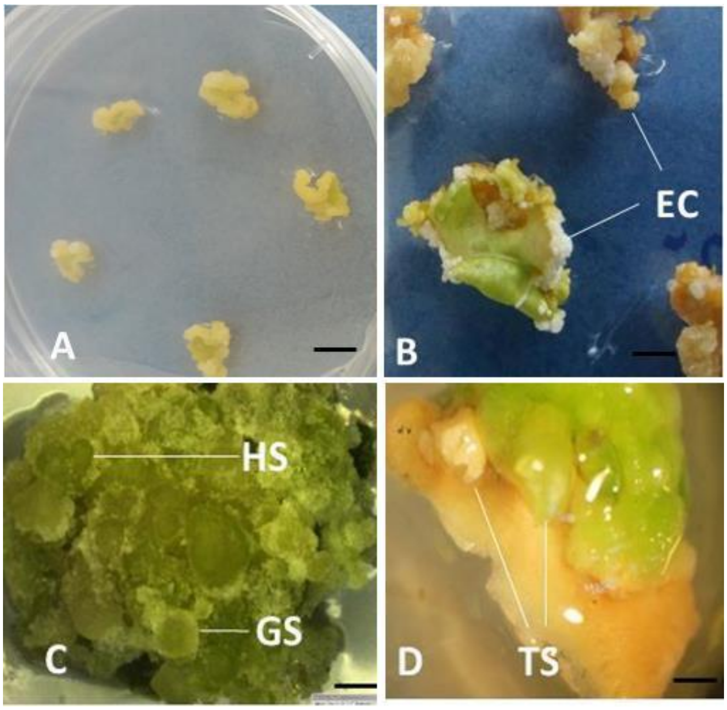


Plate 4.10: Effect of 2, 4-D on callus induction and somatic embryogenesis on leaf discs of purple passion fruit and KPF 4. A, loose non-embryogenic cream yellow callus from purple passion fruit leaf explants on MS supplemented with 4.0 mg L^{-1} (Bar = 1 cm) ; B, white friable embryogenic callus (EC) from leaf explants of KPF 4 on MS supplemented with 8 mg L^{-1} 2, 4-D (Bar = 1 cm); C, Globular (GS) and heart shaped (HS) stages of somatic embryos of KPF 4 on medium supplemented with 8 mg L^{-1} 2, 4-D (Bar = 1 mm); D, torpedo stage (TS) of somatic embryos from KPF 4 leaves on medium supplemented with 8 mg L^{-1} 2, 4-D (Bar = 1 mm).

Table 4.15: Effect of 2, 4-D on callus induction and somatic embryogenesis from leaf explants of KPF 4 and purple passion fruit genotypes after 6 weeks of culture

PGR	**KPF 4**		**PURPLE**	
2,4-D (mg/l)	**% No. of explants with embryogenic callus**[X]	**% No of callus with somatic Embryos**[X]	**% No of explants with non embryogenic callus**[X]	**% No of callus with somatic embryos**[X]
0.0	24.4.±4.4^{d}	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0
0.5	75.6±5.6^{c}	0.0±0.0^{d}	51.1±5.6^{b}	0.0±0.0
1.5	81.1±4.9^{bc}	0.0±0.0^{d}	60.0±4.5^{b}	0.0±0.0
2.5	97.8±1.5^{a}	0.0±0.0^{d}	86.7±3.9^{a}	0.0±0.0
4.0	96.7±1.8^{a}	46.7±3.6^{b}	85.5±3.8^{a}	0.0±0.0
6.0	97.8±2.2^{a}	53.3±6.6^{b}	93.3±2.3^{a}	0.0±0.0
8.0	100.0±0.0^{a}	91.1±4.3^{a}	90±0.7^{a}	0.0±0.0
10.0	92.2±2.8^{ab}	82.2±4.5^{a}	86.7±2.7^{a}	0.0±0.0
12.0	93.3±2.2^{ab}	38.9±6.3^{bc}	90±3.3^{a}	0.0±0.0
16.0	100.0±0.0^{a}	20.0±3.6^{c}	86.7±3.9^{a}	0.0±0.0

[X] Values represent mean ± standard error of 6 replicates per treatment in three repeated experiments. Means having the same letter within a column are not significantly different at $P > 0.05$ according to Tukey's Honest significant difference test.

When embrogenic callus was transferred to hormone free MS medium, MS supplemented with 1% activated charcoal, MS supplemented with 1 mg L^{-1}BAP and MS supplemented with 0.1 mg L^{-1}ABA they turned green but later turned brown as from the 15th day of culture and finally died. Frequent subcultures only preserved the green coloration with no further development.

4.3.5 Effect of 2, 4-D and TDZ on callus induction and somatic embryogenesis from immature seeds of KPF 4 and purple genotypes of passion fruit

Immature seeds from KPF 4 and purple passion fruits inoculated on MS basal salts and B5 vitamins supplemented with 4 mg L^{-1}**,** 8 mg L^{-1}**,** 16 mg L^{-1} and 32 mg L^{-1} in combination with 1 mg L^{-1} TDZ yielded white friable embryogenic callus as from the 5th day of culture (Plate 4.11 A). Most callus appeared from the basal end of the seed while a few masses of callus appeared from the surface of the seed. The highest frequency of callus induction (95.5% for KPF 4 and 93.3% for purple cultivar) was obtained from callus initiation medium augmented with 8 mg L^{-1} 2,4-D in combination with 1 mg L^{-1} TDZ (Table 4.16). These frequencies were significantly ($p \leq 0.05$) different from other treatments. Small callus was also observed on hormone free MS medium (control) after one week.

After 6 weeks of culture on callus induction medium, the callus was transferred to hormone free MS basal salts with B5 vitamins augmented with 1% activated charcoal. Callus from hormone free callus initiation medium did not produce somatic embryos for both genotypes tested. Similarly, no somatic embryos were observed from callus obtained from purple passion on initiation and maturation medium. The callus lost the green colour after 5 weeks. However, on subculturing to fresh medium with 1% activated charcoal, the green colour was retained for a longer time.

On the other hand, for KPF 4 genotype, callus induced in increasing concentrations of 2, 4-D formed different stages of somatic embryos after one week of transfer to MS basal salts and B5 vitamins supplemented with 1% activated charcoal (Plate 4.11 B, C & D). Callus from initiation medium containing 8.0 mg L^{-1} 2,4-D and 1.0 mg L^{-1} TDZ produced the highest frequency of somatic embryos which was not significantly ($P > 0.05$) different from other treatments. All callus transferred to 0.1 mg L^{-1} ABA did not generate somatic embryos.

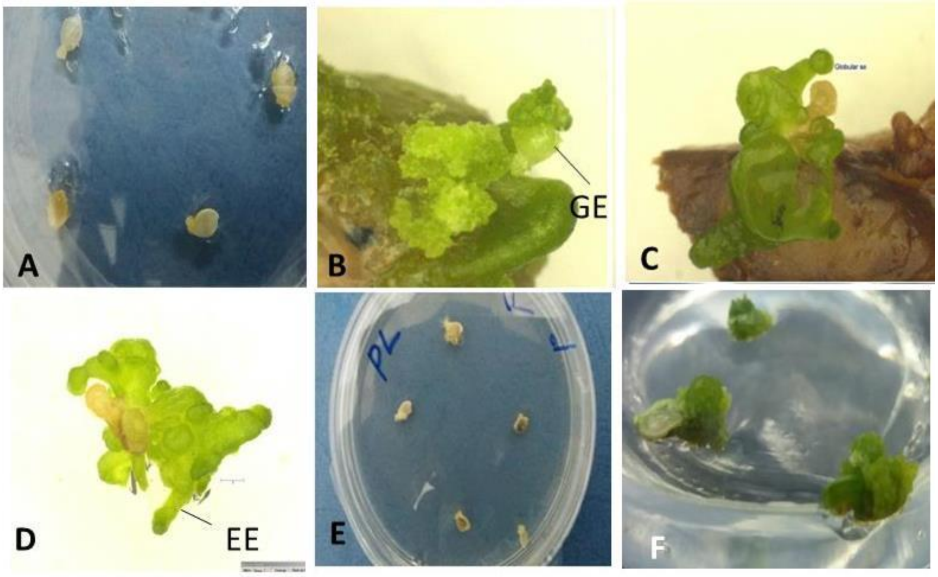


Plate 4.11: Effect of 2, 4-D and TDZ on induction of somatic embryos from immature seeds of KPF 4 and purple passion fruits. A, Six days old callus from immature seeds of KPF 4 on MS supplemented with 8 mg L^{-1} 2,4-D and 1 mg L^{-1} TDZ (Bar = 1 cm); B, Eight weeks old callus of KPF 4 seeds on 1% activated charcoal showing globular stage of somatic embryos (GE, globular embryos) (Bar = 1 mm); C, Ten weeks old embryogenic callus of KPF 4 passion displaying secondary somatic embryogenesis (Bar = 1 mm); D, Eleven weeks old callus of KPF 4 passion showing further development of somatic embryos (EE, elongating embryos) (Bar = 1 mm); E, Six days old little callus from immature seeds of purple passion on MS supplemented with 8 mg L^{-1} 2,4-D and 1 mg L^{-1} TDZ (Bar = 1 cm); F, Eight weeks old callus (without somatic embryos) from immature seed explants of purple passion on MS medium supplemented with 0.5 mg L^{-1} BAP (Bar = 1 cm).

Table 4.16: Effect of 2, 4-D and TDZ on somatic embryogenesis from immature seed explants of KPF 4 and purple passion fruits

PGR		**KPF 4 Variety**		**Purple Variety**	
2, 4-D (mg/l)	**TDZ (mg/l)**	**% No. of explant with callus induction^{X}**	**% No. of callus with somatic embryos^{X}**	**% No. of explants with callus induction^{X}**	**% No. of callus with somatic embryos^{X}**
0.0	0.0	80.0 ± 3.9^{c}	0.0 ± 0.0^{d}	56.6 ± 4.0^{c}	0.0 ± 0
4.0	1.0	86.6 ± 3.6^{bc}	77.8 ± 3.9^{c}	72.0 ± 2.9^{bc}	0.0 ± 0
8.0	1.0	95.5 ± 2.0^{a}	91.1 ± 1.8^{a}	93.3 ± 2.3^{a}	0.0 ± 0
16.0	1.0	90.0 ± 3.7^{b}	85.6 ± 2.9^{ab}	74.4 ± 4.2^{b}	0.0 ± 0
32.0	1.0	92.2 ± 2.8^{b}	75.5 ± 4.7^{bc}	73.3 ± 3.9^{b}	0.0 ± 0

^{X}Values represent mean ± standard error of 6 replicates per treatment in three repeated experiments. Means having the same letter within a column are not significantly different at $P > 0.05$ using Tukey's Honest significant difference test.

4.3.6 Conversion of somatic embryos to form plantlets

Only somatic embryos from immature seeds of KPF 4 previously cultured on MS augmented with 8.0 mg L^{-1} 2,4-D and 1 mg L^{-1} TDZ and then transferred onto MS supplemented with 1% (w/v) activated charcoal and thereafter into germination medium (MS with 0.5 mg L^{-1} BAP) formed plantlets. The micro shoots were from somatic embryos (Plate 4.12). A total of nine plantlets were obtained after 8 weeks of culture. The rest of the embryos retained their green colour, for as long as they were subcultured into fresh medium, without further development. Of the nine plantlets weaned and hardened for 4 weeks in small pots, 7 plants survived giving a survival rate of 77.8%.

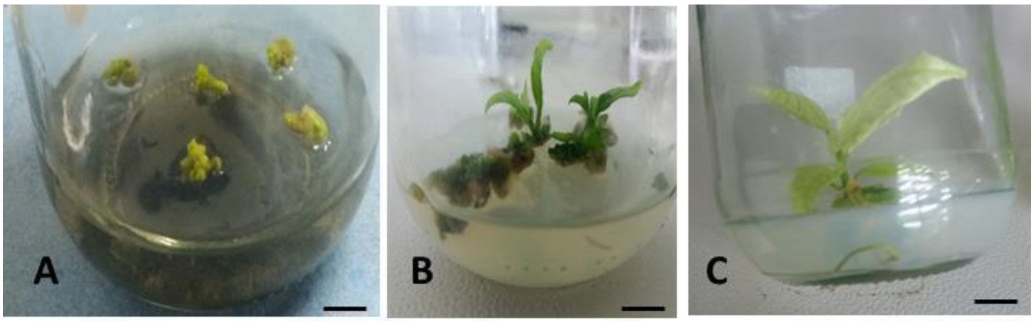


Plate 4.12: Microshoot formation from somatic embryos originating from immature seeds of KPF 4 cultured on MS augmented with 8.0 mg L^{-1} 2,4-D and 1 mg L^{-1} TDZ (Bars = 1 cm). A, Three weeks old callus with somatic embryos on MS medium supplemented with 1% activated charcoal B, Six weeks old shoots on MS medium supplemented with 0.5 mg L^{-1} BAP ; C, Eight weeks old plantlet on MS medium without plant growth regulators.

4.3.7 Assessment of genetic fidelity of *in vitro* regenerated KPF 4 plants

Seven SRAP primer combinations produced a total of 1,570 scorable bands ranging from 173 to 260, with a mean of 224 bands per primer (Table 4.17). The amplicons ranged in size from 130 to 2000 bp. The identical banding pattern in majority of the *in vitro* regenerated plants with the mother plant (Plate. 4.13) for each of the 7 primer combinations confirmed the genetic fidelity of the *in vitro* regenerated plants. A similarity matrix based on Jaccard's coefficient demonstrated that the pairwise value between the mother plants and the *in vitro* regenerated plants ranged from 88.5% (0.885) and 100% (1.0) (Appendix V).

Table 4.17. Details of SRAP primer combinations used with the number and size of amplified fragments generated in mother plants and *in vitro* regenerated plants

Primer code	**Forward sequence (5' to 3')**	**Reverse sequence (5' to 3')**	**No. of scorable bands**	**Range of band sizes (bp)**
me1 – em 9	TGAGTCCAACC GGATA	GACTGCGTA CGAATTCAG	234	400 – 800
me 5 - em 7	TGAGTCCAAAC CGGAAG	GACTGCGTA CGAATTCAA	260	300 – 2000
me2 - em 10	TGAGTCCAAAC CGGAGC	GACTGCGTA CGAATTCAG	205	300 – 2000
me 1- em 12	TGAGTCCAACC GGATA	GACTGCGTA CGAATTCTC	208	250 – 2000
me11- em11	TGAGTCCAAAC CGGTCC	GACTGCGTA CGAATTCCA	260	400 – 1000
me1 - em 15	TGAGTCCAACC GGATA	GACTGCGTA CGAATTGTC	230	130 – 650
me2 - em 12	TGAGTCCAAAC CGGAGC	GACTGCGTA CGAATTCTC	173	150 – 600
Average			**224**	

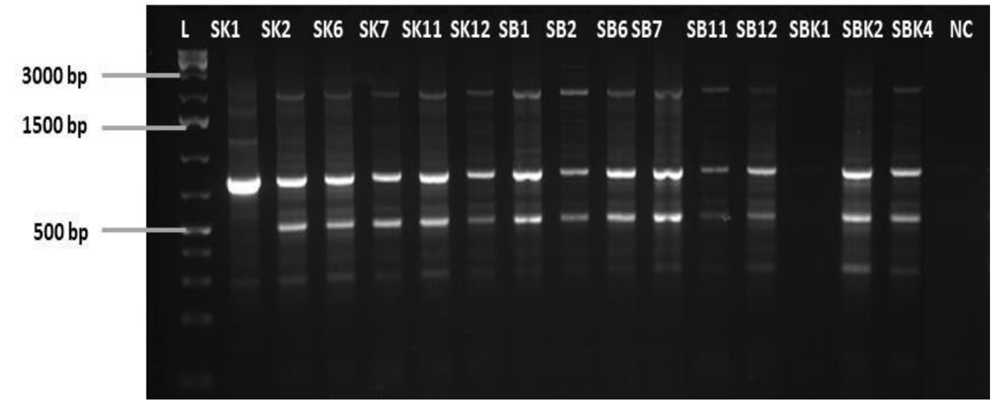


Plate 4.13: SRAP amplification profiles of mother plants and randomly selected *in vitro* regenerated passion fruit plants of KPF 4 genotype (from nodal explants) using primers me 2 and em 12. Lane L, 0'GeneRuler^{TM} 1 kb DNA plus DNA ladder (Thermo Scientific); Lanes, SK1 and SK2, are plantlets micropropagated on MS + 1 mg L^{-1} KIN; SK6 and SK7 are plantlets micropropagated on MS + 2 mg L^{-1} KIN; SK11 and SK12 are plantlets micropropagated on MS + 3 mg L^{-1} KIN; SB1 and SB2, are plantlets micropropagated on MS + 1 mg L^{-1} BAP; SB6 represents a mother plant; SB7, is a plantlet micropropagated on MS + 2 mg L^{-1} BAP; SB11and SB12 are plantlets regenerated on MS + 3 mg L^{-1} BAP; SBK1 and SBK 2 are plantlets micropropagated on MS augmented with 2 mg L^{-1} BAP + 0.5 mg L^{-1} KIN; SBK4 represents a mother plant; NC, Negative control (master mix and nuclease- free water).

Dendrogram analysis based on the Jaccard's similarity coefficient revealed three clusters whose similarity was above 99% (Figure 4.8). *In vitro* regenerated plantlets; SK1, SK2, SB2 and SB13 were in cluster A, SK3, SK4, SK5, SK6, SK7, SK8, SK11, SK12, SK13, SB1, SB3, SB7, SB8, SB9, SB11, SB12, SB14, SBK1 and SBK3 and mother plants (SB6 and SBK4) were in cluster B while SBK2 was in cluster C (Figure 4.8). The dendrogram generated from SRAP data showed that the *in vitro* regenerated plants SK3, SK4, SK5, SK6, SK7, SK8, SK11, SK12, SK13, SB1, SB3, SB7, SB8, SB9, SB11, SB12, SB14, SBK1 and SBK3 were identical to the mother plants (SB6 and SBK4) since they are in the same cluster (Figure 4.8). The pairwise value of a similarity matrix based on Jaccard’s coefficient was 88.5 (0.885) to 100% (1) indicating significant similarity (Appendix V).

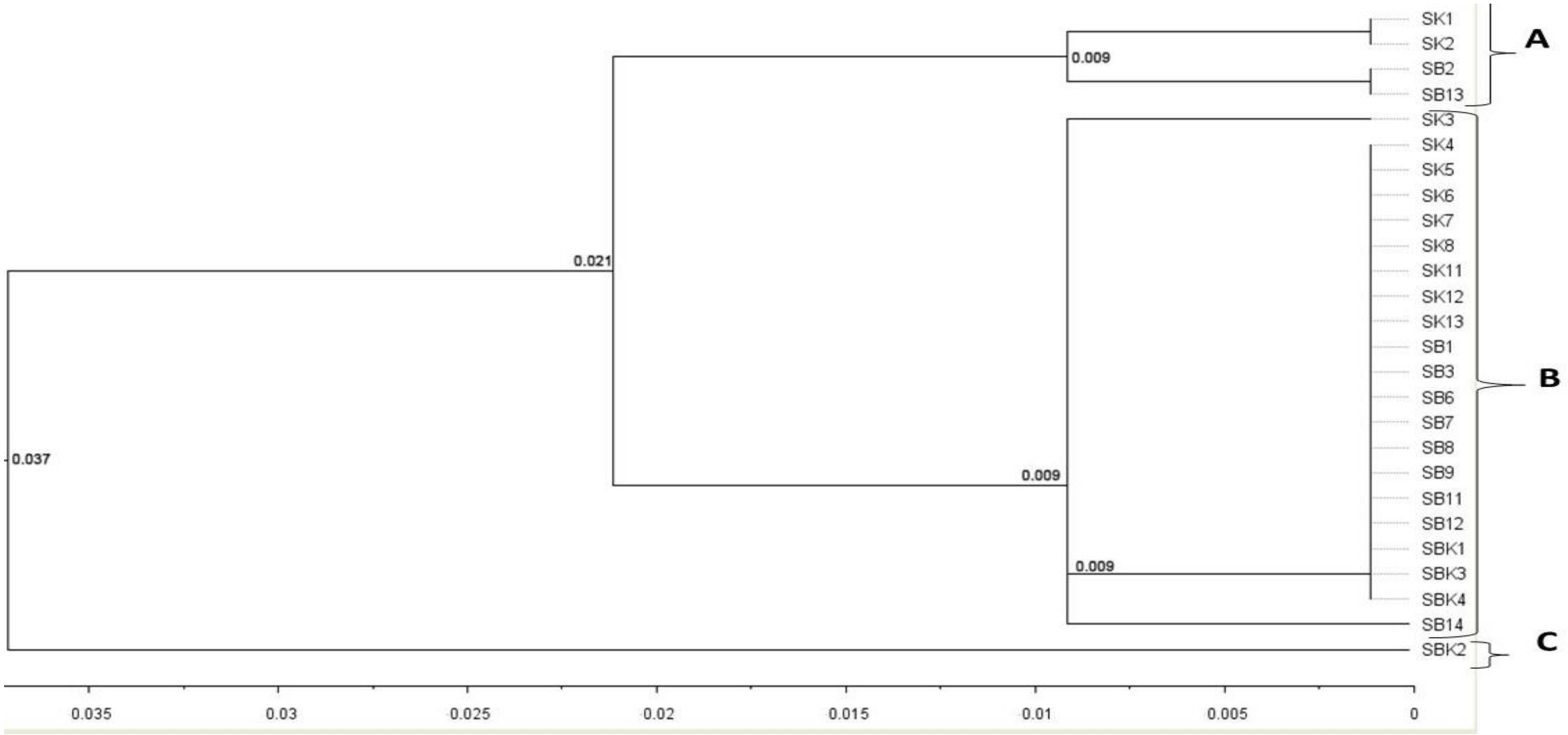


Figure 4:8 Dendrogram illustrating coefficient similarity among *in vitro* regenerated plants (from nodal explants) with mother plants (SB6 and SBK4) of passion fruit KPF4 by UPMGA cluster analysis of SRAP data set showing genetic relationship. SK1, SK2, SK3, SK4 and SK5 represent plants micropropagated on MS augmented with 1 mg L^{-1} KIN.SK6, SK7 and SK8 represent plants micropropagated on MS augmented with 2 mg L^{-1} KIN. SK11, SK12 and SK13 represent plants micropropagated on MS supplemented with 3 mg L^{-1} KIN. SB1, SB2 and SB3 represent plants regenerated on MS augmented with 1 mg L^{-1} BAP. SB6 is mother plant; SB7, SB8 and SB9 represent plants regenerated on MS augmented with 2 mg L^{-1} BAP. SB11, SB12, SB13 and SB14 represent plants micropropagated on MS augmented with 3 mg L^{-1} BAP. SBK 1, SBK2 and SBK3 represent plants regenerated on MS augmented with 2 mg L^{-1} BAP and 0.5 mg L^{-1} KIN while SBK4 represents the mother plant.

4.3.8 Assessment of genetic fidelity of putrescine treated plants

Among the SRAP primer combinations tested for amplification of two genotypes of passion fruit subjected to different concentrations of putrescine, only 7 combinations generated clear and distinct bands. The sizes of bands generated varied from 250 to 2000 bp. The average number of bands amplified by the seven combinations of primers ranged from 8 and 11 bands per primer combination. Besides, the bands generated for donor plants and putrescine treated derivatives were monomorphic (Plate 4.14). A similarity matrix based on Jaccard's coefficient revealed that the pair-wise value between the donor plants and the putrescine treated nodal explants ranged from 79.2% (0.792) to 98.6% (0.986) and 80.7% (0.807) to 98.7% (0.987) for purple (Appendix VI) and KPF 4 varieties (Appendix VII) respectively.

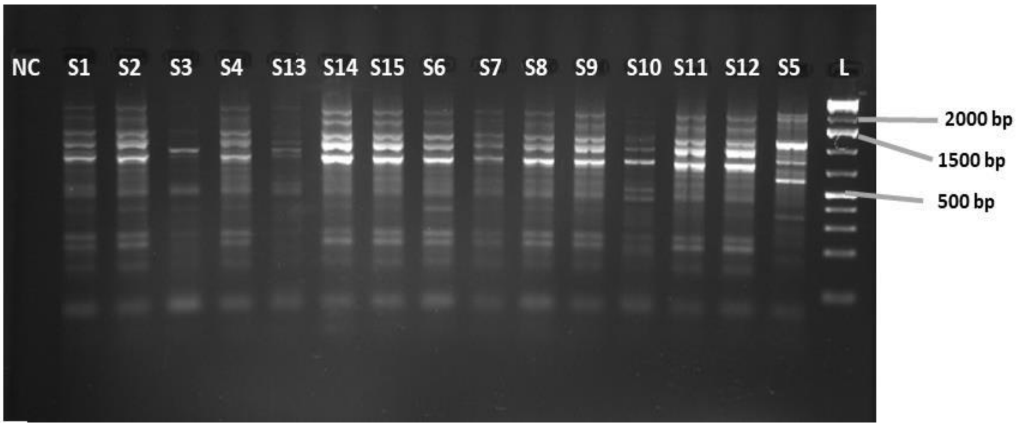


Plate 4.14: Assessment of genetic fidelity of putrescine treated plants using primers me11 and em11. Lane L- 0'GeneRuler^{TM} 1 kb DNA plus DNA ladder (Thermo Scientific); NC, Negative control (master mix and nuclease- free water). S1, purple passion mother plant; Lanes S2, S3 and S4 purple passion plants treated with 0.5% putrescine; Lanes S13, S14 and S15 purple passion plants treated with 2% putrescine; Lanes S6, S7 and S8 KPF 4 plants treated with 0.5% putrescine; Lanes S9, S10, S11 and S12 KPF 4 plants treated with 2% putrescine; Lane S5, KPF 4 mother plants.

which was high (Figure 4.9). S1 was in cluster A, S2, S3, S4, and S13 were in cluster B while S14 and S15 in cluster C.

Dendrogram analysis of KPF 4 passion fruit, based on the Jaccard's similarity coefficient revealed two clusters (A and B) whose similarity was 99.9% which was high (Figure 4.10). S6, S7 and S10 were in cluster A while S5, S8, S9, S11 and S12 were in cluster B.

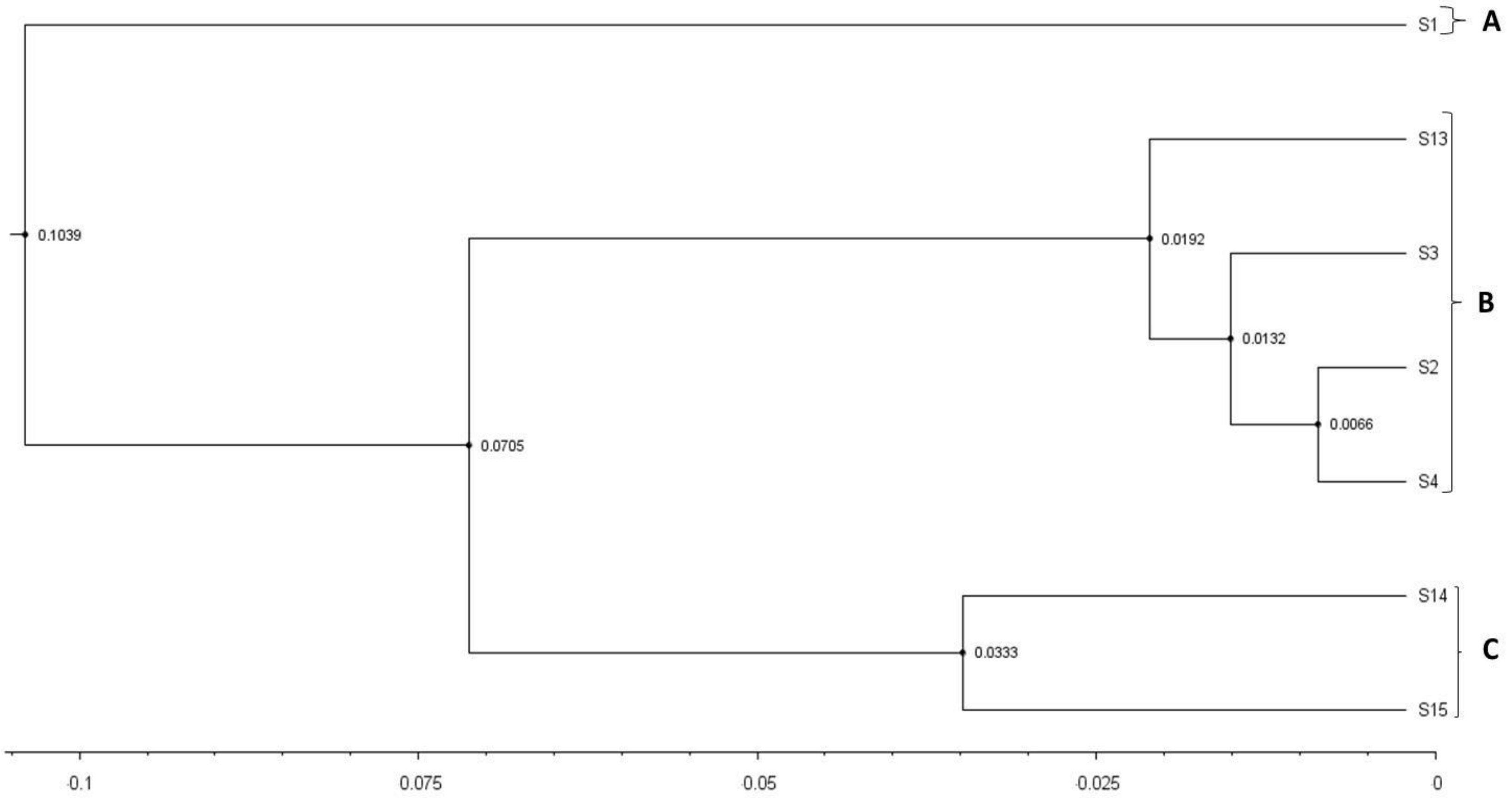


Figure 4.9: Dendrogram displaying genetic relationships between purple passion fruit mother plants (S1) and putrescine treated plants by UPMGA cluster analysis of SRAP data. S2, S3 and S4 represent plants treated with 0.5% putrescine while S13, S14 and S15 represent plants treated with 2% putrescine.

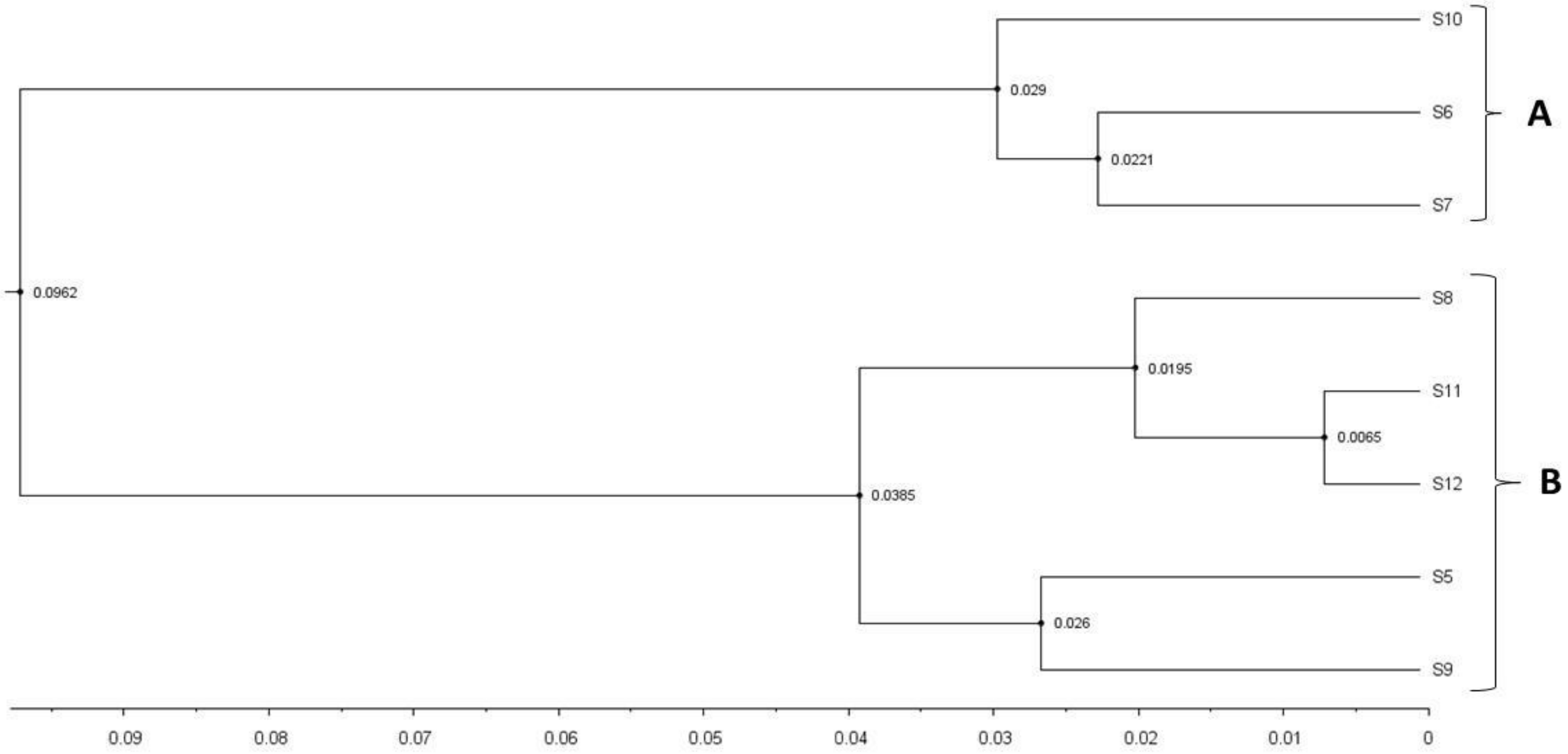


Figure 4.10: Dendrogram displaying genetic relationships between KPF 4 mother plants (S5) and putrescine treated plants by UPMGA cluster analysis of SRAP data. S6, S7, S8 represent plants treated with 0.5% putrescine while S9, S10, S11, S12 represent plants treated with 2% putrescine.

4.4 Factors affecting the development and optimization of an *Agrobacterium*-medited transformation system using *gus* reporter gene

4.4.1 Effect of LBA 4404 cell density

Results indicated that while LBA cell densities (OD_{600}) of 0.1 generated a blue coloration, increasing the cell density to 0.5 resulted in a substantial increment in the number of leaf discs displaying *gus* activity (Figure 4.11). Further increases in OD_{600} beyond 0.5, however, led to a substantial reduction in the number of leaf explants harboring the blue coloration (Figure 4.11). *Agrobacterium tumefaciens* (LBA 4404, harboring pCAMBIA 1301) densities had a significant ($p \leq 0.05$) effect on the number of explants displaying *gus* activity. The best density for delivery of the transgene into plant tissues was 0.5.

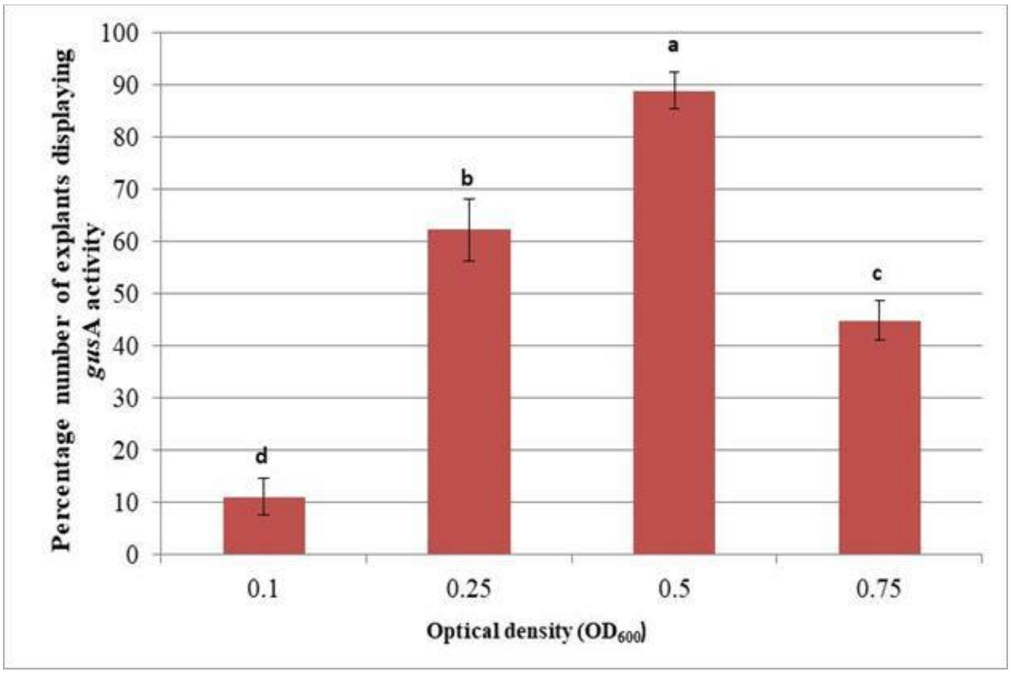


Figure 4.11: Effect of LBA 4404 cell density on the percentage number of *gus* positive leaf explants of KPF 4 passion fruit. Means having the different letters above bars are significantly different at $P \leq 0.05$ using Tukey's Honest significant difference test.

4.4.2 Effect of duration of infection of leaf on transient *gus* expression on leaf explants of KPF 4 passion fruit

The duration of infection of KPF 4 leaf explants had an influence on transient *gus* expression (Figure 4.12). At the optimum cell density (OD_{600}=0.5) the highest percentage of explants displaying *gus* activity was 80.0 obtained at 30 minutes infection time (Figure 4.12). When inoculation time was increased at the optimal cell density, *gus* expression increased significantly ($p \leq 0.05$) in terms of the frequency of responding explants showing *gus* activity. On the other hand, increasing the duration of inoculation beyond 30 minutes, decreased the frequency of *gus* expression.

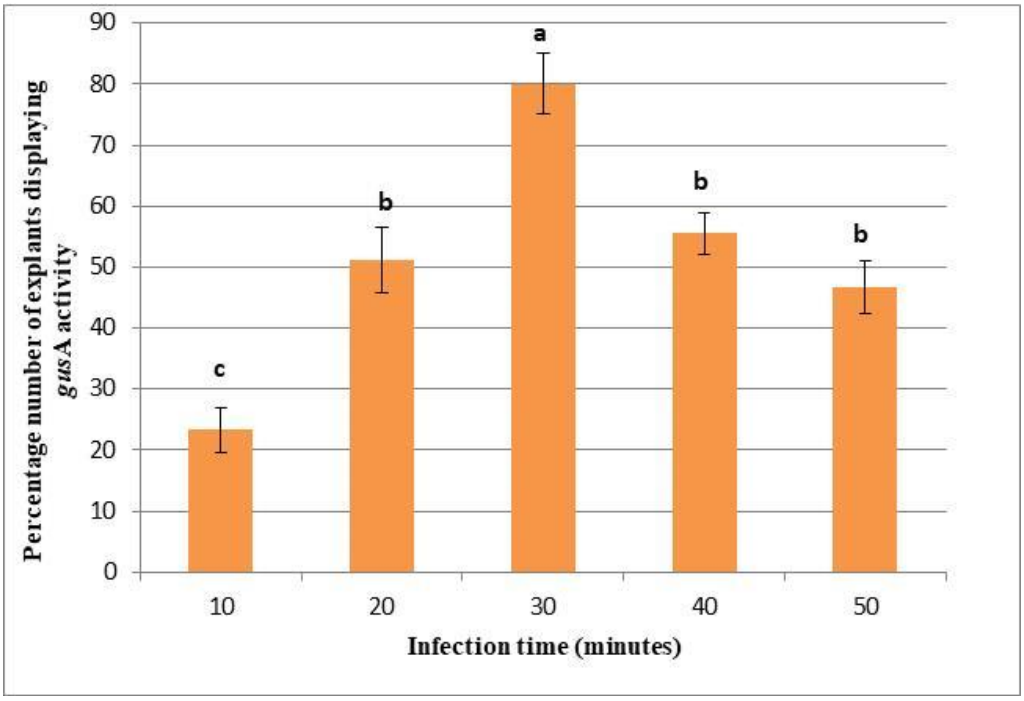


Figure 4.12: Effect of infection time on transient *gus* expression of leaf explants of KPF 4 passion fruit. Means having the same letters above bars are not significantly different at P > 0.05 using Tukey's Honest significant difference test.

4.4.3 Effect of co-cultivation period on transient *gus* expression

In these experiments transient *gus* expression assays after co-cultivation period (0-5 days) displayed blue coloration an indication of transient expression of *gus* reporter gene in leaf explants of KPF 4 passion fruit (Plate 4.15). However, there were visible variations in the blue coloration among the explants co-cultivated for different periods. The percentage number of *gus* positive leaf explants varied from 5.56 to 86.67 (Figure 4.13).

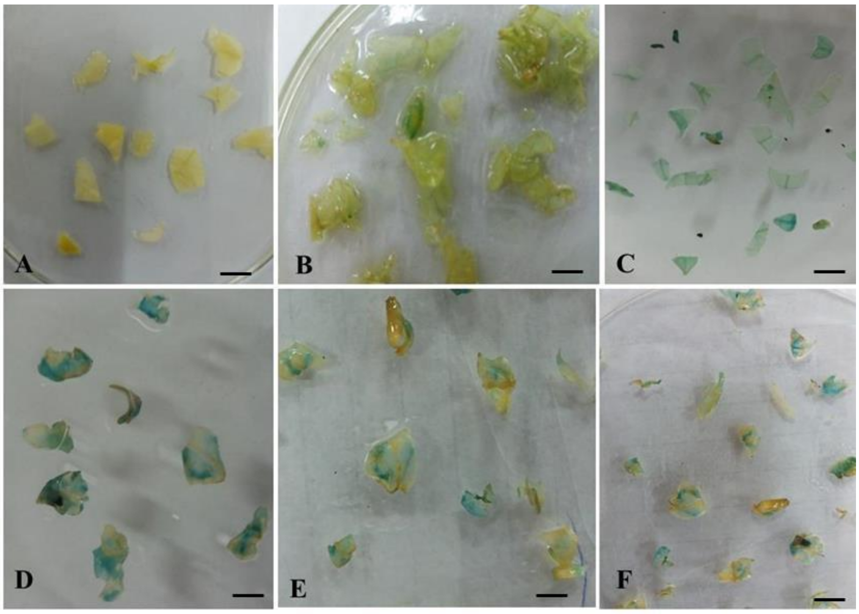


Plate 4.15: Transient *gus*A gene expression in leaf tissues of KPF 4 passion fruit after co-cultivation with LBA 4404, harboring pCAMBIA 1301 (Bar = 1 cm). A, non-transformed leaf tissues (control); B, transient *gus*A expression in leaf tissues after 1 day of co- cultivation; C, transient *gus*A expression in leaf tissues after 2 days of co-cultivation; D, transient *gus*A expression in leaf tissues after 3 days of co-cultivation; E, transient *gus*A expression in leaf tissues after 4 days of co-cultivation; F, transient *gus*A expression in leaf tissues after 5 days of co-cultivation.

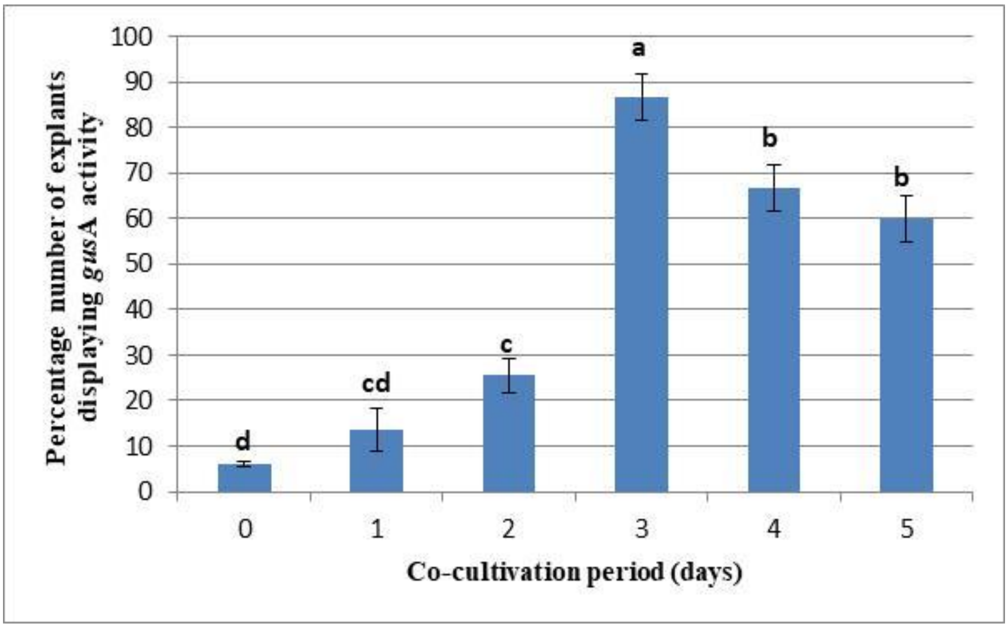


Figure 4.13: Effect of co-cultivation period on the percentage number of *gus* positive explants of KPF 4 passion fruit. Means having the same letters above bars are not significantly different at P > 0.05 using Tukey's Honest significant difference test.

The percentage number of *gus* positive leaf explants increased with co-cultivation period with an optimum being at 3 days yielding the highest mean of 86.67 which was significantly ($p \leq 0.05$) different from other co-cultivation periods. After three days, there was a reduction in the frequency of leaf explants with *gus* activity recorded. At five days of co-cultivation an overgrowth of *Agrobacterium* was observed in which the explant became necrotic.

4.4.4 Effect of acetosyringone on transient *gus* expression

Results of the six different concentrations of acetosyringone (0, 50, 100, 150, 200, 250 µM) tested on freshly cut leaf explants of KPF 4 passion fruit, indicated that *gus* positive results improved following an increase in the concentration of acetosyringone with optimum *gus* expression being at 200 µM (Figure 4.14). The concentration of acetosyringone had a significant ($p \leq 0.05$) effect on transient *gus* expression. The expression reduced when the concentration of acetosyringone was increased above 200 µM (Figure 4.14). However, *gus* expression at 200 µM acetosyringone was not significantly ($p > 0.05$) different from 150 µM.

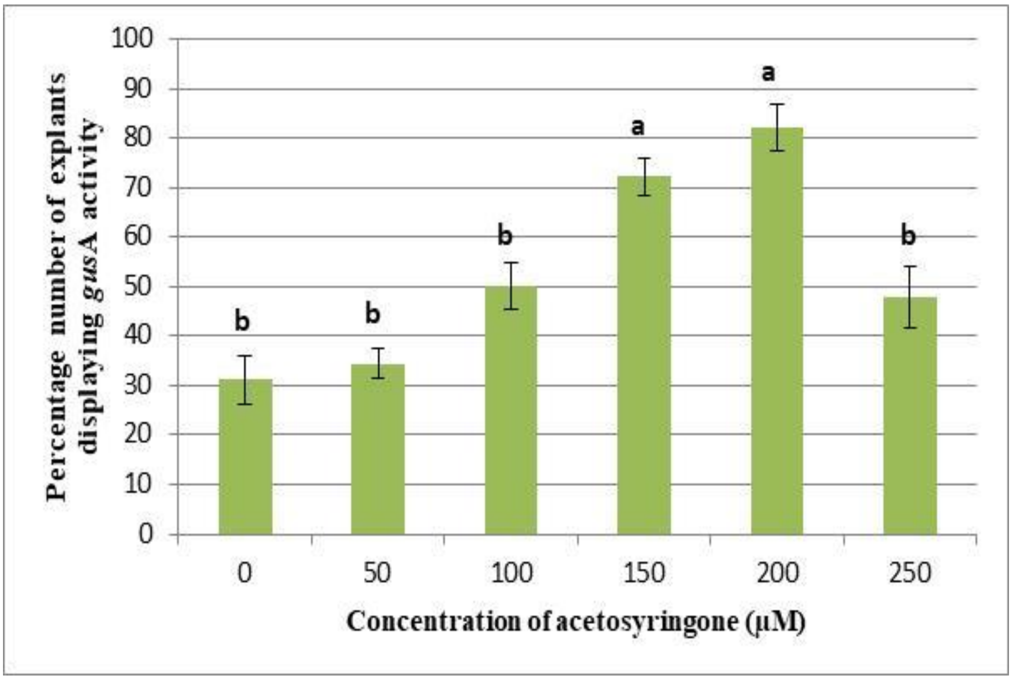


Figure 4.14: Effect of acetosyringone on the percentage number of *gus* positive explants of KPF 4 passion fruit. Means having the same letters above bars are not significantly different at $P > 0.05$ using Tukey's Honest significant difference test.

4.4.5 Regeneration of putatively transformed leaf explant of KPF 4 passion fruit

4.4.5.1 Infection, co-cultivation and resting period

Successful transformation was recorded using the optimized conditions for transient gene expression. A total of 1,800 explants were inoculated in 6 different experiments with 300 explants in each experiment. All leaf explants inoculated with LBA 4404 harboring pCAMBIA 1301, followed by co-cultivation for 3 days, retained their green coloration (Plate 4.16A). This observation was recorded across all experiments. After co-cultivation, explants exposed to a resting period for 4 days (in MS medium augmented with 450 mgL^{-1} cefotaxime but lacking selection agent, hygromycin] also retained their green color (Plate 4.16B).

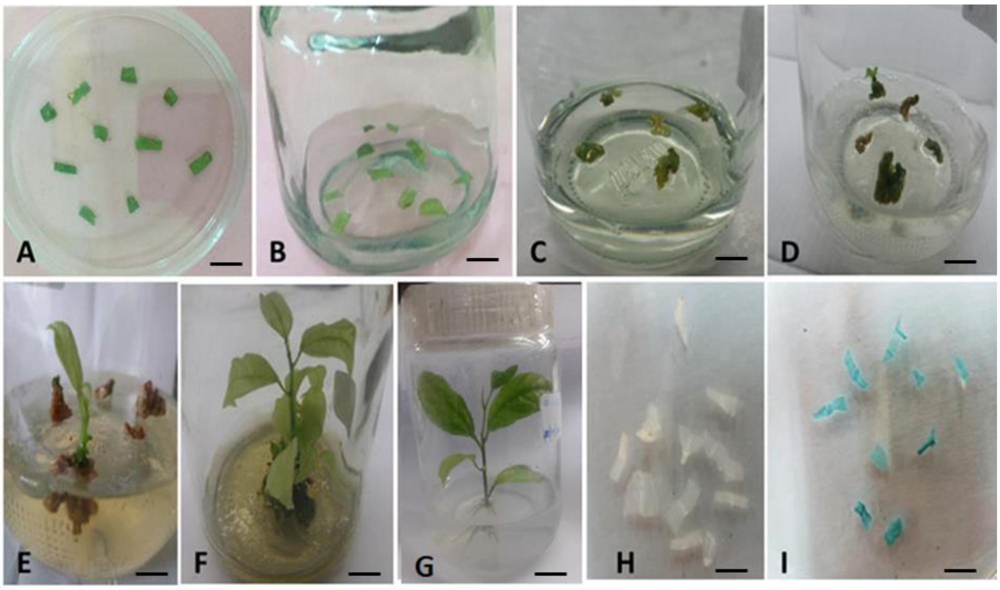


Plate 4.16: Infection, regeneration and *gus*A gene expression in leaf tissues of putative transgenic KPF 4 passion fruit (Bars = 1 cm). A, 3 days old leaf explants in co-cultivation medium; B, 3 days old leaf explants on resting stage; C, 1 week old explants on selection medium; D, 4 weeks old explants on selection medium; E, 6 weeks old explants with a regenerants on regeneration medium; F. 8 weeks old regenerants on regeneration medium; G, Six days old plantlet in root induction medium; H, non-transformed leaf tissues (control); C, *gus* expression in leaves from putative transgenic plants.

4.4.5.2 Selection and regeneration of putative transgenic lines

On transfer to selection medium, leaf explants became brown after 3 weeks of culture (Plate 4.16C). Majority of the leaf explants yielded to hygromycin selection pressure, with the antibiotic killing untransformed tissues of the leaf disc within 3 to 12 weeks (Plate 4.16D). In several leaf explants, some patches (cells) turned brown and ultimately died while the rest of the tissue survived the selection process. The browning occurred in patches of different magnitudes. Regeneration of the putative transgenic shoots from the green parts of the transformed leaves began from the 4th week of culture. The initiated shoot buds began producing shoots after 6 weeks of culture on selective/ regeneration medium augmented with the same antibiotics (450 mg L^{-1} cefotaxime and 7.5 mg L^{-1} hygromycin) and with subculturing every 2 weeks (Plate 4.16D). The non transformed leaf explants turned brown and finally died on transfer to selective medium (Plate 4.16E).

When micro shoots were transferred onto MS augmented with 0.1 mg L^{-1} BAP, cefotaxime (450 mg L^{-1}) and hygromycin (7.5 mg L^{-1}), further development of shoots was recorded after 1 week of culture (Plate 4.16E). In the first experiment, out of the 300 explants inoculated, 11 were lost to infection. In experiment 1, 2 and 5 no PCR positive plants were obtained and hence a transformation efficiency of 0.00% (Table 4.18). A transformation efficiency of 0.67% and 0.33% was recorded in experiment 3 and 4 respectively. All the plants were PCR positive for *gus*A gene. Experiment 6 recorded the highest transformation efficiency of 1.0% with three shoots that were PCR positive for *gus*A gene. The 6 regenerants were pale greenish yellow but were able to root in the root initiation medium with the same antibiotics (Plate 4.16G). The six putative transgenic lines were PCR positive for *gus*A gene giving an overall transformation efficiency of 0.33%.

Table 4.18: Regeneration and transformation efficiency of KPF 4 passion fruit

Experiment No.	No. of explants Infected	No. of explants survived selection	No. of explants forming shoot buds	No. of plants regenerated	No. of PCR positive plants	Transformation efficiency (%)
1	300	14	6	2	0	0.00
2	300	21	3	1	0	0.00
3	300	18	5	3	2	0.67
4	300	29	6	4	1	0.33
5	300	15	4	2	0	0.00
6	300	37	7	3	3	1.0

4.4.5.3 Root induction

When shoots (3 cm in height) were transferred to root induction medium, only six shoots developed roots. The shoots began to form roots after 9 days of culture in root initiation media. All the PCR positive shoots rooted in root induction medium. Of the six plants that were weaned and hardened for 4 weeks in small pots, five plants survived giving a survival rate of 83.3%. One of the KPF4 plants successfully weaned is indicated in Plate 4.18.

Plate 4.17: A representative of regenerated four-week- old KPF 4 passion fuit plant growing in a plastic pot. Bar = 10 cm.

4.4.5.4 *Gus*A histochemical assay for putative transgenic lines

No blue coloration was recorded in non-transformed plants (control) (Plate 4.16H). On the other hand, a uniform blue coloration was recorded on all leaf discs of putative transgenic plants (Plate 4.16I).

4.4.5.5 Polymerase chain reaction analysis of putative transgenic lines

An amplicon of size 500 bp equivalent to the internal fragment of the reporter gene *gus*A was amplified from genomic DNA of all putatively transformed plants using *gus*A primers (Plate 4.17), an indication of the presence of *gus*A gene in the transgenic plants. There was no band detected in the control.

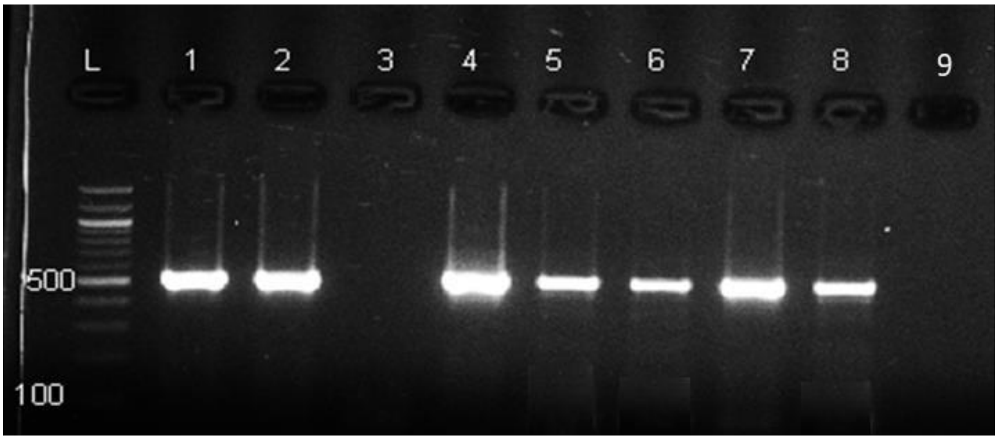


Plate 4.18: PCR amplification of *gus*A gene in DNA isolated from KPF 4 plants using *gus*A primers. L, Quick-Load® 100 bp DNA Ladder (New England Biolabs); Lane 3, non transformed plant (control); Lane 4, pCAMBIA1301 plasmid DNA containing the *gus*A gene (positive control); Lanes 1,2,5,6,7&8, putative transgenic plants; Lane 9, negative control (master mix and nuclease- free PCR water only).

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Determination of prevalence, incidence and severity of passion fruit woodiness disease in Kilifi and Kwale Counties

Findings from the present study showed that passion fruit woodiness disease (PWD) is widespread with a prevalence of 100% in both Kwale and Kilifi Counties in the coastal lowlands of Kenya. This finding is consistent with studies done in Murang'a, Uasin Gishu, Nakuru, Embu, Trans Nzoia, Nyeri, Bungoma, Kirinyaga, Kiambu and Meru Counties in Kenya that indicated wide distribution of PWD among farmers' fields (Kilalo *et al.*, 2013). It has also been reported that PWD is the most widely distributed virus disease infecting passion fruit worldwide (Nascimento *et al.*, 2006; Ochwo-Ssemakula *et al.*, 2012). Symptoms observed during surveys in all the locations in Kwale and Kilifi Counties included yellow foliar mosaic, leaf mottling, misshapen fruits with corky rinds, leaf curling and distortion, foliar bunchiness and stunting which have been previously reported in Kenya, Uganda, Nigeria and Brazil (Nascimento *et al.*, 2006; Ochwo-Ssemakula *et al.*, 2012; Kilalo *et al.*, 2013; Arogundade *et al.*, 2018). This indicates that PWD symptom development on passion fruit plants is independent of agro-ecology in sub-Saharan Africa and Brazil.

Viral disease incidence in Kwale and Kilifi Counties ranged from low (32.50% and 33.75% for Kwale and Kilifi, respectively) to moderate (51.43% and 59.16% for Kwale and Kilifi, respectively). In contrast, very high disease incidence ranging between 70 and 100% has been previously reported in other Counties in Kenya including Uasin Gishu, Trans Nzoia, Embu, Kirinyaga Thika, Meru, Gatundu and Nakuru Counties (Kilalo *et al.*, 2013). The difference in PWD incidence in the present study and reports from other regions could be attributed to the different passion fruit genotypes cultivated. Kwale and Kilifi Counties were dominated by the cultivation of the yellow passion fruit, which are probably more tolerant to PWD compared with other passion fruit genotypes including purple and KPF 4 found in other Counties. The disparity in disease incidence could also be due to the difference in agro-ecological zones.

Kwale and Kilifi Counties are located in the coastal lowlands of Kenya. While the Counties previously reported with high disease incidences were located in the upper midland and lower highland agro-ecological zones (Kilalo *et al.*, 2013) hence different environmental factors which may have had an influence on vector population dynamics and virus transmission rate (Hull, 2009). Similarly, high incidences (71.8% and 73.1%) in passion fruit vines have also been reported in Brazil (Gioria *et al.*, 2000). High variations in disease incidence have also been reported in Uganda in the range of 0% to 100% with a mean of 32% (Ochwo-Ssemakula *et al.*, 2012).

Disease severity in Kwale County ranged from 2.40 to 2.77 while that of Kwale County ranged from 2.65 to 3.18. The difference could be due to the differential susceptibility of the passion fruit genotypes grown in the two Counties. In Kwale County, all farms surveyed had the local yellow passion fruit genotype while the farms surveyed in Kilifi County had KPF 4 passion fruit genotype in addition to the largely cultivated yellow passion fruit genotype. In contrast, findings by Kilalo *et al.* (2013) in Uasin Gishu, Nakuru, Embu, Trans Nzoia, Nyeri, Bungoma, Kirinyaga, Kiambu and Meru reported a disease severity in the range of 2.4 to 3.7. This disparity could be due to the different genotypes cultivated in Kwale and Kilifi Counties and the Counties in the upper midland agroecological zones and the lower highland agro ecological zones (HCDA, 2017).

The observed prevalence, incidence and severity levels of PWD in Kwale and Kilifi Counties could be exacerbated by poor management practices such as non-sterilization of pruning tools, intercropping with target crops such as cucurbits and cowpeas and crop rotation with the same target crops. According to Fischer and Rezende (2008), leguminous crops and cucurbits that harbor passion fruit woodiness virus complex should not be intercropped with passion fruit. In addition, lack of knowledge on proper identification of the woodiness disease symptoms amounts to inability of farmers to rogue infected plants. Use of already infected seedlings obtained from non-certified local nurseries or their own nurseries could also heighten disease incidence and prevalence levels.

5.1.2 Screening of selected passion fruit genotypes for reactions to PWD under greenhouse and field conditions

Identification of reliable sources of resistance to virus diseases is an important aspect of plant breeding. In this study, under greenhouse conditions, inoculated plants of all the genotypes tested did not display PWD symptoms until three weeks after inoculation. This could be attributed to the low virus replication rates and concentrations in the plants, in addition to the duration required for visual expression of characteristic PWD symptoms. Similar findings were reported by Gonçalves *et al.* (2017), in which no symptoms of *Cowpea Aphid Borne Mosaic Virus* infection were recorded until 20 days after inoculating different genotypes of passion fruit grown in Brazil.

There was significant variation in disease progression in the tested genotypes with purple passion and KPF 4 displaying a higher disease progression compared to yellow passion, sweet granadilla and banana passion fruit genotypes. This shows that purple passion fruit was more susceptible to passion fruit woodiness disease compared to the other passion fruit genotypes. The findings in this study are in agreement with reports by Cerqueira-Silva *et al.* (2015) indicating varying susceptibilities of different passion fruit varieties to woodiness disease and a broad genetic variability for *Passiflora edulis* and wild *Passiflora* species. This probably shows that the variation in disease progression could be due to the genotypic effect. Although disease progression was observed in all the tested genotypes, plants of banana passion fruit (BNN) showed delay symptom expression with the first symptoms appearing in most of the plants at 35 days post-inoculation. The delayed symptom observed in banana passion fruit may be due to the presence of resistance genes which are providing a certain degree of PWD resistance.

Plants of all the tested passion fruit genotypes inoculated with PWD exhibited significantly reduced growth compared to the non-inoculated plants. These results clearly indicate the high potential damage that passion fruit woodiness disease (PWD) can cause to passion fruit plants, especially when young plants are infested. These findings are consistent with previous report by Gichimu *et al.* (2013), indicating that passion fruit woodiness disease affects plant growth and lifespan of passion fruit vines.

Passion fruit genotypes were found to differ greatly in the symptoms caused by PWD and in the severity of symptom expression in the field. The Area Under Disease Progress Curve (AUDPC) for the five passion fruit genotypes varied significantly among them. The differential susceptibility to PWD may be due to the genetic variability within the screened genotypes as reported by Cerqueira-Silva *et al.* (2008) and Freitas *et al.* (2015). Studies conducted by Schuerger and Hammer (1995) also revealed that the genetic background might influence the apparent relative effectiveness of the resistant genes of the plant, resulting in many genotypes becoming susceptible to a virus attack.

ACP ELISA assays using universal potyvirus antiserum (Agdia Inc., Elkhat, IN) confirmed that the observed characteristic symptoms of woodiness disease were as a result of potyvirus infection. This is consistent with findings by Fukumoto *et al.* (2013) and Cerqueira-Silva *et al.* (2014b) describing different types of potyviruses as potential pathogens of passion fruit woodiness (PWD) in Africa, Asia and Brazil.

5.1.3 *In vitro* regeneration system

5.1.3.1 Responses of leaf explants of KPF 4 and purple passion fruit genotypes to BAP and kinetin concentrations

The induction of shoot organogenesis from leaf explant was successfully established for Kenya passion fruit 4 (KPF 4). The presence of BAP in the induction medium was essential for induction of shoot buds and development of shoots. The *in vitro* regeneration studies in other passion fruit species such as *P. edulis f. flavicarpa*, *P. caerulea*, *P. alata* and *P. setacea* have shown the role of BAP in shoot formation from leaf explants (Busilacchi *et al.*, 2008; Pacheco *et al.*, 2012; Trevisan and Mendes, 2005; Vieira *et al.*, 2014; Rosa *et al.*, 2016).

When leaf discs of purple passion fruit were cultured on MS with all the concentrations of BAP tested, no shoot buds were induced. The results obtained from this study indicate genotype based disparities between the two genotypes tested. These observations demonstrate that there might be an underlying genetic control in the capability of a given genotype to induce bud and shoots in tissue culture. In addition, this response can be attributed to the different levels of endogenous phytohormones. The genotypic effect on the shoot organogenesis of passion fruit genotypes tested in this study was also reported by Amugune *et al.* (1993) and Mukasa *et al.* (2016). This suggests that regeneration process needs to be optimized and standardized for each passion fruit variety.

5.1.3.2 Responses of nodal explants of KPF 4 passion fruit genotype to BAP and kinetin concentrations

Shoot multiplication from nodal explants of KPF 4 passion fruit grown in Kenya was successfully established. The inclusion of BAP in the shoot multiplication medium was necessary for multiplication of shoots in nodal explants of KPF 4 which is consistent with the reports of Busilacchi *et al.* (2008) and Ozarowski and Thiem (2013) on *Passiflora caerulea*.

BAP has the potential to shift the apical dominance toward the development of lateral buds, which causes cell division to occur in meristem cells hence enhancing the number of branches and amplifying the rate of cell division in the lateral buds of nodal explants (Gomez-Leyva *et al.*, 2008).

From the results of the present study, BAP was found to be more superior to kinetin for multiplication of shoots. This is consistent with findings documented by Ragavendran *et al.* (2012) who demonstrated that compared to other cytokinnins and KIN, BAP is more efficient in explant regeneration in *Passiflora foetida.*

5.1.3.3 Effect of putrescine on root initiation

In the present study, roots were successfully induced on both concentrations of putrescine suggesting that polyamines play a significant role in promoting root initiation in passion fruit varieties grown in Kenya. This is in agreement with Viu *et al.* (2009), Vasudevan *et al.* (2017) and Ghehsareh and Khosh-Khui (2019) who reported the involvement of polyamines in adventitious and lateral root formation and that putrescine enhances root initiation and development. On putrescine-free control treatment, roots were recorded only on purple passion fruit nodal explants but no roots were recorded on KPF 4 nodal explants, indicating genotypic specificity. The number and length of roots in the putrescine treatment were significantly higher compared to the roots regenerated in putrescine-free treatment. The differences of the two passion fruit varieties in rooting response following putrescine treatment is an indication of genotypic specificity for specific polyamine requirement or might be due to variation in endogenous putrescine levels and the inherent genetic characteristics of the varieties.

Polyamines are growth regulators present in all plant tissues and participate in many important processes such as root formation and development, organogenesis and regeneration (Besford *et al.*, 1993; Arena *et al.*, 2005; Kusano *et al.*, 2008). The role of polyamines in micro-shoot rooting has been reported in different plant species (Arena *et al.*, 2005), in which putrescine has been reported to show a better response compared to other polyamine substances in playing a key role in root initiation and development in hard-to-root plants (Wu *et al.*, 2010). The present study demonstrates that nodal explants of *in vitro* derived shoots of passion fruit can be exploited for mass multiplication of plantlets accompanied by better rooting with the utilization of polyamines in the glasshouse.

5.1.3.4 Responses of leaf explants of KPF 4 and purple passion fruit genotypes to 2, 4-D concentrations

Callus was initiated from a few leaf explants of KPF 4 genotype on hormone free medium, an indication of endogenous hormones in the leaf explants. Auxins have been reported widely as being critical in regulating cell divion and differentiation (Feher *et al.*, 2003; Jimenez and Thomas, 2006). Findings from the present study show that growth of callus on media containing increasing concentrations of 2,4 D can be attributed to the effect of auxins in eliciting production of endogenous cytokinins (Pernisová *et al.*, 2009) while combinations of auxin and cytokinnin both endogenous and exogenous, stimulate cell division (Mohr and Schopher, 1995). This is also in agreement with Braybrook and Kuhlemeier (2010) that auxins and cytokinnins are essential for callus induction.

For purple variety of passion fruit, loose non embryogenic callus without further development into somatic embryos was recorded on different concentrations of 2,4 D. The KPF 4, leaf explants inoculated on MS medium supplemented with the different concentrations of 2,4 D formed friable callus wich developed further into somatic embryos. These results indicate genotype specificity in callus induction and somatic embryogenesis in passion fruit. The genotypic effect on the response of passion fruit varieties to *in vitro* regeneration has also been reported by Amugune *et al.* (1993) and Mukasa *et al.* (2016).

5.1.3.5 Callus induction and somatic embryogenesis responses of immature seeds of KPF 4 and purple passion fruit gynotypes to 2, 4-D concentrations

Different concentrations of 2, 4-D alone or in combination with TDZ were able to initiate callus and different stages of somatic embryos. Similar results were reported by Ferreira *et al.* (2015) in wild species of passion fruit, *Passiflora miniata* and *Passiflora speciosa,* when media was supplemented with 2, 4-D. In many *in vitro* embryogenic models, 2, 4-D is necessary for the initiation of cell programming through somatic embryogenesis (Raghavan, 2004; Fehér, 2005; Pinto *et al.*, 2011; Rocha *et al.*, 2015).

In most species of plants studied in which growth regulators are needed for induction of somatic embryogenesis, auxins and cytokinins are significant factors defining the embryogenic response, possibly due to their involvement in the regulation of cell cycle, division and differentiation (Fehér *et al.*, 2003; Gaaj, 2004; Fehér., 2005). The isolated utilization or interaction of 2,4-D with other auxins and cytokinins, for the initiation of somatic embryogenesis through tissue culture of mature and immature zygotic embryos, seeds, leaves and cotyledons for several species has been reported (Amugune *et al.*, 1993; Pinto *et al.*, 2011; Cerqueira-Silva *et al.*, 2011; Rocha *et al.*, 2015).

Conversion of somatic embryos, from leaf explants of KPF 4 passion fruit, into plantlets was not achieved. However, nine somatic embryos from immature seeds of KPF 4 passion fruit were converted into normal plantlets. This can be attributed to the genotypic effect and type of explant on somatic embryogenesis. The findings of the present study are consistent with previous findings by Pinto *et al.* (2011) reporting abnormal somatic embryos and no conversion of cotyledonary stage somatic embryos, obtained from zygotic embryo explants of *Passiflora edulis* Sims, into plantlets. The somatic embryos became necrotic after 10 days of culture. In contrast, Ferreira *et al.*

(2015) reported conversion of somatic embryos from immature zygotic embryos of *Passiflora miniata* and *Passiflora speciosa* into normal plantlets.

5.1.3.6 Assessment of genetic fidelity of *in vitro* regenerated KPF 4 plants cultured on MS supplemented with BAP and kinetin

Genetic fidelity of *in vitro* regenerated and macropropagated plants is an essential step in the utilization of the protocol for mass multiplication of planting materials and as a pre-requisite for genetic transformation for improved agronomic traits. In this study, SRAP profiles of *in vitro* regenerated plantlets tested were monomorphic. This suggests genetic stability of majority of the plantlets. Lack of polymorphism in majority of the *in vitro* regenerants indicates that the plantlets were true-to- type. The minimal variations may be attributed to somaclonal variation ascribable to the *in vitro* regeneration system. Vidal and Garcia (2000) reported shoots initiation from nodal explants as superior for clonal propagation due to the preserved genetic stability as the plantlets are less prone to genetic changes that occur during cell development under *in vitro* conditions.

5.1.3.7 Assessment of genetic fidelity of KPF 4 and purple genotypes of passion fruit treated with putrescine

In this specific study, all SRAP profiles of putrescine treated plants displayed minimal variation. The plants (S5, S8, S11 and S12) with relatively high variation (similarity coefficient of 75.6%) would be explained by the difference in genotypes and other stresses originating from the tissue culture (Karp, 1994; Sato *et al.*, 2011; Smulders and de Klerk, 2011).

5.1.4 *Agrobacterium* mediated transformation

5.1.4.1 Factors affecting transient *gus* expression

Findings from the present study show that, at low cell densities, the degree of colonization and the frequency of transformation is low. Above an optimum value (OD_{600} of 0.5), however, further increments in cell density reduced the *gus* positive response, possibly as an outcome of a reduction in cell viability. These observations can be explained by the hypothesis that each plant cell binds to a limited number of *Agrobacterium* (Gutlitz *et al.*, 1987). Beyond this optimum, cell viability seems to be compromised, causing reduced numbers of *gus* positive leaf explants.

Ascertaining the optimum inoculation intensity is critical because at very high levels, the leaf tissues are almost entirely colonized by *Agrobacterium*, eradication of which becomes more hectic during the subsequent stages namely, resting, selection and regeneration. Commonly this would require use of higher levels of antibiotics which, in themselves have injurious effects on the development of plant tissue (Amoah *et al.*, 2001). The optimum inoculation intensity comprised of ($OD_{600}=0.5 \leq OD < 0.7$). Successful transformation has also been documented in yellow passion fruit at an OD_{600} of 0.4 (Alfenas *et al.*, 2005). Similar results have been reported by Mondal

(2001) on tea (*Camellia sinensis*) where OD_{600} values higher than 0.8 were found to be unsuitable for genetic transformation. Moreover, extensive tissue damage was reported at OD_{600} values higher than 1.0 due to bacterial overgrowth. This is also in agreement with Pena *et al.* (1995) that a higher OD inhibited the regeneration of plant tissues in Citrus as a result of *Agrobacterium* induced stress. In addition, management of bacterial overgrowth following co-cultivation becomes hectic. According to Archilletti *et al.* (1995) an OD_{600} of 0.6 was most effective in giving great efficiencies of transformation in almond.

Varying the inoculation periods had an influence on the transfer of T-DNA from *Agrobacterium* to the host genome. Extending the infection time above 30 minutes caused recurrence of the *Agrobacterium* and ultimate death of the leaf explant. This is in agreement with reports by Trevisan *et al. (*2006) in which successful transformation of yellow passion fruit was achieved when the infection period was 20 minutes. Similar results were also reported by Jha *et al.* (2011) and Zhao *et al.* (2011) on other plant species recommending an optimum infection period of 30 minutes in *Pennisetum glaucum* and Chinese upland rice respectively. Diverse infection periods have also been reported by various research works on different plants; Howe *et al.* (2006) and Ishida *et al.* (2007) in sorghum and maize, respectively, reported 5 minutes as optimal infection period while Sarker and Biswas (2002) reported an optimum infection time of 50 minutes. This is an indication that infection time varies with the species of plants under investigation.

Transferring leaf explants right into the resting medium (no cocultivation period) lowered the frequency of transient *gus*A expression. Extending the duration of co-cultivation increased the frequency with an optimum result being obtained at 3 days. Less days (1 to 2 days) of co-cultivation produced fewer *gus* positive explants owing to inadequate time for maximum transfer of *Agrobacterium* T-DNA into the host genome (Rahman *et al.*, 2011). Beyond 3 days of co-cultivation transformation frequency was significantly reduced due to suffocation of explants and damage by too much bacterial growth (Rahman *et al.*, 2011). A period of co-cultivation is necessary for transformation of passion fruit. This is in agreement with Trevisan *et al.* (2006) who reported transformation in yellow passion fruit after 3 days of co-cultivation. Similar results were recorded on other crops such as yam (Nyaboga *et al.*, 2013), *P. hexandrum* (Rajesh *et al.*, 2013) and *Hybanthus enneaspermus* **(**Sivanandhan, 2016). In contrast, Huang and Wei (2005) reported no transformation in immature embryo of maize without a period of co-cultivation. They also indicated in their reports that extended co-cultivation period reduces frequency of transformation of immature embryo due to overgrowth of bacterium.

Addition of acetosyringone in the infection medium for transformation of leaf explants enhanced the success of transient *gus* expression. All explants were also wounded prior to infection. Wounding of plants causes secretion of phenolics including acetosyringone and α-hydroxy acetosyringone which at very low concentrations, are effective chemo attractants and therefore improve the attachment of *Agrobacterium* cells to the wounded sites (Potrykus, 1990; Zambryski, 1992). These phenolics also induce the expression of vir genes located on the Ti plasmid of *Agrobacterium* (Escobar and Dandekar, 2003). Detection of these phenolic compounds is by the VirA/VirG sensor–transducer system, which subsequently induces all vir loci encoding constituents of the protein machinery for transfer of T-DNA (Zupan *et al.*, 2000). Although dicots such as passion fruit are well-known to naturally secrete phenolics from wound sites, addition of exogenous acetosyringone was found to enhance the transformation frequency in KPF 4 passion fruit variety. Similar findings were reported by Trevisan *et al.* (2006) indicating enhanced transformation of yellow passion fruit using acetosyringone. This is also in agreement with reports from other plant species such as sweet potato (Xing *et al.*, 2007), *Phalaenopsis violacea* orchid (Subramaniam and Xavier, 2010) and banana (Subramanyam *et al.*, 2011).

Agrobacterium tumefaciens –mediated transformation is the most commonly used method of genetic transformation (Sandal *et al.*, 2007) due to its simple operation. To distinguish transformed from non-transformed plants, *gus*A is commonly used as a reporter gene to ratify putative transformed lines (Duque *et al.*, 2007). Amongst the factors defining the reporter gene response is cell density of inoculum, infection time, co-cultivation period and use of acetosyringone whose influence was recorded in the experiments conducted in the present study.

5.1.4.2 Transformation and regeneration of putatively transformed plantlets of KPF 4 genotype of passion fruit

Findings of the present study show that genetic transformation of KPF 4 passion fruit grown in Kenya can be achieved through *Agrobacterium tumefaciens* (LBA 4404). However, the transformation efficiency was low (0.33%). Similar results were obtained by Trevisan *et al.* (2006) who obtained a transformation efficiency of 0.11% and 0.21% in IAC-275 and IAC-277 passion fruit varieties grown in Brazil. Similarly, Monteiro *et al.* (2011), documented low transformation efficiencies of 0.67% and 0.19% in the same Brazilian varieties. A relatively higher transformation efficiency (0.89%) was recorded by Correa *et al.* (2015) in *Passiflora alata*. Tuhaise *et al.* (2019) reported a transformation efficiency of 0.456% in Ugandan yellow varieties of passion fruit. The low efficiency of genetic transformation recorded in the present study could be attributed to genotype and effects of antibiotics used in the study. Antibiotics usually used to get rid of *Agrobacterium* from explant tissues, have been demonstrated to have an injurious effect on plant tissues by affecting the regeneration potential of the explants (Okkels and Pedersen, 1988; Lin *et al.* 1995; Ling *et al.*, 1998; Ahmed *et al.*, 2007). According to Ahmed *et al.* (2007) cefotaxime inhibited regeneration of infected leaf explants of Evola cultivar of lettuce.

In plants, hygomycin is very toxic (Rashid, 2017). Consequently, it has been reported for efficient selection of transformed and non transformed explants with extremely low frequency of escapes (Vanjildorj *et al.*, 2005; Dias *et al.*, 2006; Deng *et al.*, 2005; Deng *et al.*, 2007). Since only a restricted number of leaf explant cells are normally transformed after cocultivation with *Agrobacterium tumefaciens*, this causes chimerism in leaf tissues. The observed extensive necrosis of transformed explants in the present study could be attributed to chimerism where sections of the explant tissue that were not transformed yielded to hygromycin selection leading to death of the entire leaf tissue. Antibiotics substantially reduce the relative density of viable cells by killing nontransformed cells deterring growth of surviving transformed cells as only a minor section of cells are transformed in many experiments (Winkler and Quoirin, 2002). Hygromycin has also been used in selection of other crops varieties like banana (Maziah *et al.*, 2007), cassava (Nyaboga *et al.*, 2014) and pigeon pea (Kumar *et al.*, 2004). From the *gus* histochemical assays, the blue coloration was evenly distributed throughout the leaf discs used. This is a clear indication that there were no chimeras.

In this specific study, no escapes were detected from hygromycin selection through PCR analysis. This shows that hygromycin is efficient for selection of transgenic plants. This confirms previous reports in other studies that hygromycin is efficient for selection (Olhoft *et al.*, 2003; Li *et al.*, 2013).

5.2 Conclusions

- Passion fruit woodiness disease is widespread in Kwale and Kilifi Counties with low to moderate disease incidence and severity.
- Response of passion fruit genotypes to woodiness viruses was genotype dependent.
- Variation of cytokinnins and auxins led to an efficient and rapid regeneration system for passion fruit from leaf and nodal explants.
- Exogenous application of polyamine (putrescine) plays a key role in promoting induction of roots on stem nodal explants of passion fruit. The regeneration system led to plantlets that were genetically identical to the mother plants.
- All factors tested which include *Agrobacterium* cell density, infection time, co-cultivation period and concentrations of acetosyringone, were found to be fundamental in determining the efficiency of a transformation system.
- A simple and reproducible *Agrobacterium* mediated transformation system for KPF 4 genotype of passion fruit grown in Kenya was established.

5.3 Recommendations

- There is need to sensitize farmers on passion fruit woodiness disease and their management strategies such as acquiring disease free seedlings from certified nurseries.
- Since response of passion fruit genotypes to woodiness viruses was genotype dependent, more genotype should be screened to identify resistant genotypes.
- Since the plants regenerated through *in vitro* culture system were genetically identical to the mother plant, the system can therefore be used for propagation of passion fruit.
- There is need for more research to be carried out to optimize regeneration protocols for purple passion fruit and other genotypes grown in Kenya because response to tissue culture was genotype specific.
- There is need for further studies to be carried out to establish if the minimal variations detected in the present study will be inherited in the subsequent generations both in the greenhouse and in the field.
- The *Agrobacterium* mediated transformation system can be used to introduce beneficial traits such as resistance to woodiness disease and other agronomically important traits into KPF 4 passion fruit
- More research should be carried out to optimize *Agrobacterium* mediated transformation systems for different passion fruit varieties grown in Kenya, since in this study only KPF 4 genotype was tested.

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APPENDICES

Appendix I: Questionnaire

Interviewer: Lydia Asande, a PhD student at Kenyatta University

I am carrying out a survey on the incidence and severity of passion fruit woodiness disease in coastal Kenya for intervention purposes. Kindly respond to the questions below. The information you will give will remain confidential and will only be used for the research purposes.

1. Details of the farmer

(a) Name of the farmer (Optional)____________________

(b) Gender____________________

(c) Occupation________________________

2. Location details

GPS reading__________ Total farm acreage______________________

County_______________________ Division_________

Location___________ Sub location ___________

Altitude_______________

Topography: 1 = Steep slope 2 = Gentle slope 3 = Valley 4= Hill top 5 = flat

1. List the varieties of passion fruit in your farm

2. How old are your plants?________________

3. What is the total number of plants in your farm?___________

4. For how long have you grown passion fruits?__________

5. Name your preferred varieties_________________________________

Reason____

6. What is the source of your passion frut seedlings?
   1 = Your own

   2 = Local nursery

   3 = Research Institution

   4 = Other (specify)
7. Do you practice
   a) Small scale farming
   b) Large scale farming

8. Is it (a) your own enterprise (b) contracted

9. What crop were you growing before shifting to passion fruit?_____________________

10. Is there an incidence of passion fruit woodiness disease in your farm? [Yes] [No]

If yes, how would you rate the infection

1= Wide spread

2=Scanty

11. How do you control woodiness disease in your farm?

12. Tick the maintenance practices of passion fruit carried out in your farm
    a) Pruning

    b) Weeding [Manual] [herbicides]

    c) Spraying with pesticides

    d) Mulching

e) Irrigation

f) Application of foliar feeds or fertilizers

g) Crop rotation

Other (specify)___________________________

If you practice pruning, do you sterilize your apparatus? [Yes] [No]

If yes, how do you sterilize them?

10. Do you practice intercropping?

If yes, with which crops in order of preference

13. What other diseases do you experience in your farms

14. Are pests a problem in your farm? [Yes] [No]

a) If yes how do you control them?

b) List the pests

15. What are the other challenges of passion fruit farming do you face?

Appendix II: Buffers formulations used in serological tests

Buffer	Composition	Quantity
Indirect Sample extraction buffer (1X)	Sodium carbonate (anhydrous)	1.59 g
	Sodium bicarbonate	2.93 g
	Sodium azide	0.2 g
	Polyvinylpyrrolidone (PVP) MW 24-40,000	20.0 g
	Dissolved in distilled water	1000 ml
	pH 9.6	
	Storage 2-8 °C	
PBST Buffer (Wash buffer) (1X)	Sodium chloride	8.0 g
	Sodium phosphate, dibasic(anhydrous)	1.15 g
	Potassium phosphate monobasic (anhydrous)	0.2 g
	Potassium chloride	0.2 g
	Tween-20	0.5 g
	Distilled water	
	pH 7.4	1000 ml
ECL buffer (1 X)	Bovine serum albumin (BSA)	2.0 g
	Polyvinylpyrrolidone (PVP) MW 24-40,000	20.0 g
	Sodium azide	0.2 g
	Distilled water	1000 ml
	pH 7.4	
	Storage 2-8 °C	
PNP Buffer (substrate) (1X)	Magnesium chloride hexahydryte	0.1 g
	Sodium azide	0.2 g
	Diethanolame	97.0 ml
	Distilled water	1000 ml
	pH 9.8	
	Storage 2-8 °C	

Appendix III: Composition of various media used for regeneration of leaf disc and immature seed explants

Medium	Composition	Quantities
Shoot induction medium (SIM)	MS salts + vitamins (premix)	4.4 g L^{-1}
	Sucrose	3 %
	Gelrite	0.24 %
	BAP	1.0 - 3.0 mg L^{-1}
	KIN	0.5 mg L^{-1}
	pH (5.8)	
Shoot elongation medium (SEM)	MS salts + vitamins (premix)	4.4 g L^{-1}
	Sucrose	3 %
	Gelrite	0.24 %
	BAP	0.1 mg L^{-1}
	pH (5.8)	
Rooting medium	MS salts + vitamins (premix)	4.4 g L^{-1}
	Sucrose	3 %
	Gelrite	0.24 %
	NAA	0.1 mg L^{-1}
	pH (5.8)	

Appendix IV: Composition of various media used for somatic embryogenesis of leaf disc and immature seed explants

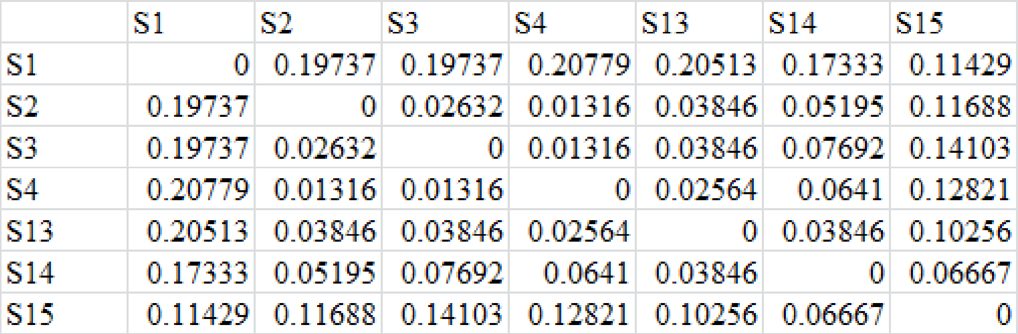
Medium	Composition	Quantities
Somatic embryogenesis (For leaf explants)	MS salts + vitamins (premix)	4.4 g L^{-1}
	Sucrose	3 %
	Gelrite	0.24 %
	2,4 D	0.5-16 mg L^{-1}
	pH (5.8)	
Maturation medium for leaf explants	As for somatic embryogenesis medium (For leaf explants)	
	Without 2,4 D	
	Activated charcoal or	1 %
	ABA	0.1 mg L^{-1}
Somatic embryogenesis (For immature seeds)	MS salts	4.3 g L^{-1}
	B5 vitamins	0.112 g L^{-1}
	Sucrose	3 %
	Gelrite	0.24 %
	Glutamine	200 mg L^{-1}
	Casein hydrolysate	500 mg L^{-1}
	2,4 D	4.0-32.0 mg L^{-1}
	TDZ	1.0 mg L^{-1}
Maturation medium for immature seeds	As somatic embryogenesis medium (For immature seeds) without 2,4 D	
	Activated charcoal or	1 %
	ABA	0.1 mg L^{-1}
Germination medium (somatic embryos)	MS salts +vitamins (premix)	4.4 g L^{-1}
	Sucrose	3 %
	Gelrite	0.24 %
	without hormones or BAP	1.0 mg L^{-1}
	pH (5.8)	

Appendix V: Similarity matrices of mother plants of KPF 4 passion fruit variety and their *in vitro* regenerated plants based on Jaccard's similarity coefficient of SRAP data

	SK1	SK2	SK3	SK4	SK5	SK6	SK7	SK8	SK11	SK12	SK13	SB1	SB2	SB3	SB6	SB7	SB8	SB9	SB11	SB12	SB13	SB14	SBK1	SBK2	SBK3	SBK4
SK1	1	1	0.967	0.951	0.951	0.951	0.951	0.951	0.951	0.951	0.951	0.951	0.983	0.951	0.951	0.951	0.951	0.951	0.951	0.951	0.983	0.934	0.951	0.885	0.951	0.951
SK2		1	0.967	0.951	0.951	0.951	0.951	0.951	0.951	0.951	0.951	0.951	0.983	0.951	0.951	0.951	0.951	0.951	0.951	0.951	0.983	0.934	0.951	0.885	0.951	0.951
SK3			1	0.984	0.984	0.984	0.984	0.984	0.984	0.984	0.984	0.984	0.951	0.984	0.984	0.984	0.984	0.984	0.984	0.984	0.951	0.967	0.984	0.918	0.984	0.984
SK4				1	1	1	1	1	1	1	1	1	0.967	1	1	1	1	1	1	1	0.967	0.984	1	0.934	1	1
SK5					1	1	1	1	1	1	1	1	0.967	1	1	1	1	1	1	1	0.967	0.984	1	0.934	1	1
SK6						1	1	1	1	1	1	1	0.967	1	1	1	1	1	1	1	0.967	0.984	1	0.934	1	1
SK7							1	1	1	1	1	1	0.967	1	1	1	1	1	1	1	0.967	0.984	1	0.934	1	1
SK8								1	1	1	1	1	0.967	1	1	1	1	1	1	1	0.967	0.984	1	0.934	1	1
SK11									1	1	1	1	0.967	1	1	1	1	1	1	1	0.967	0.984	1	0.934	1	1
SK12										1	1	1	0.967	1	1	1	1	1	1	1	0.967	0.984	1	0.934	1	1
SK13											1	1	0.967	1	1	1	1	1	1	1	0.967	0.984	1	0.934	1	1
SB1												1	0.967	1	1	1	1	1	1	1	0.967	0.984	1	0.934	1	1
SB2													1	0.967	0.967	0.967	0.967	0.967	0.967	0.967	1	0.951	0.967	0.902	0.967	0.967
SB3														1	1	1	1	1	1	1	0.967	0.984	1	0.934	1	1
SB6															1	1	1	1	1	1	0.967	0.984	1	0.934	1	1
SB7																1	1	1	1	1	0.967	0.984	1	0.934	1	1
SB8																	1	1	1	1	0.967	0.984	1	0.934	1	1
SB9																		1	1	1	0.967	0.984	1	0.934	1	1
SB11																			1	1	0.967	0.984	1	0.934	1	1
SB12																				1	0.967	0.984	1	0.934	1	1
SB13																					1	0.951	0.967	0.902	0.967	0.967
SB14																						1	0.984	0.918	0.984	0.984
SBK1																							1	0.934	1	1
SBK2																								1	0.934	0.934
SBK3																									1	1
SBK4																										1

Lanes SK1, SK2, SK3, SK4 and SK5 represent plants cultured on MS supplemented with 1 mg L^{-1} kinetin. SK6, SK7 and SK8 represent plants cultured on MS supplemented with 2 mg L^{-1} kin. SK11, SK12 and SK13 represent plants cultured on MS supplemented with 3 mg L^{-1} kin. SB1, SB2 and SB3 represent plants cultured on MS supplemented with 1 mg L^{-1} BAP. SB6 is a mother plant. SB7, SB8 and SB9 represent plants cultured on MS supplemented with 2 mg L^{-1} BAP. SB11, SB12, SB13 and SB14 represent plants cultured on MS supplemented with 3 mg L^{-1} BAP. SBK1, SBK2 and SBK3 represent plants cultured on MS supplemented with 2 mg L^{-1} BAP and 0.5 kin while SBK4 represents the mother plant.

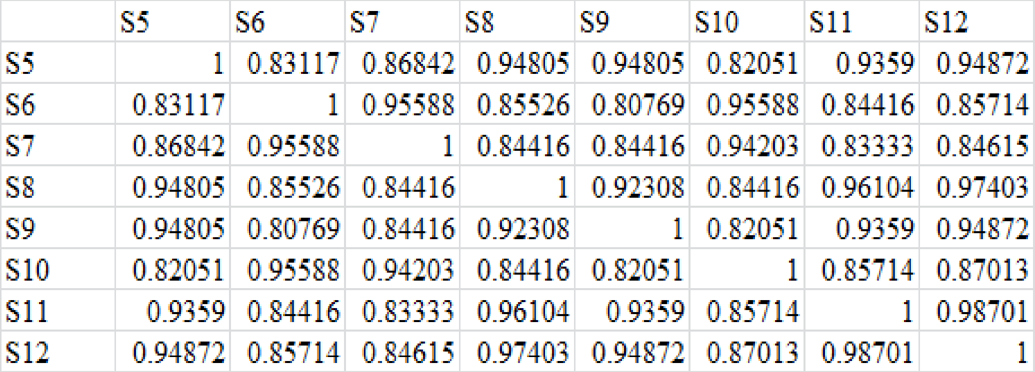
Appendix VI: Similarity matrices of mother plants of purple passion fruit variety and putrescine treated plants based on Jaccard's similarity coefficient of SRAP data.



	S1	S2	S3	S4	S13	S14	S15
S1	0	0.19737	0.19737	0.20779	0.20513	0.17333	0.11429
S2	0.19737	0	0.02632	0.01316	0.03846	0.05195	0.11688
S3	0.19737	0.02632	0	0.01316	0.03846	0.07692	0.14103
S4	0.20779	0.01316	0.01316	0	0.02564	0.0641	0.12821
S13	0.20513	0.03846	0.03846	0.02564	0	0.03846	0.10256
S14	0.17333	0.05195	0.07692	0.0641	0.03846	0	0.06667
S15	0.11429	0.11688	0.14103	0.12821	0.10256	0.06667	0

Lane S1 (purple) represents a mother plant of passion fruit varieties. Lanes S2, S3 and S4 represent plants treated with 0.5 % putrescine. Lanes S13, S14 and S15 represent plants treated with 2 % putrescine.

Appendix VII: Similarity matrices of mother plants of KPF 4 passion fruit variety and putrescine treated plants based on Jaccard's similarity coefficient of SRAP data



	S5	S6	S7	S8	S9	S10	S11	S12
S5	1	0.83117	0.86842	0.94805	0.94805	0.82051	0.9359	0.94872
S6	0.83117	1	0.95588	0.85526	0.80769	0.95588	0.84416	0.85714
S7	0.86842	0.95588	1	0.84416	0.84416	0.94203	0.83333	0.84615
S8	0.94805	0.85526	0.84416	1	0.92308	0.84416	0.96104	0.97403
S9	0.94805	0.80769	0.84416	0.92308	1	0.82051	0.9359	0.94872
S10	0.82051	0.95588	0.94203	0.84416	0.82051	1	0.85714	0.87013
S11	0.9359	0.84416	0.83333	0.96104	0.9359	0.85714	1	0.98701
S12	0.94872	0.85714	0.84615	0.97403	0.94872	0.87013	0.98701	1

Lane S5 represents a mother plant of passion fruit variety KPF 4. Lanes S6, S7 and S8 represent plants treated with 0.5 % putrescine. Lanes S9, S10, S11 and S12 represent plants treated with 2 % putrescine.

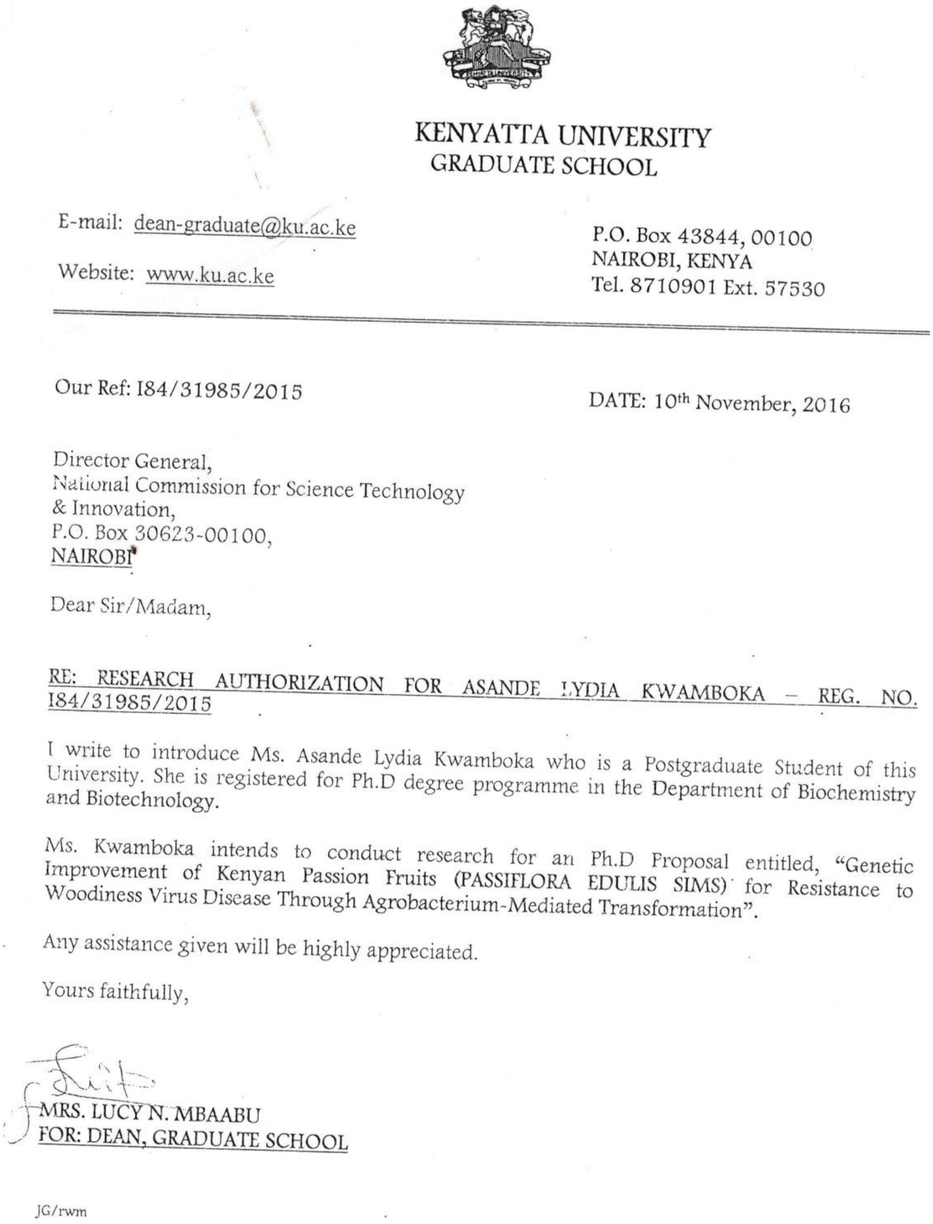
Appendix VIII: Bacterial culture media, DNA extraction and electrophoresis buffers

Medium	Composition	Quantities
LB Broth (Premix)	Tryptone	10.0 g L^{-1}
	Yeast extract	5.0 g L^{-1}
	NaCl	10.0 g L^{-1}
	pH 7.5	
LB Agar (premix)	Tryptone	10.0 g L^{-1}
	Yeast extract	5.0 g L^{-1}
	NaCl	10.0 g L^{-1}
	Agar	15.0 g L^{-1}
	pH 7.5	
TAE electrophoresis buffer (50x stock)	Tris base	242.0 g L^{-1}
	Glacial acetic acid	57.1 ml L^{-1}
	EDTA (pH 8)	0.5 M
CTAB Buffer (DNA)	CTAB	2 %
	NaCl	2 M
	EDTA (pH 8)	25 mM
	Tris – HCl (pH 8)	100 mM
	Polyvinylpyrrolidone	2 %
Loading dye (6X)	Bromophenol blue	0.25 %
	TE	50 %
	Glycerol	50 %

Appendix IX: Composition of various media used for transformation of leaf explants

Medium	Composition	Quantities
Co-cultivation medium	As SIM except for BAP	2 mg L^{-1}
Resting medium	As co-cultivation medium	
	Cefotaxime	450 mg L^{-1}
Selection medium	As resting medium	
	Hygromycin	7.5 mg L^{-1}
Rooting medium	As rooting medium	
	Cefotaxime	450 mg L^{-1}
	Hygromycin	7.5 mg L^{-1}

Appendix X: Research Authorization - NACOSTI



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Tel. 8710901 Ext. 57530

Our Ref: I84/31985/2015

DATE: 10th November, 2016

Director General,
National Commission for Science Technology
& Innovation,
P.O. Box 30623-00100,
NAIROBI

Dear Sir/Madam,

RE: RESEARCH AUTHORIZATION FOR ASANDE LYDIA KWAMBOKA – REG. NO. I84/31985/2015

I write to introduce Ms. Asande Lydia Kwamboka who is a Postgraduate Student of this University. She is registered for Ph.D degree programme in the Department of Biochemistry and Biotechnology.

Ms. Kwamboka intends to conduct research for an Ph.D Proposal entitled, "Genetic Improvement of Kenyan Passion Fruits (PASSIFLORA EDULIS SIMS) for Resistance to Woodiness Virus Disease Through Agrobacterium-Mediated Transformation".

Any assistance given will be highly appreciated.

Yours faithfully,

MRS. LUCY N. MBAABU
FOR: DEAN, GRADUATE SCHOOL

JG/rwm