OPTIMIZING MICROPROPAGATION PROTOCOLS FOR WILD BLACKBERRY (*Rubus* sp)

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and to the best of my knowledge has not been previously presented for the award of a degree in this or any other institution.

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Recommendation

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DEDICATION

This thesis is dedicated to my; dad Kennedy Gichaba, mum Lydia Ateka, my siblings, husband Nyandoro Omuria and daughter Sally Kwamboka.

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ABSTRACT

Blackberry is a crop of great economic potential but it has not been exploited commercially in Kenya. Breeding and commercial production of blackberry requires rapid propagation which is not possible through the traditional seed and vegetative techniques. This study aimed at developing an efficient protocol for rapid multiplication of blackberry (*Rubus* sp). The study comprised of seven separate experiments to establish the best sterilization protocol, explant size, source of explants, callus induction, shoot induction and elongation and rooting experiments using varying plant hormone concentrations. The experiments were laid out in a Completely Randomized Design with three replications. Data was collected on percent contamination and survival rates, callus fresh weight, callus color and morphology, shoot length, number of shoots, root length, number of roots, and root fresh weight. The data were subjected to analysis of variance (ANOVA) and means separated using Tukeys' Honestly Significant Difference Test (Tukeys' HSD) at 5% level of significance. For sterilization experiments, washing with tap water, Caberndezim for 10min, 5% NaOCl for 2 min and 70% ethanol for 2 min gave the least contamination of 11.1% and the highest survival rate of 5.1 explants per culture vessel. Shoot tips had the highest survival rates of explants per vessel with *Rubus fruiticosus* giving 96% survival. There were significant effects explant sizes of *R. apatelus* on survival rates. The 2.1-3.0 cm sized explant gave the highest survival rate of 93.3%. There were significant differences in the fresh weight of callus of the blackberry species and the hormone concentration: 2.0 and 2.5 mg/l 2,4-D gave the highest fresh weight for *R. fruiticosus* species of 60.2 and 57.5 mg respectively. Treatments with 2.5mg/l BAP and 0.5 mg/l NAA showed the highest number of leaves of 12.6, 12.3 and 11.7 for *Rubus fruiticosus*, *Rubus apatelus* and *Rubus volkenisis* respectively and fresh weight of 1.8mg, 1.7mg and 1.8 mg *Rubus fruiticosus*, *Rubus apatelus* and *Rubus volkenisis* respectively. Maximum shoot initiation and growth was achieved with 2.5mg/l BAP supplemented with 0.5 mg/l NAA while that for rooting in all the species was achieved on MS medium supplemented with IBA at 3.0 mg/l. In conclusion, washing the explants with tap water followed by dipping in caberndezim for 10min, then 5% NaOCl for 2 min and 70% ethanol for 2 min gave the least contamination of 11.1% and the highest survival rate of explants per vessel of 5.1 explants. Two and 2.5 mg/l 2, 4-D gave a white green friable callus. Two and a half milligrams per liter of BAP supplemented with 0.5 mg/l NAA showed rapid shoot proliferation and 3 mg/l IBA for root proliferation. These concentrations should be adopted for mass seedling propagation in breeding and commercialization ventures.

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LIST OF ABBREVIATIONS AND ACRONYMS

2, 4-D	2, 4- Dichlorophenoxy acetic acid
ANOVA	Analysis of Variance
BA	Benzylalanine
BAP	6- Benzylaminopurine
FeNaEDTA	Iron sodium Ethelenediaminetetraacetic Acid
IAA	Indole Acetic Acid
IBA	Indole 3 Butyric Acid
Kin	Kinetin
MS	Murashige and Skoog
NAA	Napthyl Acetic Acid
PGR	Plant Growth Regulator
POP	Peroxidase
PPO	Polyphenol oxidase
PAL	Phenylalanine ammonia lyase

CHAPTER ONE
INTRODUCTION

1.1 Background Information

Blackberries (*Rubus* L sub-genus *Rubus*) are included in the group of small fruits referred to as 'brambles' or 'caneberries' (Finn and Clark, 2011). The crop is a shrub with erect, semi-erect or creep growing habit, and most cultivars have thorny stems (Strik, 1992). It is a native crop of Europe in Armenia (Hummer and Janick, 2006) and a naturalized species in South America (Finn and Clack, 2011). According to Strik *et al.,* (2008). Mexico was the leading producer of blackberry by 2009 while Africa was the least in production. Wild *Rubus* species are mostly found in forest edges. Besides forest edges, many members of the wild genus *Rubus* can be found in vineyards, extensive orchards, garden hedges, pastures as well as in neglected meadows (Dujmović, *et al*., 2008). Owing to long-lasting natural selection processes, wild *Rubus* species have adapted to the ecological conditions of their habitats and developed natural resistance mechanisms to biotic and abiotic environmental stress factors. In Kenya, R*ubus* species are found in most counties although there are limited studies on the crop (Chittarajan, 2011).

Blackberry fruits contain high amounts of phenolic flavonoid phytochemicals such as anthocyanins, allagic tannin, guercetin, gallic acid, cyanidins, pelargonidins, catechnis, kaempferol and salicylic acid (Lee, 2017). Phenolics have been reported to have health benefits to humans (Lee, 2017). Phenolics and flavonoids possess diverse biological activities, for instance, antiulcer, anti-inflammatory (Araujo and Leon, 2001) antioxidant (Ghasemzadeh and Ghasemzadeh, 2011), cytotoxic and antitumor, antispasmodic (Ammon and Wahl, 1991) and antidepressant activities (Yu *et al*., 2002). Worldwide, there is a rising demand in blackberry phenolics owing to their health benefits (Lee, 2017).

The crop is commercially propagated by the classical vegetative methods, which include hard wood and soft wood cuttings, layering and bush division. However, successful application of these methods is limited to a certain extent. For example, propagation by tip layering requires a sizeable layering bed, few tips are available per plant, and weed control among the layers is a problem. Propagation by hardwood stem cuttings is simple but rooting is not always satisfactory or guaranteed. Softwood cuttings root readily, but require considerably more care for successful plant multiplication. Grafting and budding require suitable rootstalks (Busby and Himelrick,

1998). Breeding programs require thousands of plant materials of a particular species for evaluation, which is difficult for vegetatively propagated plants and blackberries are no exception. This is because blackberry seeds exhibit dormancy that last up to ten years hence erratic germination. This problem can be solved using biotechnology.

Plant biotechnology is a landmark achievement of science and technology in the twentieth century and it has a huge role in the development and progress of modern agriculture and horticulture in particular in ensuring production of food and nutritional security. The technique of growing sterile plant cells, tissue or organs separate from the mother plant on artificial medium is called plant tissue culture. Plant tissue culture i.e. micropropagation is a process which allows fast and rapid production of genetically identical plant species using a relatively small amount of planting materials, space and time (Odutayo *et al*., 2004; Fira *et al*., 2014). *In vitro* propagation through the development of axillary buds eliminates the seasonal limitations encountered with the classical vegetative methods and needs a small quantity of starting material. *In vitro* propagated plants are uniform and true to type.

Aseptic conditions are practiced in plant tissue culture. Microbes can be present in the explants or can be introduced from poor aseptic handling, unhygienic conditions in the laboratory or from laboratory instruments and apparatus. Microbial contamination is a constant problem, which often compromises development of *in vitro* cultures (Webster *et al*., 2003). These microbes compete adversely with plant tissue cultures for nutrients, and their presence often results in increased culture mortality or can also result in variable growth, tissue necrosis, reduced shoot proliferation and rooting. It is important to establish sterile explants for *in vitro* cultures because the success of tissue culture depends majorly on effective and efficient elimination of both exogenous and endogenous pathogens (Buckley and Reed, 1994). The surfaces of living plants are naturally infested by microorganisms from the environment more especially while dealing with woody plants. Surface sterilization is usually done using chemical solutions whose concentration and duration of exposure must be critically determined. Although various surface sterilization in protocols have been described, there is no protocol for wild species in Kenya. In this study different concentrations and exposure times of sodium hypochlorite and ethanol were studied.

Different explant sources respond differently to *in vitro* conditions. According to Chern *et al*. (1993), different explant sources have different growth potential due to differences in age, endogenous metabolic status and differential genome. Therefore, different source of explants were used in this study in order to establish the most suitable explants source for rapid multiplication. In addition, the size of the explants determines the rate of survival of the explants (Chern *et al*., 1993). Long-sized stems of nodal sections explants have been used and have shown to be most responsive *in vitro* in terms of regeneration on other crops. Despite this the ideal explants size for *Rubus apatelus* has not been established.

Plant growth regulators are inevitable for *in vitro* regeneration of crop plants in any artificial medium. Generally, shoot proliferation is aided by cytokinins while root proliferation is favoured by and auxins. Nevertheless, their requirement depends on the variety of blackberry. Therefore, different levels of plant growth regulators were used in this study to determine the most suitable concentration for rapid multiplication of wild blackberry species tested. For multiplication, generally, MS medium supplemented with plant growth regulators was used, especially BAP at various concentrations (0.5-2.5 mg/l), and the auxins (NAA at 0.5 mg/l). BAP 2.5mg/l showed the highest shoot multiplication rate while IBA at 2.5 mg/l also showed the best root induction rate for the wild blackberry species tested (Zawadzka and Orlikowska, 2006).

Application of *in vitro* propagation has been recorded in some commercial blackberry cultivars (Meng *et al*., 2004; Najaf-Adadi and Hamidoghli, 2009). Despite these studies, no protocol for the rapid proliferation of wild blackberry species has been developed.

1.2 Statement of the Problem

Blackberry is a common wild fruit in many parts of Africa including Kenya. Currently there is an increase in demand for the fruit due to its health benefits However, the rising rates in deforestation for settlement and the search for new land for cultivation may lead to extinction of the wild species mostly in forested areas. Breeding and commercial production of blackberry requires rapid propagation procedures, which is not possible through the traditional seed and vegetative techniques. While technologies in production of high yielding, disease free planting material are important, a rapid multiplication protocol is lacking if wild blackberry is to be bred and commercialized in Kenya.

1.3 Objectives of the Study

1.3.1 Broad Objective

To contribute towards increased blackberry growing by providing an efficient *in vitro* Micro-propagation protocol for wild blackberry species in Kenya.

1.3.2 Specific Objectives

The specific objectives were to:

i. Determine the effects of different *in vitro* sterilization protocols, sources of explant and size of explants on the responses of different wild blackberry species
ii. Determine the effect of different concentrations of phytohormones on callus, shoot and root formation of wild blackberry species *in vitro*

1.4 Hypotheses

i. Different sterilization protocols, sources of explant and explant sizes have no effects on the responses of different wild blackberry species to *in vitro*
ii. Different concentrations of phytohormones have no effect on callus, shoot and root formation of wild blackberry species *in vitro*.

1.5 Justification

Blackberry is a crop of great economic potential and has been shown to have health benefits to humans. Blackberry wild species have been reported to have high levels of vitamins, minerals and antioxidants. They also are tolerant to cold and hot weather conditions besides their capacity to grow in areas with low soil fertility. They are prolific and well adapted to many agro-ecological zones. Research has shown that they are native to many counties in Kenya. Despite their economic importance the wild species have not been given much attention in Africa including Kenya. With the current trends of deforestation for settlement, the crop faces danger as it may become extinct. Fruit scientists in Kenya are on the move to protect the crop. To achieve this, rapid propagation is necessary. Conventional propagation methods could still be used but they have a number of limitations. To begin with the crop is scarce thus may not offer enough materials for propagation. Grafting and budding is a challenge as a suitable rootstalk has not yet been identified. Since these species are less cultured now, the difficulty regarding the production of planting material can be an impediment for establishing commercial plantation. Tissue culture

offers a means for efficient multiplication and conservation of the crop. It is expected that this study will provide blackberry scientists with a micropropagation protocol that will result in production of enough planting materials. The results of this study will add to the already existing knowledge on *in vitro* propagation of blackberry fruits**.**

CHAPTER TWO
LITERATURE REVIEW

2.1 Botany of Blackberry

Blackberry belongs to the Rosaceae family, Rubus (Tourn) L. genus and Rubus (formally Eubatus) subgenus (Clark and Finn, 2011). The genus Rubus is a highly diverse taxon that includes more than 750 species. Blackberry is a shrub with erect, semi-erect or creep growing habit, and most cultivars have spiny stems (Strik, 1992). Blackberries are perennial plants which typically bear biennial stems ("canes") from the perennial root system (Clark *et al*., 2007). In its first year, a new stem called the primocane, grows vigorously to its full length of 3–6 m (in some cases, up to 9 m), arching or trailing along the ground and bearing large palmate compound leaves with five or seven leaflets (Clark *et al*., 2007). In its second year, the cane becomes a floricane and the stem does not grow longer, bust the lateral buds break to produce flowering laterals (which have smaller leaves with three or five leaflets). First- and second-year shoots usually have numerous short-curved, very sharp prickles that are often erroneously called thorns. In Kenya flowering and fruiting occurs during the rainy season. The flowers and fruits are born in a panicle (Hummer and Janick, 2006). The flower receptacle has multiple ovaries, styles and stigmas and is surrounded with white or pink petals. After fertilization an aggregate fruit is produced that consists of central torus (receptacle) surrounded by a number of fleshly drupelets that each contain a seed (pyrene) (Clark *et al*., 2007).

2.2 Origin of Blackberries

Blackberries have long been a favourite wild fruit, as many species are native to several countries worldwide and are picked for personal or commercial use. The shrub is believed to have its origin in Armenia, and is now distributed throughout Europe, Asia, Oceania and North and South America (Pullaiah, 2006; Hummer and Janick, 2006). Blackberries were domesticated in Europe by the seventeenth century and in North America during the nineteenth century (Jennings, 1988). In Africa, South Africa has been the only country reporting significant commercial blackberry production with 100 hactares in 2005 (Table 1). About 60% of the area with blackberry is planted with the cultivar known as ‘Young’ trailing blackberry. 60% of the young trailing blackberry fruits produced in South Africa are exported and the rest are processed.

Other varieties grown in S. Africa are ‘Hull Thornless’, ‘Loch Ness’, ‘Choctaw’ and ‘Arapaho’ with 50% of their production being marketed fresh.

2.3 World Production of Blackberry

The blackberry industry continues to expand around the world, and an estimated 20,035 Ha are planted and cultivated (wild plants harvested not included) (Strike *et al*., 2008) (Table 1). While it is hard to point to one predominant reason for the continual expansion, some of the reasons include: blackberries are a new crop to many areas of the world, they share many similarities to red raspberry and as raspberry production develops in an area blackberry often follow. Blackberries are less expensive to produce than red raspberries because they do not have to be replanted as often and commonly do not have as many pest control inputs. Improved cultivars have also been developed that ship better, taste better, are spineless, and the growing awareness of the nutraceutical value of blackberries. Other than black raspberries, blackberries have higher levels of anthocyanins than many other widely available fruits (Clark *et al*., 2007). Blackberry has been ranked fourth in production after strawberries (*Fragaria X ananassa* Duchesne), blueberries (*Vaccinium sp*) and red raspberries in the USA and some parts of Europe.

Worldwide, Mexico is the leading producer of blackberries, with nearly the entire crop being produced for export into the off-season fresh markets in North America and Europe. The Mexican market is almost entirely from the cultivar 'Tupy' (often spelled 'Tupi', but the EMBRAPA program in Brazil from which it was released prefers the 'Tupy' spelling). In the US, Oregon was the leading commercial blackberry producer, producing 19323.02 tonnes 2502.02 hactares (25.0 km^2), in 1995and 25446.51 tonnes on 2834 Ha^{-1} (28 km^2) in 2009.

Reports indicate that in New Zealand, there were about 260 Ha^{-1} of ‘Boysenberry’ producing 3350 tonnes (t) annually. Blackberry production was estimated in China at 1400–1600 Ha^{-1} with the bulk of the production in Jiangsu (1000 Ha^{-1}), (Hu, *et al.*, 2004). In Europe, The United Kingdom, Romania, Poland, and Germany each have about 100 Ha^{-1} of blackberry, while Croatia has about 180 Ha^{-1}. South Africa had limited blackberry production with total area estimated to be 100 Ha^{-1}. ‘Youngberry’ is grown for processing into jam and juice and makes up the majority of the production, while the remainder is for local and export fresh production (Strike *et al*., 2006).

Table 1: Worldwide Area and Production of Blackberries, 2005

Region	Area planted (Ha^{-1})	Production (Tons)
Europe	7695.1	47,386
North America	1761.9	65,154
Central America	1640.9	1,752
South America	1597.6	7,031
Asia	1550.6	29,038
Oceania	297.2	4,022
Africa	100	220
World total	20044.1	154,603

Source: Strik *et al*., (2008).

2.4 Nutritive Value and Importance of Blackberries

Blackberry (*Rubus* sp.) fruit contains high levels of anthocyanins and other phenolic compounds, mainly flavonols and ellagitannins, which contribute to its high antioxidant capacity and other biological activities (Sellappan *et al*., 2002) (Table 2). A lot of studies show that they are a source of vitamin C, dietary fiber (Bushman *et al*., 2004). Anthocyanin in blackberries is responsible for their rich dark color. Berry extracts, rich in polyphenols, have a range of biological effects that can have beneficial outcomes on human health (Battino *et al*., 2009). Phytochemical components of blackberries, salicylic acid and allergic acid have been associated in preliminary research with toxicity to cancer cells including breast cancer cells (Papoutsi *et al*., 2005).

Table 2: Berry Fruits Nutritive Value Compared

MINERALS (mg)	Blackberry	Raspberry	Strawberry	Cloudberry	Bilberry	Cranberry	Sea buckthorn
Sodium	0.5	0.7	0.7	1.5	0.3	0.9	3.5
Salt	1.3	1.8	1.8	3.8	0.8	2.3	8.9
Potassium	340	220	190	170	110	25	133
Magnesium	24	25	15	29	9	8	30
Calcium	72	35	21	16	19	13	42
Phosphorus	58	37	30	36	20	10	8.6
Iron	1.2	1.1	0.5	0.7	0.6	0.7	0.4
Zinc	0.3	0.4	0.1	0.6	0.2	0.2	0.0
VITAMINS							
Vitamin A(μg)	8.2	1.1	0.9	14.4	3.9	1.8	2.6
Vitamin E(μg)	2.2	0.9	0.6	3.0	1.9	0.9	3.0
Vitamin K(μg)	30	10.2	5.5	9.0	9.0	9.0	11.3
Vitamin C(μg)	120.0	38.0	60.0	100.0	15.0	20.0	165.0
Vitamin B1(mg)	0.05	0.01	0.03	0.06	0.04	0.05	0.18
Folate (μg)	7.7	33.0	35.6	30.0	11.5	2.0	10.0
Carotenoids (μg)	542.2	95.9	44.5	240.0	310.5	50.0	158.6

Source; http://www.fineli.fi/foodlist.php?foodname=A%andlang=en (retrieved 12/04/2018)

2.5 Conventional Propagation Techniques and their Limitations

Plant propagation is defined as the science and art of multiplication of plants by either sexual or asexual means. Conventional methods that have always been used have limitations. Tip layering propagation for instance requires a sizeable planting for the layering bed, few tips are available per plant, and weed control among the layers is a problem. Propagation by hardwood stem cuttings is simple but rooting is not always satisfactory. Softwood cuttings root readily, but require considerably more care for successful plant production (Broome and Zimmerman, 1978). Breeding is difficult since attaining sufficient germination of hybrid seeds is a challenge (Clark *et al*., 2007).

Rubus species seeds tend to acquire deep dormancy after the drying process they undergo when stored in cold rooms of germplasm banks. The release of this mode of dormancy may take place under cold storage conditions over months or years, probably setting a significant contrast with initial seed viability (Wada, 2009). In 'Castilla' blackberry seeds, the highest germination counts were recorded between 16 and 21 hours, when the scarification effect seems to be at an optimum point. Longer times were apparently harmful for the embryo (Diaz Diez *et al*., 2013).

2.6 Plant Tissue Culture Technology

2.6.1 Importance of Tissue Culture

Tissue culture is the culture and maintenance of plant cells or organs in sterile, nutritionally and environmentally supportive conditions *in vitro.* Tissue culture is used for many purposes in biologicals research areas such as propagation, transformation tools, and elimination of pathogens especially viruses, cryopreservation, and breeding. The importance of micropropagation and its main advantages as compared to classical vegetative multiplication include: (1) The possibility to obtain pathogen-free, especially virus-free plants by meristem culture; (2) Immense efficacy and productivity, due to rapid multiplication rates, in geometric progression, in several cycles per year; (3) Potential role in plant breeding, due to the fact that a valuable selection can be propagated extremely rapidly and then introduced into large scale culture; (4) It does not depend on season and weather, due to the fact that it is done in areas with controlled environment; (5) Space is economized; (6) It offers the possibility for the conservation of valuable genotypes in small spaces; (7) It offers the possibility for the easy transportation of a large amount of germplasm independent of phytosanitary quarantine; (8) It

offers the means for the effective multiplication of some species where propagation by traditional methods is difficult and uneconomical; (9) it offers real possibilities for saving and propagating rare plant species (Diallo *et al.,* 2008, Fira *et al*., 2012, Diaz*, et al.,* 2013)

2.6.2 Micropropagation

Micropropagation is the practice of using tissue culture methods to rapidly multiply stock plant material on nutrient suitable media; the technique offers the possibility of large scale multiplication of plant material. Micropropagation involves culturing meristematic tissue or somatic cells *in vitro* under controlled aseptic physical conditions to produce a large number of progeny plantlets that are genetically identical to the parent (Fira *et al*., 2012). The micropropagation process can be divided into five different stages:

Stage 0: growing mother plants under hygienic conditions. It involves the production of stock plants in greenhouse.

Stage I: initiation of culture. The purpose of this stage is to initiate axenic cultures. It involves the selection of explants, disinfestations and the cultivation under aseptic conditions.

Stage II: rapid regeneration and multiplication of numerous propagules (multiplication phase). Masses of tissues are repeatedly sub-cultured under aseptic conditions onto new culturing media that encourage propagule proliferation. The culture can supply shoots for the subsequent propagation phases as well as material that is required to maintain the stock.

Stage III: elongation and root induction or development (rooting phase). This phase is designed to induce the establishment of fully developed plantlets. It is the last period *in vitro* before transferring the plantlets to ex vitro conditions.

Stage IV: transfer to ex vitro condition (acclimatization). Acclimatization is defined as the climatic or environmental adaptation of an organism, especially a plant that has been moved to a new environment.

Micropropagation involves collection and sterilization of explants which may include leaves, stem tips, anthers and other plant tissues, which are then placed on a growth media containing sucrose and plant growth regulators. Establishment is followed by multiplication which entails taking of tissue samples produced during the first stage and increasing their number. Through

repeated cycles of this process, a single explant may be increased to hundreds or thousands of plants. This step is then followed by preparation of the above developed shoots for their eminent transfer to soil. This stage involves elongation of shoots, the transfer of these shoots onto rooting medium containing auxin and pre-hardening cultures to improve survival. Pre-transplanting stage is then ushered in where plantlets that were previously being cultured on media are grown in soil. This is referred to as hardening and it involves growing the plantlets in sterilized soil under greenhouse environment and it is aimed at acclimatizing plantlets to natural growth conditions. Finally, the plantlets are transferred to soil for continued growth by conventional methods in the field (Mineo, 1990). Micropropagation has a number of advantages over traditional plant propagation techniques that include the production of many plants that are clones of each other, production of disease-free plants, multiplying sterile plants which do not have the ability to reproduce using seeds.

2.7 Factors Affecting *in vitro* Propagation

2.7.1 Media

Influence of media on plant regeneration from different parts of a plant has been reported (e.g. Chan, 2004). Various basal media including White medium, Nitsch and Nitsch medium, B5 medium and Gamborg medium for micropropagation (Diallo *et al*., 2008), have been used, but the most commonly used culture medium is Murashige and Skoog (MS) (1962) medium; the reason being that most of the plants respond favorably to MS medium since it contains all nutrients that are essential for plant growth *in vitro*. Selection, combination and strength of media, are also some of the important parameters for optimizing the regeneration protocol (Diallo., 2008).

Minerals are important components of the culture medium. There is a large choice of combinations of macro- and micro-salt mixtures. However, due to the high salt content, these salts nutrient solution is not necessarily always optimal for growth and development of plants *in vitro*. For that reason, the use of dilute media formulations has generally promoted better formation of roots, since high concentration of salts may inhibit root growth, even in presence of auxins in the culture medium. The ability of rose explants to produce shoots and initiate roots was studied by Kim *et al*. (2003). They concluded that optimum shoot proliferation was obtained in full-strength MS salts, while rooting improved with 1/4 strength. Sauer *et al*. (1985) reported

that 1/3 strength MS salts proved to be suitable on rooting of rose. For globe artichoke, 1/2 strength MS salts have been used in the rooting medium (Lauzer and Vieth, 1990).

Culture media can be classified as liquid or solid. The liquid media have the advantage of faster (and cheaper) preparation than the solid ones. Furthermore, liquid media are more homogeneous, since gradients of nutrients may appear during tissue growing in solid media. This phenomenon is not observed in liquid media. Furthermore, it has been shown that the propagation ratio of some species is higher in liquid than in solid media (Debergh *et al*., 2000; Pateli *et al*., 2003). One serious disadvantage of using liquid media for shoot growth and multiplication is that shoots, which are perpetually submerged in liquid cultures, may become hyperhydric and will then be useless for micropropagation (Debergh, 2000).

Ebrahim and Ibrahim (2000) reported that solid medium should be used to overcome the production of vitrified shoots of *Maranta leuconeura* and to insure obtaining vigorous plants with higher chlorophyll content. Agar has traditionally been used as the preferred gelling agent for tissue culture, and is very widely employed for the preparation of semi-solid culture media (Torres *et al*., 2001). It is a polysaccharide extracted from 68 species of red algae which are collected from the sea (Torres, 2001). The cv. 'Motrea' preferred higher concentrations of agar (7 g L^{-1}). At this concentration, completely developed shoots were formed. The cv. 'Sweet Promise', in turn, showed the best results with extremely low concentrations (4 g L^{-1}). Paques (1991) pointed out that there is a strong connection between culture medium hardiness, proliferation ratio and hyperhydration.

Normally, an increase in the agar concentration promotes a reduction in the occurrence of hyper hydration symptoms in plants. However, the propagation rate can be drastically reduced and, consequently, the efficiency of micro-propagation (Debergh, 2000). The concentration of agar in the medium may also affect the formation of roots. Rahman *et al*. (1992) reported that rooting performance of rose decreased with increasing agar concentration (from 6 to 15 g L-1). At 6 g L-1, optimal rooting induction was achieved. An alternative to agar is the use of a gelling agent named gelrite. Gelrite is a gellan gum, a hetero-polysaccharide produced by the bacterium *Pseudomonas elodea* (Kang *et al*., 1982).

Gelrite is an attractive alternative to agar for plant tissue culture because its cost per liter of medium is lower, and it produces a clear gel which facilitates the proper observation of cultures and their possible contamination. Williams and Taji (1987) found that several Australian woody plants survived best on a medium gelled with gelrite rather than agar.

According to Silva and Scherwinski-Pereira (2011), in the *in vitro* conservation under minimal growth conditions, an excessive elongation of the propagules is unwanted because besides promoting the filling of the cultivation test tubes, it also causes the depletion of the nutrient media, and may lead plant material to death. The maintenance of the shoots in culture medium with the salts reduced by half, associated with the decreasing of the temperature to 20° C, was sufficient to lower the growth rates and to achieve survival rates close to 100%, after 15 months in minimal growth conditions.

2.7.2 Type of Explant

Type of explant is among the most important factors in optimizing the tissue culture protocols. Explants such as petiole, cotyledonary leaf, embryo, root and internode respond differently to the tissue culture process (Kumar *et al*., 2011b). This may be as a result of the different levels of endogenous plant hormones that are present in the various plant parts. The most commonly used explant for regeneration is the leaf because of the larger surface area available (Tyagi *et al*., 2010). Tyagi *et al*. (2010) used root, shoot, and leaf explants and the observation made was maximum regeneration efficiency from leaf expalnts in *Cajanus cajan*. Sujatha and Mukta (1996) employed different explants like hypocotyle, leaf and petiole. The observation was a maximum regeneration from the leaf explant of Jatropha curcas. Alagumanian *et al*. (2004) used leaf and stem explants and maximum regeneration efficiency was observed from stem explant in *Solanum trilobotam*. Gubis *et al*. (2003) used hypocotyle, epicotyle, petiole, internode cotyledons, leaf, and a maximum response was obtained from hypocotyle in Tomato. Ali and Mirza (2006) used root, stem, leaf and petiole but maximum responses were observed from stem explant in *Citrus jambhiri Lush*.

2.7.3 Genotype

Genotype is also one of the most important factors affecting regeneration (Kumar and Reddy, 2010). Genotypic effect on shoot regeneration and elongation has been explained well in many

species, and could be due, in part, to differences in the levels of endogenous hormones, particularly cytokinins (Bhatia *et al*., 2005).

The blackberry genotypes showed different responses to the *in vitro* conservation process, both for multiplication rate and number of dead leaves (Gomes *et al*., 2017). For shoot height, no differences were observed among the genotypes. As for the multiplication rate, 'Xavante' developed a number of buds per explant lower than the results observed for 'Guarani', 'Caingangue' and 'Ébano'. As to the number of dead leaves, 'Caingangue' and 'Ébano' showed lower senescent leaves per shoot than 'Xavante' and 'Guarani' (Gomes *et al*., 2017). In contrast, for the number of roots, no significant differences were observed among the studied genotypes. Reed (2003) also observed that in *in vitro* germplasm conservation of Rubus under minimal growth induction, the morphogenesis and development of the cultures significantly varied among the different genotypes analyzed. These results agree with those by Engelmann (2011), who reported that different genotypes have different potentials for the absorption and metabolization of the compounds of the nutrient medium, thereby presenting different responses to the *in vitro* cultivation. Therefore, for gene bank purposes, it is essential to standardize and simplify the culture conditions for plant conservation, not only for the involved high costs, but also for the *in vitro* collections that often maintain several accessions of the same species (Reed, 2003; Engelmann, 2011).

Additionally, shoot formation and multiplication rate generally can have significantly higher averages in the last subculture, thus showing that carrying out five subcultures in the micropropagation of blackberry is possible, without affecting the multiplication rates thereof. This fact contradicts Vujović *et al*, (2012) who claim that during *in vitro* propagation of nodal segments, a decrease of potential shoot growth can be seen in the last subcultures due to successive cuttings, and to the cumulative effect of cytokinins, which, over time, can cause damage to the tissues in cultivation. Thus, the high-multiplication rates obtained in our study show that large-scale propagation of blackberry can be successfully performed, after short and medium-term storage under minimal growth conditions, which is a rare, or nonexistent result in the literature, especially regarding the time of *in vitro* conservation evaluated without subcultures.

2.7.4 Source of Explant

Source of explant i.e. *in vitro* and *in vivo* is also necessary for regeneration (Reddy *et al*., 2008; Kumar *et al*., 2010a). *In vitro* explant is taken to be the most suitable for organogenesis (Reddy *et al*., 2008). The fact that original source of explant has different capacity of regeneration are well documented and established (Feyissa *et al*., 2005). *In vitro* explant generally has better potential to organogenesis as compared to in vivo explant (Reddy *et al*., 2008). The difference could be due to the amount of endogenous hormones present in the explant. Seedling explant is more responsive or meristematic than mature plants (Feyissa *et al*., 2005) due to different level of plant hormones present in the plants.

In a study by Gonzalez *et al*., (2000) on micropropagation of three berry fruit species using nodal segments from field-grown plants, differences were observed, since explants from ëWillametteí plants showed higher survival rates (54%) than those from ëGradinaí (42%) after 15 days of culture in basal MS medium. During the next 30 days in MS medium with 4 mm BA and 0.25 mM IBA all ëWillametteí explants became necrotic, while some ëGradinaí explants survived, and then they were cultured in a modified MS medium (see material and methods). After 30 days of culture in these new conditions, 1.9 ± 0.2 buds per explant were observed.

2.7.5 Carbon Source

Sucrose is by far the most used carbon source, mainly because it is readily available, cheap, relatively stable to autoclaving, and readily assimilated by plants. Other carbohydrates such as glucose, maltose and galactose as well as the sugar-alcohols glycerol and sorbitol can also be used (Fowler, 2000). The carbohydrates added to the culture medium supply energy for metabolism. The addition of a carbon source in any nutrient medium is essential for *in vitro* growth and development of many species, because photosynthesis is insufficient, due to the growth taking place in conditions unsuitable for photosynthesis or without photosynthesis (in darkness). Normally, green tissues are not sufficiently autotrophic under *in vitro* conditions and depend on the availability of carbohydrates in the growing medium. The effect of the carbon source for blackberry plants has not been published.

2.7.6 Growth Regulators

Growth regulators are organic compounds naturally synthesized in higher plants, which influence growth and development. Apart from the natural compounds, synthetic chemicals with similar

physiological activities have been developed which equate to the natural. There are several classes of plant growth regulators, as e.g. cytokinins, auxins, gibberellins, ethylene and abscisic acid. Growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied in the medium, and the growth substances produced endogenously (George, 1993). The equilibrium between auxin and cytokinin is most often required for the establishment of adventitious shoots and roots. In tobacco cultured *in vitro*, it was discovered that the establishment of roots and shoots depended on the ratio of auxin to cytokinin in the culture medium.

High levels of auxin relative to cytokinin stimulated the formation of roots, whereas high levels of cytokinin relative to auxin led to the formation of shoots (Taiz and Zeiger, 1991). The proportionality of growth regulators depends on the objective of the culture *in vitro* (as e.g. shoot, root, callus or suspension culture) and on the micro-propagation phase considered (initiation, multiplication or rooting).

In the multiplication phase, the level of cytokinins should be normally higher than of auxins. In the rooting phase, the use of cytokinin is, in some cases, not necessary and the culture medium can be supplemented with higher levels of auxins (Torres *et al*., 2001). Cytokinins are derived from adenine (amino purine) and play a vital role in the *in vitro* manipulation of plant cells and tissues (Torres *et al*., 2001). Cytokinins stimulate plant cells to divide, and they were shown to affect many other physiological and developmental process. These effects include the delay of senescence in detached organs, the mobilization of nutrients, chloroplast maturation, and the control of morphogenesis. Added to the culture medium, these compounds overcome apical dominance and release lateral buds from dormancy (George, 1993). The most common cytokinins used are kinetin, BA and 2Ip. Also auxins (IAA, IBA, NAA or 2,4-D) are often added to the culture medium to promote the growth of callus, cell suspensions or organs, and to regulate morphogenesis, especially in combination with cytokinin (George, 1993).

Auxins are involved in the regulation of several physiological processes, as e.g. apical dominance and formation of lateral and adventitious roots. This growth regulator generally causes cell elongation and swelling of tissues, cell division (callus formation) and the formation of adventitious roots as well as the inhibition of adventitious and axillary shoot formation. Normally, the concentration of auxin used in the culture medium varies between 0.01 and 10 mg

L-1 (Torres *et al*., 2001). The IAA is a natural auxin, whereas 2-4-D and NAA are synthetically produced and have similar effect in comparison to natural-occurring auxins.

According to most of the studies that have been published concerning the effect of auxin type and concentration in rose, low concentrations of this growth regulator should be used in the culture medium. The rooting of rose shoots was improved with IAA (considered a weak auxin) supplementation at 1.0 mg L-1 (Kim *et al*., 2003), 0.1 mg L-1 NAA or even in absence of auxin (Ibrahim and Debergh, 2001).

Application of *in vitro* propagation has been recorded in great number of blackberry cultivars (Meng *et al*., 2004; Ruzic and Lazic, 2007). Simala, (2006) working on microclonal propagation of *vaccinium* sp. and *rubus* sp. and detection of genetic variability in culture *in vitro* found out that in the high-bush blueberry and the lingonberry, shoots were regenerated from isolated meristems and dormant buds and cultivated on modified Anderson's rhododendron (AN) medium. Shoot formation was induced on medium containing 0.5 mg l-1 zeatin. In the high-bush blueberry, the cultivar with the highest shoot proliferation intensity was 'Brigitta', with 14.2 shoots per primary explant. Research done by Najaf-Abadi and Hamidoghli. (2009) and Fira *et al*. (2011) showed success of tissue culture for thorn-less and trailing blackberry respectively. Mihaljevic and Salopek Sondi, (2012) reported that rooting of microcuttings of high bush blueberries was enhanced using all examined concentrations and types of auxins. The greatest percentage of shoots were rooted after treatment with 4.9 mmol/L (1.34 g/L) IBA-Ala (93.3%), and only slightly weaker (about 85%) with 4.9 mmol/L or 4.9 µmol/L (1 g/L or 1 mg/L) IBA . Treatment with no auxin resulted with lower rate of rooting (60%) although the difference was not significant.

In blackberry cultivar Gazda, the cytokinin CPPU (N-(2-Chloro-4-pyridyl) N'-Phenylurea) gave optimal results in the multiplication stage and the shoots were rooted directly *ex vitro* in Jiffy7 pellets (Vescan *et al*., 2013). MS media supplemented with 0.5 mg/l BAP and gelled either with wheat starch or agar proved to be very effective for the multiplication of blackberry cultivar 'Loch Ness' (Fira *et al*., 2012).

2.8 Effects of Phytohormones on Callus Formation *in vitro*

The term "callus" originates from the Latin word *callum*, which means hard. "Callus" in the early days of plant biology referred to the massive growth of cells and accumulation of calli associated with wounding. Today the same word is used more broadly, and disorganized cell masses are collectively called callus. However, auxin and cytokinin have been by far the most extensively used and studied hormones in the context of callus formation and subsequent organ regeneration. Callus culture is important because; callus is the starting material for the suspension culture which cells are separated. It helps in the production of secondary plant products. It is useful for the synthesis of starting compounds that are subsequently modified to yield the desired product. It is the starting materials for vegetative propagation of plants.

2, 4-Dichlorophenoxyacetic acid (2, 4-D) has been shown to be the most convenient auxin for the induction and sub-culturing of morphogenic callus in *Iris ensata* thumb (Boltenkov *et al*., 2007). In an experiment with *Hymenocallis littoralis* plants the optimum concentration for callus initiation was obtained by using 13.50µM of 2, 4-D and 4.50µM of BAP with 93.75% of callus induction rate. However, concentration 22.50µM of 2, 4-D and 9.50µM of BAP produced the lowest callus induction at12.50%. The earliest time period, which is needed to initiate callus from the meristematic tissue was obtained at 15 days on semi-solid MS media with 13.50µM of 2, 4-D and 4.50 µM of BAP. A study carried out by Afshari *et al*. (2011) for rapeseed (*Brassica nupus*) by revealed that auxins had an inhibitory effect on chlorophyll formation, whereas cytokinin tended to promote it. The results also showed that 2, 4-D when combined with cytokinin (BA), callogenesis and cell division were stimulated faster and better in rapeseed cultivars whereas NAA (with BA or without it) stimulated root formation and rhizogenesis (Afshari *et al*., 2011). Increasing NAA as an auxin the callus inductions rates increased in *Ephedra procera*. Also kinetin as a cytokine was more suitable than BAP along with NAA, and fresh weight increased significantly when kinetin was used as a cytokine along NAA compared with BAP. It was found that half strength MS basal medium supplemented with 2, 4-D and BA (0.5mg/l and 1.0 mg/l, respectively) and 6% sucrose was best for biomass production of leaf callus and enhancement of alkaloid accumulation in *C. roseus* (Verma *et al*., 2012).

Use of 2, 4- D has shown best results for callus inductions in many plants like, E*clipta alba* L. (Sharma *et al*., 2013), *Arnica Montana* (Petrova *et al*., 2011) and *Ionidium suffruticosium* Ging.

(Sonappanavar and Jayraj, 2011). MS media supplemented with 0.5 mg L -1indole acetic acid + 0.5 mg L Naphthalene acetic acid was found to be finest for mean callus induction (62.66%) as well as callus mediated shoot regeneration with mean percentage response (56) and number of shoot per culture (5) of *Rauvolfia serpentine* L. (Rani *et al*., 2014). The best callusing was observed on MS media supplemented with BAP 0.5 mg L-1 + NAA 1.5 mg L-1in *W. somnifera* (Adhikari and Pant 2013). A good callus growth was observed on MS medium for *Rauwolfia serpentina* stem explants using a combination of BAP (1.0 - 2.5 mg L-1) and IAA (0.1 – 0.2 mg/L. poor growth of callus is observed on MS media on shoot apex explants using a combination of BAP (0.5,1.0) and KIN (0.5,1.0) (Malik *et al*., 2012).

Studies on *in vitro* callus induction from adult tissues of peach (*Prunus persica* L. Batsch) showed that woody plant medium supplemented with 2, 4-D and KN significantly increased the rates of callus induction in the majority of treatments (Pérez-Jiménez *et al*., 2013). And no significant differences among the *P. persica* genotypes were found. The explants derived from the stem and calyx produced up to 85 and 96% callus induction, respectively. Nodal explants of *Centella asiatica* isolated and cultured on MS medium fortified with 0.5 -1.0 mg/l 2, 4- D alone and in combination with BAP induced profuse, compact, light green to greenish colored calli (Panathula *et al*., 2014).

2.8.2 Effects of Phytohormones on Rooting and Shooting *in vitro*

Auxin is a plant growth regulator which plays a key role in regulating the following functions: cell cycling, growth and development, formation of vascular tissues (Davis, 1995) and pollen and development of other plant parts (He *et al*., 2005). The growth and development of different plant parts, including the embryo, leaf and root is believed to be controlled by auxin transport (Benjamins and Scheres, 2008; Popko *et al*., 2010). Another important function defined for auxin is elongation of cell, which is done non-transcriptionally with the help of ABP1 activating the expression of AUX/IAAs genes. In tissue culture auxins also play key functions. There are reports that rooting of microcuttings of high bush blueberries was enhanced using all examined concentrations and types of auxins. The greatest percentage of shoots were rooted after treatment with 4.9 mmol/L (1.34 g/L) IBA-Ala (93.3%), and only slightly weaker (about 85%) with 4.9 mmols/L or 4.9 μmol/L (1 g/L or 1 mg/L) IBA. Treatment with no auxin resulted with lower

percentage of rooting (60%) although the difference was not significant according the ANOVA (Mihaljevic and Salopek Sondi, 2012).

Cytokinins are derived from adenine molecules in which there is a side chain at the N6 position. Cytokinins were discovered as factors promoting cell division in tobacco tissue cultures. They have been used in various experiments in tissue culture. All the BA treatments increased the number of shoots formed per explant in comparison with the control treatment for clones of Liatris. The highest shoot proliferation rates were attained in higher concentration of BA tested. High level of Kinetin (4-10ml/L) induced variable amount of shoot multiplication from nodal segment of *Catharanthusss roseus* with best concentration being 6ml/L (Rajora *et al*., 2013). In studies done for trailing blackberry 2mg/l IBA gave a greater number of roots and maximum root length than 0, 0.5 or 1.0mg/l. root length average of 7.83 cm were produced. An overall good rooting response of 60% was observed at 2.0 mgl-1 NAA+0.5 mgl-1 BAP respectively. Significant results ($p< 0.05$) were obtained in this concentration with highest number of roots (4.75±0.48) per shoot and maximum root length (4.85±0.25) (Najaf-Abadi and Hamidoghli, 2009). In fact, higher and very low values of BAP were inhibitory for roots (Kumud *et al*., 2015). Best shoot induction was obtained when the leaf explants of *Citrus limon* L. Burm cv. 'Primofiore' were cultured on Murashige and Tucker media supplemented with 3.5 mg·L-1BAP (Kasprzyk-Pawelec *et al*., 2015).

In other studies, low concentration of BA is more effective for mass propagation of the studied three strawberry clones (Biswas *et al*., 2008). The greatest shoot proliferation in strawberry was achieved in MS medium (full strength) containing 0.5mg/L BAP. The explants in MS medium with different concentrations of KN taken alone for shoot initiation showed an average shooting response (11–54%) in KN with best shoot induction at 3.5 mgl-1 (6.50±0.65 number of shoots per explant with shoot length of1.70±0.12 cm) but this value was not significantly different from shoot number and shoot length values at 2.5 mgl-1 Higher concentrations of KN were inhibitory for both the parameters taken for study (Nankali and Azghandi, 2008).

Highest percentages (>80%) of shoot proliferation of Shoot-tip cultures of *Pyrus elaeagrifolia* Pallas were obtained in the mediums supplemented with 9.0 µM BA and 0.5 µM indole- 3-acetic acid. In the subcultures, the highest shoot proliferation rates were obtained in the medium

containing 4.5 and 9.0 µM BA. The shoot proliferation rates ranged from 91.1 ± 2.4 to 96.4 ± 2.0% in the second subculture and from 76.7 ± 7.8 to 89.4 ± 3.3% in the third subculture. In the second subculture, the shoots grown on 9.0 µM BA without auxin produced the best proliferation (10.6 ± 1.6). In a study carried out for *Camellia sinensis* (L) O. Kuntze, best treatment for nodal segment multiplication in terms of the number of shoot per explant and shoot elongation was obtained using 3 mg/L BAP in combination with 0.5 mg/L GA_3 (Aygun and Dumanoglu, 2015).

TDZ was found to be inappropriate for multiplication of tea clone Iran 100 as it resulted in hyperhydricity especially at concentrations higher than 0.05 mg/L. Healthy shoots treated with 300 mg/L IBA for 30 min followed by transfer to 1/2 strength MS medium devoid of PGR resulted in 72.3% of shoots producing roots and upon transferring them to acclimatization chamber 65% survival was obtained prior to field transfer (Gonbad *et al*., 2014). In species *Rubus laciniatus*, cultivar 'Thornless Evergreen' BAP concentrations lower than 1 mg/l were tested, without the use of auxins and very high multiplication rates were achieved (Fira *et al*., 2010).

2.9 Influence of Phenolic Exudates in Tissue Culture

One of the major problems for many tissue culture systems is oxidized lethal browning and subsequent death of the cultured explants that usually depend on the phenolic compounds and the quality of the total phenols (Ozyigit, 2008). Phenolic compounds are secondary metabolites released from plants, which are present in high amounts. Browning in plants occurs mainly due to the oxidation of phenolic compounds by phenol oxidase (Kefeli *et al*., 2003). This phenomenon occurs when the compartmentalized phenolic compounds are released during explant incision and henceforth react with phenolic oxidases and release quinone (Kefeli *et al*., 2003). Quinone has negative effect on cell growth and can result in death/necrosis of cells. Phennolic compounds occur as secondary metabolites in all plant species and they are generally characterized by a benzene ring and one hydroxyl group (Antolovich, 2000: Kefeli *et al*., 2003). Plant phenolics are classified into major groupings distinguished by the number constitutive carbon atoms in conjuction with the structure of basic phenolic skeleton (Robards *et al*.,1999: Antolovich, 2000). Many phenolics are rather reactive compounds and as long as no steric inhibition due to additional side chains occurs, they form hydrogen bonds (Robards *et al*., 1999).

The composition and synthesis of phenolics in plants tissue may be determined by genetic and environmental condition like oxidative reaction during culturing, processing and storage (Lux-Endrich *et al*., 2000). It appears that there is a relation between chemical compound of media and phenolic exudation, media discoloration, rooting deficiencies and explant browning and death. It was noticed that plant phenolic increased the rigidity of plant cell wall and acted as a molecular bridges between cell wall components (Ozyigit, 2008). During micropropagation, the exudation is very common and it often influences the results. Phenolic secretions and other exudates in plant tissue culture systems lessen explant initiation, growth, and development

CHAPTER THREE
MATERIALS AND METHODS

3.1 Experimental Site

The study was conducted in the Crop Molecular Research laboratory at Egerton University, Kenya. The laboratory lies at latitude of 0º 20' S and longitude 35º56'40'' E. The average (60 years) minimum and maximum temperatures of the area where the laboratory lies are 9º C and 24º C respectively (Kenya Meteorological Services, 2006). The average room temperature where the laboratory is located is 18º C and 6º C maximum and minimum respectively.

3.2 Plant Material

Shoot tips were harvested from a mature crop in the Horticultural Teaching and Research Fields of Egerton University. The species used were *Rubus fruiticosus*, *Rubus apatelus* and *Rubus volkensis*. The wild species were selected because; *Rubus apatelus* was the most vigorous in terms of growth and it produces more fruits per season thus a better choice for commercialization. Its fruits have a weight of 1.2 mg, a diameter of 11.19 mm and a length of 11.37 mm. *Rubus volkensis* had good fruit quality attributes such as a weight of 3.6 mg, a diameter of 16 mm and 16.37 mm length. The species *Rubus fruiticosus* was selected because it had a fruit weight of 5.17 mg, a fruit diameter of 20.09 mm and a length of 22.93 mm (Omondi *et al*., 2018) and it has also been adopted for commercialization in Kenya.

3.3 Surface Sterilization

Surface Sterilization was carried out by first switching on for 15 minutes the Laminar air flow cabinet (SW-CJ-2E manufactured by Suzhou purification equipment company China). Then the Laminar air flow cabinet surfaces were wiped with 70% ethanol. Before any experiment was set, all the apparatus were sprayed with 70% ethanol and placed inside the cabinet. After arranging all the tools that were to be used, the UV light was switched on for 20 minutes after which it was switched off. The laminar air flow cabinet remained on until all culturing and sub-culturing was completed.

3.5 Stock Solution and Media Preparation

Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium was prepared by dissolving the appropriate amount of macro and micro nutrients and organic supplements in distilled water

(Table 3). Similarly, stock solutions of growth regulators were prepared at the ratio of 1mg plant hormone: 1ml double distilled water and stored in refrigerator at 6°C until use. The MS culture medium was prepared from its respective stock solutions using the 30g/l sucrose. The pH of the culture medium was adjusted to 5.8 before autoclaving at 121°C for 20 minutes. 8g/l agar was used for solidification.

Table 3: Composition of Modified MS Basal Medium

Constituent	**Murashige and Skoog (1962) – MS**
KCl	-
$MgSO_4 . 7H_2O$	370
$NaH_2PO_4 . H_2O$	-
$CaCl_2 . 2H_2O$	440
KNO_3	1900
Na_2SO_4	-
NH_4NO_3	1650
KH_2PO_4	170
$Ca(NO_3)_2 . 4H_2O$	-
$(NH_4)2SO_4$	-
$FeSO_4 . 7H_2O$	27.8
$MnSO_4 . 4H_2O$	22.3
$MnSO_4 . H_2O$	-
Kl	0.83
$CoCl_2 . 6H_2O$	0.025
$ZnSO_4 . 7H_2O$	8.6
$CuSO_4 . 5H_2O$	0.025
H_3BO_3	6.2
$Na_2MoO_4.2H_2O$	0.25
$Fe_2(SO_4)_3$	-
EDTA disodium salt	37.3
EDTA-Na ferric salt	-
m-inositol	100
Thiamine HCl	0.1
Pyridoxine HCl	0.5
Nicotinic acid	0.5
Glycine	2
Cysteine	-
Sucrose	30,000

* All the reagents used were analytical grade

3.6 *In vitro* Conditions

The explants were placed in culture vessels containing 20 mls of culture medium. MS basal salts composition was as indicated in Table 3. The pH of all media was adjusted to 5.8 with 1 N NaOH or 1 N HCl prior to autoclaving at 1.05 kg/cm^2, 121° C for 20 min (LDZH-100KBS manufactured in ShangHai, China). Cultures were maintained at 25±1° C air temperatures in a culture chamber (PRX-600C manufactured in ShangHai, China) with a 16 hour photoperiod under an illumination of 20 mmol m^2/s photosynthetic photon flux density provided by cool-white fluorescent tubes.

3.7 Sterilization of the Explants

To establish the best sterilization procedure, about 5 cm long shoots were washed under running tap water for 30 min. Shoots were then cut into 1.0 cm pieces containing axillary buds. To ensure that the disinfectant made good contact with explant, tween 20 was added. After decontamination treatments, all treatments were rinsed three times with distilled sterile water. Nodal sections with axillary buds were isolated and inoculated in culture medium containing the MS macro nutrients (Murashige and Skoog, 1962). They were placed in 90mm x 15mm sterile petri dishes and later used for different treatments as shown in the (Table 4). Each treatment comprised of nine explants per vessel. The experiment was laid out in a completely randomized design (Figure 1)

Table 4. Sterilization Treatments

	Treatment
T1	**Wash with tap water**
	5% NaOCl for 5 min
	70% ethanol for 5 min
T2	**Tap water**
	50% ethanol for 20 min
	10% NaOCl for 10 min
T3	Tap water with tween 20
	25% ethanol for 12 min
	2.5% NaOCl for 12 min
T4	70% ethanol for 5 min
	2.0% NaOCl for 5min
T5	2.0% NaOCl for 15 min
T6	2.5% Ethanol for 10 min
	2.5% NaOCl for 10 min
T7	25% Ethanol for 5 min
	2.5% NaOCl for 5 min
T8	Control – without sterilization
T9	Wash with tap water
	Caberndezim for 10 min5%
	NaOCl for 2 min
	70% ethanol for 2 min

* All the reagents used were analytical grade

T7	T4	T7	T8	T9	T9	T5	T6	T1
T5	T9	T2	T3	T1	T6	T7	T8	T8
T3	T4	T2	T3	T1	T2	T6	T5	T4

Figure 1; experimental layout for sterilization experiment

3.8 Experiment on the Effects of used for Micro propagation for Wild Blackberry Species

Different parts of blackberry plants were evaluated, these included nodal sections with mature stem sections and apical stem sections and auxiliary buds only (Table 5). The experiment comprised of three treatments replicated three times with five explants per culture vessel. The experiment was laid out in Completely Randomized Design (figure 2).

Table 5: Plant Parts used for Micro propagation of Wild Blackberry Species

Treatment	Source of explants
T1	Nodal sections with mature stem sections
T2	Nodal sections with apical stem sections
T3	Auxiliary buds without stem sections

T3	T1	T1
T3	T2	T2
T1	T2	T3

Figure 2; Experimental layout source of explant

3.9 Experiment on the Effect of Explant Size on Survival rate of *Rubus apatelus in vitro*

The effect of explant size on axillary bud formation (proliferation) was evaluated by culturing explants of different sizes (0.7- 1.0cm, 1.1- 2.0 cm and 2.1- 3.0 cm) on the proliferation medium. The experiment comprised of three treatments replicated three times with five explants per culture vessel(Table 6).. The experiment was laid out in a Completely Randomized Design (Figure 3)

Table 6: Treatments of the Different Sizes of *Rubus apatelus* Used *in vitro*

Explant size	Treatment
0.7- 1.0cm of stems of nodal sections	T1
1.1- 2.0 cm of stems of nodal sections	T2
2.1- 3.0 cm of stems of nodal sections	T3

T2	T3	T1
T1	T2	T2
T1	T3	T3

Figure 3: experimental layout for the size of explant

3.10 Callus Induction using different concentrations of 2 4-D

Stem explants of about 0.5 cm long were cultured on MS medium in 90 mm x15mm petri dishes. Different concentrations of 2, 4-D were applied for callus induction and growth (Table 7). Three blackberry stem explants were used in each petri dish. The experimental layout was in A Completely Randomized Design with three replications (figure 5).

Table 7: Concentrations of 2, 4 -D used in callus formation of wild blackberry species

2, 4-D concentrations (Mg/l)	Treatment
0.5 2,4-D +1.0Kin	T1
1.0 2,4-D +1.0Kin	T2
1.5 2,4-D +1.0Kin	T3
2.0 2,4-D +1.0Kin	T4
2.5 2,4-D +1.0Kin	T5
0 2,4-D +1.0Kin	T6

T6	T2	T3	T6	T3	T4
T1	T3	T2	T5	T2	T4
T6	T4	T1	T5	T5	T1

Figure 4; Experimental layout for callusing experiment

3.10 Shoot Induction

Apical buds were subjected to Murashige and Skoog medium supplemented with 0.5mg/l IAA and 6-benzylaminopurine (BAP) as cytokinin source at different concentrations (0.5, 1.0, 1.5. 2.0 and 2.5mg/l) for shoot induction (Table 8). The treatments were laid out in completely Randomized design with three replications (Figure 5).

3.11 Shoot Multiplication

The induced shoots (1cm) long from the shoot induction medium were sub-cultured for further growth in a shoot multiplication medium. This medium consisted of MS basal salts with different concentrations of benzylaminopurine (BAP) (0.5, 1.0, 1.5, 2.0 and 2.5 mg /L) and supplemented with 1.0mg/l NAA. (Table 8). Shoots were cultured and maintained at *in vitro* conditions. The treatments were laid out in a completely Randomized design with three replications (Figure 5).

3.12 Shoot Elongation

The shoots from the shoot multiplication medium were sub-cultured after 8 weeks for further growth in shoot elongation media. This medium consisted of MS basal salts supplemented with benzylaminopurine (BAP) at 0.5, 1.0, 1.5, 2.0 and 2.5 mg /L) and 1.0 mg/l NAA (Table 8) Shoots were cultured and maintained *in vitro*. The treatments were laid in a Completely Randomised Design with three replications as shown in (Figure 5)

Table 8: Treatment shoot regeneration experiments

BAP+NAA concentrations	Treatment
0.0+0.0	T1
0.5+0.0	T2
1.0+0.0	T3
1.5+0.0	T4
2.0+0.0	T5
2.5+0.5	T6
0.5+0.5	T7
1.0+0.5	T8
1.5+0.5	T9
2.0+0.5	T10
2.5+0.5	T11

T6	T5	T2	T5	T8	T1	T11	T9	T7	T5	T2
T4	T8	T4	T3	T9	T10	T7	T3	T6	T4	T7
T9	T1	T1	T2	T11	T11	T6	T10	T3	T8	T10

Figure 5: Experimental layout of shoot regeneration experiments

3.13 Experiment on Rooting

The multiplied shoots were left to developed up to a length of 3.0 cm and above, then they were taken to the laminar clean bench where they were cut and transferred to rooting MS medium supplemented with indole-3-butyric acid (IBA) at 0.0, 0.5, 1.0, 2.0, and 3.0 mg/l (Table 9). Experiment was laid out in a completely randomized design with three replications (Figure 6).

Table 9: Treatment root regeneration experiments

IBA (mg/l Concentration	Treatment
0.0	T1
0.5	T2
1.0	T3
2.0	T4
3.0	T5

T4	T5	T2	T3	T5
T4	T2	T5	T1	T3
T3	T2	T4	T1	T1

Figure 6 : Experimental layout of root regeneration experiments

3.14 Data Collection

3.14.1 Sterilization

The average percentage contamination rates were determined by counting the number of explants that were contaminated divided by the total explants used multiplied by 100. Also the percentage survival rates were determined by counting the number of explants that survived divided by the total explants used multiplied by 100.

3.14.2 Explant source

The average percentage bud break rates were recorded by counting the number of plants that formed buds divided by the total explants used multiplied by 100.

3.14.3 Explant size for *Rubus apatelus*

The average Percentage Response to Growth by the different sizes of *Rubus apatelus* were detemined by counting the number of plants that survived divided by the total number of explants used multiplied by 100.

3.14.4 Callus Formation Parameters

The following callus formation variables were recorded:

3.14.4.1 Calli Morphology

Callus type was scored as no callus, compact or friable.

3.14.4.2 Onset of Callus Induction

The number of days elapsed for callus to be induced on explants was recorded. This was done through observation.

3.14.4.3 Percentage of Callus Induction

The percentage callus formation was calculated by the formula microcuttings that formed callus divided by total microcuttings in the glass jar x 100.

3.14.4.4 Callus Fresh Weight

Callus were collected after 24 weeks of culturing and weighed using an electric weighing balance (JA10003, manufactured in Zuzhou, China) to obtain the fresh weight.

3.15.5 Root and shoot Proliferation Parameters

3.15.5.1 Root Length

The lengths of emerged roots were measured in centimeters from the point of emergency to the tip using a linear meter and the average length from four sampled rooted shoots was used for analysis.

3.15.5.2 Root fresh weight

Fresh weights of five explants were measured in grams using an electric weighing balance (JA10003, manufactured in Zuzhou, China) and their average recorded and used for analysis.

3.15.5.3 Root Numbers

The newly grown roots from five sampled rooted shoots were counted and the average root number was recorded and used for analysis.

3.15.5.4 Shoot Length

The length of emerged shoots were measured in centimeters from the point of emergency to the tip using a linear meter and the average length from four sampled shoots was used for analysis.

3.15.5.5 Shoot fresh weight

Fresh weight of five explants was measured in grams using an electric weighing balance (JA10003, manufactured in Zuzhou, China) and their average recorded and used for analysis.

3.15.5.6 Leaf Number

Newly grown leaves from five sample shoots were counted and the average leaf number was recorded and used for analysis.

3.15.5.7 Number of shoots

Newly induced shoots from five sample shoots were counted and the average number was recorded and used for analysis

3.16 Data Analysis

Data was subjected to analysis of variance (ANOVA) at $\rho \leq 0.05$ using PROC GLM code of SAS (version 9.1) and means of significant treatments were separated using Tukey's Honestly Significant Different Test at $\rho \leq 0.05$. The models to fit for the experiments were

Interaction between genotype and treatments:

$Y_{ijk} = \mu + \Upsilon_i + \beta_j + \Upsilon\beta_{ij} + \varepsilon_{ijk}$

CRD model:

$Y_{ijk} = \mu + \Upsilon_i + \beta_j + \varepsilon_{ijk}$

I=1, 2…n; j=1, 2, 3; k= 1, 2, 3;

Where: μ- overall mean, Υ_i- effect due to i^{th} hormone, β_j- effect due to j^{th} species

($\Upsilon\beta_{)ij}$- effect due to the interaction between i^{th} hormone and j^{th} species

ε_{ijk}- random error component which is normally and independently distributed about zero means with a common variance

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 Effects of Different Sterilization Procedures on Contamination and Survival of Wild Blackberry Species

There were significant effects ($p \leq 0.05$) of different sterilization procedures on the contamination and survival rates of wild blackberry species (Table 10). Washing the plant materials with tap water followed by dipping in Caberndezim for 10min, 5% NaOCl for 5 min followed by 70% ethanol for 2 min resulted in the least contamination Tap water with tween 20, 5% ethanol for 12 min 2.5% NaOCl for 12 min and the highest percent survival rates of 5.11 explants per vessel. It is important to eliminate foreign contaminants such as bacteria and fungi prior to *in vitro* culture establishment; however, it is often difficult to acquire sterile plant material. It thus, becomes problematic while dealing with woody plant material (Niedz and Bausher, 2002). Basically, woody plants are grown in soil for a number of years under ambient climatic conditions and hence are normally infected heavily with microorganisms both endogenously and exogenously, which are often difficult to control *in vitro* (Ahmad *et al*., 2003). In the present study, Carbendazim, is considered a broad-spectrum fungicide of the benzimidazole family. This family of fungicides binds to microtubules and interferes with cell division and transport (Park *et al*., 1997). The fungicide is systemic thus controls endogenous fungi. The treatment that was less contaminated (T9) gave the highest percent survival rate of 5.1 explants per culture vessel affirming the fact that pathogens compete for nutrients *in vitro* (Cassells, 2001). Ethanol and sodium hypochlorite at high concentrations and longer exposure time were effective for surface sterilization but did improve survival of explants. Similar results were observed by Zinabu *et al*., 2018 who found out that higher concentration of NaOCl gave best sterilization but lower *in vitro* responses of Elite Enset cultivar of banana.

Table 10: Means of percent survival and contaminations rates of *Rubus apatelus* blackberry species *in vitro*

	Treatment	Contamination rates per culture vessel	Survival rates per culture vessel
T1	**Wash with tap water**		
	5% NaOCl for 5 min		
	70% ethanol for 5 min	9.72±6.05^{e}	0.11±0.71^{d}
T2	**Tap water**		
	50% ethanol for 20 min		
	1.0% NaOCl for 10 min	58.33±6.05^{bc}	2.00±0.71^{bcd}
T3	Tap water with tween 20		
	25% ethanol for 12 min		
	2.5% NaOCl for 12 min	8.33±6.05^{e}	1.88±0.719^{bcd}
T4	70% ethanol for 5 min		
	2.0% NaOCl for 5min	27.78±6.05^{de}	1.77±0.71^{bcd}
T5	20% NaOCl for 15 min	29.17±6.05^{de}	3.11±0.71^{b}
T6	25% Ethanol for 10 min		
	2.5% NaOCl for 10 min	38.61±6.05^{cd}	2.22±0.71^{bc}
T7	25% Ethanol for 5 min		
	2.5% NaOCl for 5 min	75.00±6.05^{ab}	2.55±0.71^{b}
T8	Control – without sterilization	100.00±6.05^{a}	0.33±0.71^{cd}
T9	Wash with tap water		
	Caberndezim for 10 min		
	5% NaOCl for 2 min	11.11±6.05^{de}	5.11±0.71^{a}
	70% ethanol for 2 min		

Means followed by the same letter in columns are not significantly different at p≤0.05 according to Tukey's HSD test

4.2 Effects of Blackberry Species on Percentage Bud break and Survival *in vitro*

The percentage bud break at 21 day was significantly different (p≤0.05) among the three species (Table 11). Bud break was highest in *Rubus fruiticosus* with 83.33% but was not significantly

different from 61.1% of *Rubus volkensis* and lowest in *Rubus apatelus* with value 33.3%. The number of explants that survived per culture vessel was also significantly different. Four, zero and two explants per vessel were observed with R*ubus fruiticosus, Rubus apatelus* and R*ubus volkensis* respectively.

Table 11: Means of percent bud break and survival rates of wild blackberry species *in vitro*

Species	% Bud break at 21 days	Survival per vessel after 8 weeks
Rubus fruiticosus	83.3[a]*	4.2±0.2[a]*
Rubus apatelus	33.3[b]	0.0±0.2[c]
Rubus volkensis	61.1[a]	2.0±0.2[b]

*Means followed by the same letter in columns are not significantly different at p≤0.05 according to Tukey's HSD test

In this study, it was noted that the species *Rubus apatelus* was more woody thus may have released a lot of exudates into the culture medium. Most woody tropical plants are prone to phenolics oxidation also called browning or blackening of the culture medium resulting to inactivation of growth in the cultures (Amhad *et al*., 2013). Cut surfaces of woody plants produce phenols which cause activation of oxidative enzymes such as polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and peroxidase (POP) which leads to browning and hence death of the explant (Litz and Vijayakumar, 1988). Phenolics restrict nutrient availability by the explants resulting to their death (Cheema and Hussain, 2004). Phenolic compounds are secondary metabolites released from plants, which are present in high amounts. Browning in plants occurs mainly due to the oxidation of phenolic compounds by phenol oxidase (Kefeli *et al*., 2003). This phenomenon occurs when the compartmentalized phenolic compounds are released during explant incision and henceforth react with phenolic oxidases and release quinone (Kefeli *et al*., 2003). Quinone has negative effect on cell growth and can result in death/necrosis of cells.

4.3 The Interaction between Explant Source and Blackberry Species on Percentage Response *in vitro*

There were significant at ($p \leq 0.05$) interaction effects of explant source and species on the percentage survival of wild blackberry plants (Table 13). Nodal sections with apical stem sections gave the highest percentage response among all the three species with *Rubus fruiticosus*, *Rubus apatelus* and *Rubus volkensis* having 96%, 16% and 83% respectively.

Table 12: Effects of part of explant and species on the percentage survival

Source of explant	Species	% survival rates
Nodal sections from with mature stem sections	*Rubus fruiticosus*	$29.16 \pm 3.86^{d*}$
Nodal sections from with mature stem sections	*Rubus apatelus*	0.00 ± 3.86^{f}
Nodal sections from with mature stem sections	*Rubus volkensis*	16.66 ± 3.86^{e}
Nodal sections with shoot tip stem sections	*Rubus fruiticosus*	96.00 ± 3.86^{a}
Nodal sections with shoot tip stem sections	*Rubus apatelus*	16.66 ± 3.86^{e}
Nodal sections with shoot tip stem sections	*Rubus volkensis*	83.33 ± 3.86^{b}
Auxiliary buds without stem sections	*Rubus fruiticosus*	41.66 ± 3.86^{c}
Auxiliary buds without stem sections	*Rubus apatelus*	08.33 ± 3.86^{ef}
Auxiliary buds without stem sections	*Rubus volkensis*	20.83 ± 3.86^{e}

*****Means followed by the same letter are not significantly different at $p \leq 0.05$ according to Tukey's HSD test

According to the present findings, nodal sections with apical stem sections of the species *Rubus fruiticosus* gave 96% *in vitro* survival followed by *Rubus volkensis* which gave 83.33% while *Rubus apatelus* gave 16.66%. Apical sections produce little phenolic exudates. Shoot tips showed highest percent survival rates due to less production of phenolic compound. In the case of apex explants, there was a lower frequency of infections, due to their size, compared to node explants. Regarding the response in culture, the apex and node explants showed better survival response compared to the other plant parts tested. This result was in agreement with those of Nicuţă *et al*., (2014) who found out that apex explants of *Rubus hirtus* L showed a lower frequency of infections, due to their size, compared to node explants and hence had greator responses *in vitro. Rubus fruiticosus* may have survived best *in vitro* because it has undergone a lot of genetic and environmental modification. Nodal sections with mature stem sections gave

the lowest percent rate of survival probably due to production of phenolic compounds which led to oxidation of the medium resulting in availability of nutrients by the explant.

4.4 Effects of Different Apical Nodal Section Sizes on the Percentage Response to Growth of *Rubus apatelus*

There was a significant effect at ($p \leq 0.05$) of apical nodal section sizes on the percent survival rates and bud break *in vitro* experiments of *Rubus apatelus*. Different sizes were used to determine a suitable size for micropropagation. Nodal sections cut with >2 cm stem cutting had the highest response of 93.3%. Nodal sections with less than 1 cm stem cuttings gave the lowest percentage response of 6.6% (Table 14).

Table 13: Means of survival percentage for different explant sizes for *Rubus apatelus* *in vitro*

Explant size	%survival
0.7- 1.0cm of stems of nodal sections	6.6^{c}
1.1- 2.0 cm of stems of nodal sections	26.6^{b}
2.1- 3.0 cm of stems of nodal sections	93.3^{a}

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

Size of explant significantly affected shoot regeneration. The 3cm stem of nodal section explants were most responsive (93.33%). Decrease in size of the explant resulted in reduced regeneration response. These results agree with those of Frary and Earle (1996) who observed that there was a reduction in shoot regeneration response with decreasing size of explants. High amounts of phenolic compounds that were being released to the culture medium led to the higher death percentage the small size explants. According to Shen *et al*. (2010), there was no shoot proliferation in either the de-tipped or single node explants on MS medium containing TDZ at the concentrations examined.

4.5 Effects of Different Concentrations of 2, 4-D Supplemented with 1.0mg/l Kinetin on Days to Callus Induction

There were no significant effects of different concentrations of 2, 4-D supplemented with 1.0 mg/l kinetin on the number of days taken for callus induction in the *Rubus fruiticosus* (Table 15).

Within 4 days all the concentrations tested, callus formation was induced. There were significant effects of the different concentrations of 2, 4-D on *Rubus volkensis* . Callus was observed to have been induced on 2.5mg/l 2, 4-D with 1.0 mg/l kinetin concentrations. The other concentrations tests did not show callus induction. No callus was induced for *Rubus apatelus* at all the concentrations of 2, 4-D tested was observed

Table 14 Effects of different blackberry species to days for callus induction

Blackberry species	Days to callus induction
Rubus fruiticosus	4[a]
Rubus apatelus	-
Rubus volkensis	21[b]

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

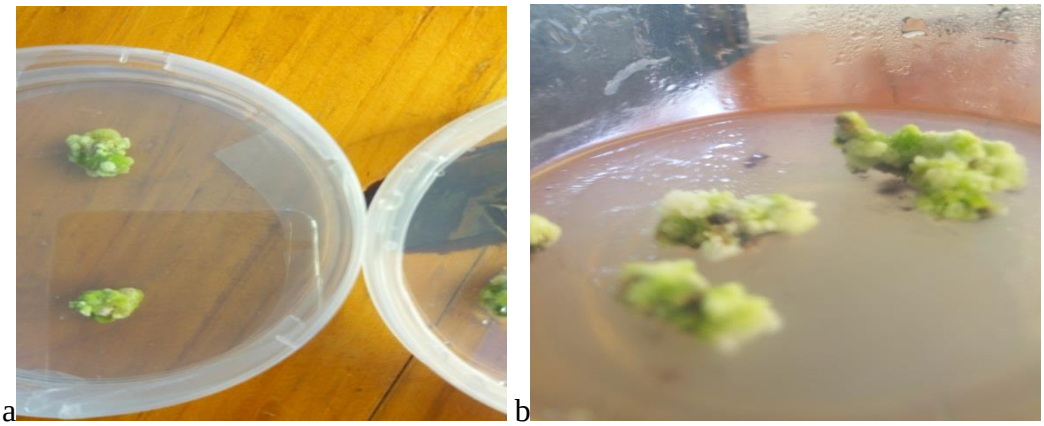
4.6 Effects of Different Concentrations of 2,4 D with 1.0mg/l Kinetin on Callus Fresh Weight, Morphology and Colour

There were significant differences in the fresh weight of the blackberry species and the hormone concentration (Table 16). At 2.0 and 2.5 mg/l 2, 4-D gave the highest fresh weight for *Rubus fruiticous* species of 60.2 and 57.5 mg respectively. 0.5mg/l 2 4-D showed green compact callus while that of 2.0 and 2.5 mg/l 2 4-D showed white green friable callus (plate 1). In the present study, all concentrations of 2, 4-D failed to induce viable callus for *Rubus apatelus*. A white friable callus was formed by *Rubus volkensis* species at 2.5 mg/l.

Table 15 Mean callus fresh weight and callus morphology and colour of different blackberry species

Hormones Mg/l	Fresh weight (mg)			Callus morphology (color-texture)			
2,4-D +1.0Kin	R. *fruiticosus*	R. *apatelus*	R. *volkensis*	R *fruiticosus*	R. *apatelus*	R. *volkensis*	
0.5	21.5[c]*	-	-	Green-compact	-	-	
1.0	34.4[b]	-	-	Green-compact	-	-	
1.5	36.6[b]	-	-	Green-compact	-	-	
2.0	60.2[a]	-	-	White Green-friable	-		
2.5	57.5[a]	-	8.6[d]	White Green-friable	-	-	Whitish friable

- *Means followed by the same letter in columns are not significantly different at p≤0.05 according to Tukey's HSD test



a b

Plate 1 Callus formed at 0.5 mg/l 2, 4-D (a) and at 2.5 mg/l 2, 4-D (b) for *Rubus fruiticosus*

For Ru*bus volkensis* calli induction was observed at 2.5 mg/l 2, 4-D concentration suggesting that low concentrations of 2, 4-D did not favour callus induction. This observation concurs with previous studies of Liu and Cantliffe (1984), and Jarret *et al*. (1984), who observed that 2, 4-D at 3.0 mg/L was effective in inducing somatic embryos in sweet potato. Chee and Cantliffe (1988) have also suggested the use of 2, 4-D at 2.2 mg/L for the induction of embryogenic callus for the apical meristem. The elevation of plant hormone concentrations was previously reported to have growth effects on the rate of callus formation in Withania somnifera (Chakraborty *et al*., 2013). Optimal callus induction could be obtained through manipulating 2, 4-D concentrations and the duration of its presence in the induction medium (Zheng and Konzak, 1999) as different genotypes respond differently to varying 2,4-D levels (Arzani and Mirodjagh, 1999).

4.7 Effects of Different Concentrations of 2, 4-D on Callus Induction of Stem Explants of Wild Blackberry Species

Rubus fruiticosus showed 100 percent callus induction for all 2, 4-D concentrations with the control (0 mg/l 2, 4-D) giving 33.3 percent (Table 17). The highest percentage of callus induction was recorded at 2.0, and 2.5 mg/L 2, 4-D, for *R. volkensis* species. *Rubus apatelus* species did not develop callus at all the treatments.

Table 16: Percentage of callus induction of stem explants for three species in different 2,-4-D (mg/l) with 1.0(mg/l) kinetin

Concentrations	*R. fruiticosus*	*R. volkensis*	*R. apatelus*
0.5 2,4-D +1.0Kin	100	0	0
1.0 2,4-D +1.0Kin	100	0	0
1.5 2,4-D +1.0Kin	100	0	0
2.0 2,4-D +1.0Kin	100	0	0
2.5 2,4-D +1.0Kin	100	0	0
0 2,4-D +1.0Kin	33.3	20	0

*****Means followed by the same letter are not significantly different at $p \leq 0.05$ according to Tukey's HSD test

The results show that the rate at which calli are induced depends largely on species and to some extent on the concentration of 2, 4-D. This implies that the capacity to produce calli depends on genotype. The genotype effect on embryogenic callus ability was also reported previously in rice

(Van Sint Jan *et al*., 1990) and in coffee (Molina *et al*., 2002). Studies show that embryogenic capacity is a stable trait since the first generations and that it would be possible to predict the embryogenic capacity of a given line by evaluating the embryogenic rate of its ancestors. For *in vitro* breeding program, selection must be followed by plant regeneration. The choice of the potential genotypes that could be improved depends mainly on their capacity to regenerate a plant.

4.8 Effects of Different Concentrations of BAP Supplemented with NAA on Shoot Variables of Blackberry Species

There were significant effects ($p \leq 0.05$) of different concentrations of BAP and NAA on the number of leaves, and shoot fresh weight of the three tested species (Table 18). Treatment with 2.5mg/l BAP with 0.5 mg/l NAA showed the highest number of leaves of 12.6, 12.3 and 11.6 for *Rubus fruiticosus, Rubus apatelus* and *Rubus volkenisis* respectively and and fresh weight of 1.8mg, 1.7mg and 1.8mg *Rubus fruiticosus, Rubus apatelus* and *Rubus volkenisis* respectively. In additin, 2.5 mg l-1 BAP in combination with 0.5 mg/l NAA showed highest results 4.26 cm than other treatments for *Rubus fruiticosus*, 3.0 cm for *Rubus apatelus* and 3.9 cm for *Rubus volkensis*. There was an increasing trend in the shoot length up to 2.5 mgl-1 BAP with 0.5 mg/l NAA.

Table 17: Means of the number of shoots, number of leaves and fresh weight of *Rubus fruiticosus, Rubus apatelus* **and** *Rubus volkenisis* **as affected by BAP and NAA concentrations and combinations**

Species	BAP+-NAA	Shoot length	No. of shoots	No. of leaves	Shoot fresh weight in g
R. fruiticosus	0.0+0.0	1.6^{f*}	0.0^{c}	3.0^{d}	0.5^{e}
	0.5+0.0	1.7^{f}	0.0^{c}	3.6^{cd}	0.5^{de}
	1.0+0.0	2.0^{ef}	0.6^{bc}	4.6^{bcd}	0.6^{cde}
	1.5+0.0	2.4^{de}	1.6^{abc}	6.3^{abcd}	0.8^{cde}
	2.0+0.0	2.5^{de}	2.6^{ab}	6.0^{abcd}	1.5^{ab}
	2.5+0.5	2.7^{cd}	2.3^{ab}	9.3^{abcd}	1.6^{ab}
	0.5+0.5	2.8^{cd}	2.0^{abc}	8.0^{abcd}	0.9^{cde}
	1.+-0.5	3.1^{bc}	1.6^{abc}	10.6^{ab}	1.1^{bcd}
	1.5+0.5	3.3^{b}	1.6^{abc}	08.3^{abcd}	1.1^{bc}
	2.0+0.5	3.9^{a}	3.3^{a}	10.3^{abc}	1.5^{ab}
	2.5+0.5	4.2^{a}	3.0^{a}	12.6^{a}	1.8^{a}
R. apatelus	0.0-0.0	0.8^{g}	0.6^{c}	3.6^{ef}	0.5^{e}
	0.5-0.0	0.9^{g}	1.0^{bc}	4.0^{ef}	0.4^{e}
	1.0-0.0	1.3^{f}	1.6^{abc}	3.3^{f}	0.6^{de}

	1.5-0.0	1.7^{ef}	2.0^{abc}	6.0^{cdef}	0.8^{cde}
	2.0-0.0	1.9^{de}	2.3^{abc}	5.6^{def}	1.1^{bcd}
	2.5-0.5	1.9^{cde}	3.0^{ab}	11.0^{ab}	1.7^{a}
	0.5-0.5	2.2^{bcd}	2.3^{abc}	7.0^{bcdef}	0.9^{cde}
	1.0-0.5	2.3^{bc}	2.6^{abc}	9.0^{abcd}	1.0^{cd}
	1.5-0.5	2.5^{b}	3.0^{ab}	8.0^{abcde}	1.3^{abc}
	2.0-0.5	3.0^{a}	3.6^{a}	10.3^{abc}	1.5^{ab}
	2.5-0.5	3.0^{a}	3.6^{a}	12.3^{a}	1.7^{a}
R. volkensis	0.0-0.0	1.4^{g}	0.6^{d}	3.3^{e}	0.5^{e}
	0.5-0.0	1.8^{fg}	1.0^{cd}	4.6^{de}	0.6^{e}
	1.0-0.0	2.2^{ef}	1.7^{bcd}	5.0^{cde}	0.7^{e}
	1.5-0.0	2.4^{e}	2.0^{bcd}	6.3^{bcde}	0.9^{cde}
	2.0-0.0	2.3^{e}	2.6^{abcd}	6.0^{bcde}	0.9^{de}
	2.5-0.5	2.6^{cde}	3.0^{abc}	9.6^{abc}	1.5^{ab}
	0.5-0.5	2.5^{de}	2.3^{bcd}	7.0^{abcde}	0.7^{e}
	1.0-0.5	2.9^{cd}	2.6^{abcd}	8.6^{abcd}	1.2^{bcd}
	1.5-0.5	3.1^{bc}	3.3^{ab}	7.6^{abcde}	1.4^{bc}
	2.0-0.5	3.5^{ab}	3.6^{ab}	10.3^{ab}	1.6^{ab}
	2.5-0.5	3.9^{a}	4.6^{a}	11.6^{a}	1.8^{a}

Means followed by the same letter in columns among species are not significantly different at $p \leq 0.05$ according to Tukey's HSD test

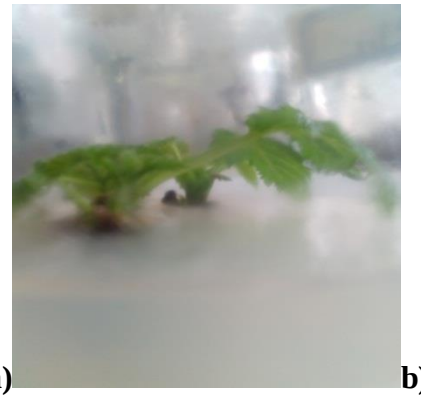
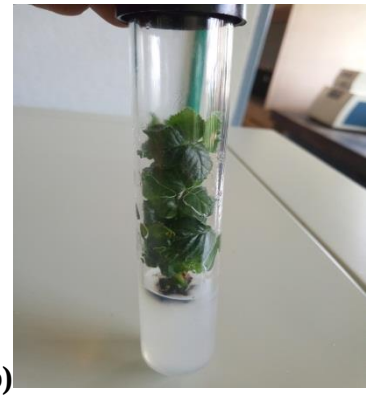
a)  **b)** 

Plate 2: Longest shoot of *Rubus fruiticosus* at day 28 in 2.0mg/l BAP and 0.5mg/l NAA (a) and day 42 at 2.5mg/l BAP and 0.5mg/l NAA (b) respectively *in vitro*

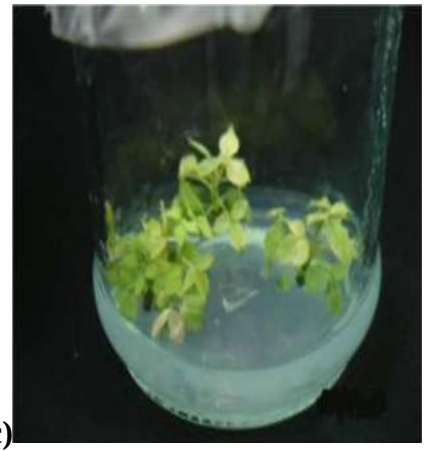
a)  b)  c) 

Plate 3: Longest shoot of *Rubus apatelus* at day 14 in 2.0mg/l BAP and 0.5mg/l NAA (a) and day 42 in 2.5mg/l BAP and 0.5mg/l NAA(c) respectively *in vitro*

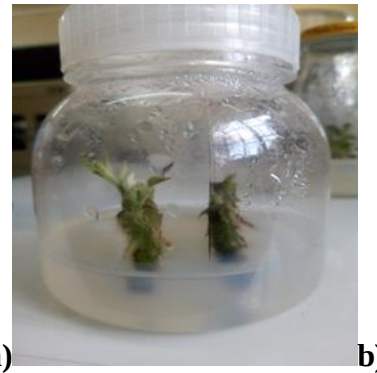
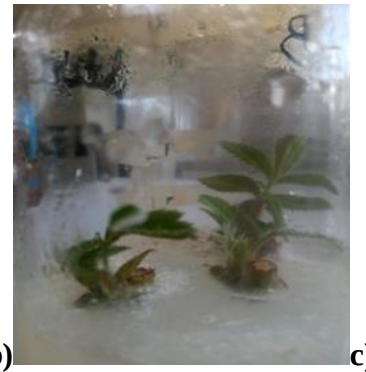
a)  b)  c) 

Plate 4: Longest shoot of *Rubus apatelus* at day 14 in 2.0mg/l BAP and 0.5mg/l NAA (a) and day 42 in 2.5mg/l BAP and 0.5mg/l NAA(c) respectively *in vitro*

(a) **(b)** **(c)** **(d)**

Plate 5: Multiplication for *Rubus fruticosus*, from callus using 2.5 mg/l + 0.5 mg/l BAP. (b)**,** (c) and (d) represents replication 1**,** 2 and 3 of 2.5 mg/l + 0.5 mg/l BAP

The maximum shoot length for *Rubus fruiticosus* (4.26 cm) (igure 5) was achieved at day 42 with 2.0 gm/l BAP supplemented with 0.5 mg/l NAA. Poor results were observed with 0.5 mg/l BAP alone across all the days the data was collected. The better response of 2.0 mg/l BAP and 0.5 mg/l NAA may be due to the fact that exogenous auxin synergistically interacted with the exogenous cytokinin which implies that at this concentration cytokinin: auxin ratio is appropriate to fulfill the requirements of shoot elongation.

Among the different treatments, 2.5 mg l-1 BAP in combination with 0.5 mg/l NAA showed the best results 4.26 cm than other treatments. There was an increasing trend in the shoot length up to 2.5 mgl-1 BAP with 0.5 mg/l NAA. However, further increase in the BAP concentration showed negative effect on shoot elongation at day 42 for *Rubus fruiticosus*. Different concentrations of cytokinin substantially influence the shoot elongation by their effect on cell division and cell expansion. Reduction in shoot length by high concentration of BAP is likely due to the toxic effects of ethylene, produced at high cytokinin concentration.

Benzyl amino purine (BAP) concentrations caused varied effects on explant multiplication. Maximum number of micro-shoots was recorded on full strength MS, BAP 2.5mg/l with 0.5mg/l NAA. In this study the best multiplication rate (3.333shoot per explant) for blackberry species was obtained using MS medium supplimented with 2.5mg/l BAP + 0.5 mg/l IAA. Bharadwaj *et*

al. (2006) similarly reported that best multiplication rate (6.9 shoots/explant) for miniature rose (*R. chinensis* Jacq. var. Minima) was obtained using MS medium fortified with 4.0 mg/l BAP + 2.0 mg/l Kn and 0.1 mg/l NAA. In nodal cultures of *Lavendula vera*, BAP (4.4 μM and 8.8 μM) augmented the potential of MS medium. In agreement with the present results, Rahman *et al*. (2004) found the maximum number of leaves (3.12 per plantlet) with 5.0 mg/l BAP at 30 DAI. Rabbani *et al*. (1996) obtained also similar results with 5.0 mg/l BAP. Thi *et al*. (2008) also demonstrated that the most suitable concentration for shoot initiation and multiplication was found on MS medium supplemented with 3 mg/l BAP. On the contrary Kim *et al*. (2003); found out that the lower concentrations of BAP (1.0 to 1.5 mg/l) stimulated the bud growth in the six rose cultivars (*R. hybrida* L. cvs. "4th of July", Graham Thomas", "Tournament of Roses", "Sequoia Ruby", "Play boy"), but higher concentrations of BAP (2.0 to 4.0 mg/l) inhibited shoot proliferation. Shoot multiplication increase resulted from increase in BAP concentration.

4.9 Effects of Different Concentrations of IBA on *in vitro* Rooting of *Rubus volkensis*

There were significant effects ($p \leq 0.05$) of different concentrations of IBA on *in vitro* rooting of *Rubus volkensis.* IBA at 3.0 mg/l showed the highest percentage rooting of 4.049. The root number was also highest at 3.0 mg/l IBA which was significantly different from all the treatments used. A 1.2 cm mean length was obtained at 3.0 mg/l which was the longest though not significantly different from 2.0 mg/l IBA concentration. In all the measured variables the lowest response was obtained the medium without plant growth regulators

Table 18: Means of the percentage rooting, root number and root length with different concentration of IBA *in vitro* of *Rubus volkensis*

IBA (mg/l)	Percentage rooting	Root number	Root length (cm)
0.0	2.9^{c}	0.1^{c}	0.1^{d}
0.5	3.1^{bc}	0.2^{c}	0.4^{c}
1.0	3.4^{ab}	0.7^{b}	0.8^{b}
2.0	3.7^{ab}	1.0^{ab}	1.1^{a}
3.0	4.0^{a}	1.3^{a}	1.2^{a}

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

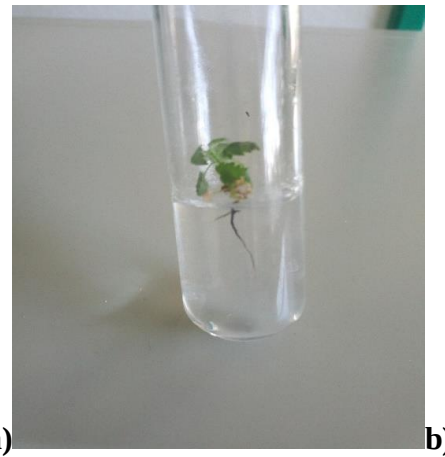
a)  b)  c) 

Plate 6: *In vitro* Rooting of *Rubus volkensis (a), Rubus apatelus(b)* and *Rubus fruiticosus (c)*

4.10 Effects of Different Concentrations of IBA on Rooting Variables of *Rubus Fruiticosus* and *Rubus Apatelus in Vitro*.

There were significant effects ($p \leq 0.05$) of different concentrations of IBA on the on the number of roots, and percentage root formation and root length of *Rubus fruiticosus* and *Rubus apatelus* (Table 17). IBA (3.0 mg/l) recorded the highest value of root length (3.5cm) and root number (1.8) for *Rubus fruiticosus* and highest value of root length (2.7cm) and root number (1.0) for *Rubus apatelus.*

Table 19: Means of the percentage rooting, root number and root length with different concentration of IBA *in vitro* of *Rubus fruiticosus* and *Rubus apatelus*

IBA			
R. fruiticosus	**Percentage rooting**	**Root number**	**Root length (cm)**
0.0	1.6^{b}	0.6^{c}	1.6^{c}
0.5	2.4^{b}	1.0^{bc}	1.7^{c}
1.0	3.4^{a}	1.5^{ab}	2.1^{bc}
2.0	3.5^{a}	1.6^{a}	2.8^{ab}
3.0	3.9^{a}	1.8^{a}	3.5^{a}
R. apatelus			
0.0	1.5^{d}	0.1^{d}	0.5^{d}
0.5	2.3^{c}	0.2^{cd}	0.8^{cd}
1.0	2.4^{bc}	0.4^{bc}	1.3^{c}
2.0	2.8^{b}	0.8^{ab}	2.0^{b}
3.0	3.5^{a}	1.0^{a}	2.7^{a}

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

According to Uddin (2006), root length and number are very important for acclimatization to ex vitro conditions, as well as water and nutrient uptake by plants. The use of auxins for *in vitro* root regeneration seems to intensify the root number by mounting the endogenous contents of enzymes (Asghar *et al*., 2011). According to Liu *et al*., (2002) auxin induces the complicated process of lateral root formation through repetitive cell division. In this study, IBA (3.0 mg/l) yielded good results of root number because it is very effective to increase endogenous auxin contents and show higher stability against catabolism and in activation by conjugation with growth inhibitors (George *et al*., 2008).

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

It was concluded that:

1. A 10 minutes washing of the explant using carbandazim followed by 5% NaOCl then 70% ethanol had least contamionation for *Rubus fruiticosus, Rubus apatelus* and '*Rubus volkensis*' nodiums. Results from this study showed that nodiums from apical sections responds best *in vitro* multiplication of wild blackberry. In this study we determined the appropriate explants size which one for high shoots proliferation.

2. The 2.5mg/l and 2.0 mg/l 2, 4-D produced white green friable for *Rubus fruiticosus* while only 2.5 mg/l 2, 4-D produced white green friable for *Rubus volkensis* which can be used to generate shoots

3. The 2.5 mg/l BAP supplemented with 0.5 mg/l NAA gave the best highest shoot multiplication from the nodal explants of (*Rubus fruiticosus*, *Rubus apatelus* and *Rubus volkensis*).

4. The 3.0 mg/l of IBA supplemented with 0.5 mg/l NAA showed the highest root multiplication rate of (*Rubus fruiticosus, Rubus apatelus and Rubus volkensis*).

5.2 Recommendations

i. The plant growth regular 2, 4-D 2.5mg/l should be used to induce callus for *Rubus fruitcosus* and *Rubus volkensis*.

The use of BAP and IBA can be incorporated in shoot and root induction and multiplication phase respectively of wild blackberry species *in vitro*

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APPENDICES

Appendix 1: Effect of different 2,4-D concentrations with 1.0 mg/l kinetin on the fresh weight of callus of wild blackberry genotype ANOVA

Source of variation	d.f.	s.s.	m.s.	F	*p*-value.
Species	2	1707065.733	853532.867	28.04	<.0001
Treatment	4	146161.644	36540.411	6.58	0.0004
Error	38	210909.822	5550.258	55.65	
Total	44	2064137.200			

Appendix 2: Effect of different concentrations of BAP with 1.0 NAA 0.5mg/l GA_3 on shoot elongation of *Rubus Fruiticous* **at day 14 ANOVA**

Source of variation	d.f.	s.s.	m.s.	F	*p*-value.
Replication	2	0.104	0.052	1.27	<.0001
Treatment	10	3.056	0.306	7.43	0.3032
Error	20	0.82			
Total	32	22.395			

Appendix 3: Effect of different concentrations of BAP with 1.0 NAA 0.5mg/l GA3 on shoot elongation of *Rubus Fruiticous* **at day 28 ANOVA**

Source of variation	d.f.	s.s.	m.s.	F	*p*-value.
Replication	2	0.010	0.005	0.17	0.8448
Treatment	10	21.815	2.182	76.50	<.0001
Error	20	0.570	0.029		
Total	32	22.395			

Appendix 4: Effect of different concentrations of BAP with 1.0mg/l NAA and 0.5mg/l GA3 on shoot elongation of *Rubus Fruiticous* at day 21 ANOVA

Source of variation	d.f.	s.s.	m.s.	F	*p*-value.
Replication	2	0.061	0.030	1.15	0.3361
Treatment	10	6.552	0.655	24.91	<.0001
Error	20	0.526	0.026		
Total	32	7.139			

Appendix 5: Effect of different concentrations of BAP with 1.0mg/l NAA 0.5mg/l and GA3 on shoot elongation of *Rubus Fruiticous* at day 28 ANOVA

Source of variation	d.f.	s.s.	m.s.	F	*p*-value.
Replication	2	0.010	0.005	0.17	0.8448
Treatment	10	21.815	2.182	76.50	<.0001
Error	20	0.570	0.029		
Total	32	22.395			

Appendix 6: Effects of different sterilization procedures on survival rates of wild blackberry species *in vitro* ANOVA

SOURCE	D.F	S.S	M.S.S	F	Pr>F
Replication	2	6.691	3.346	2.16	0.1259
Species	2	249.284	124.642	80.37	<.0001
Treatment	8	157.877	19.735	12.73	<.0001
Species*treatment	16	190.272	11.892	7.67	<.0001
Error	52	80.642	1.551		
Total	80	684.765			

Appendix 7: Effects of different sterilization procedures on percentage contamination of wild blackberry species *in vitro* ANOVA

SOURCE	D.F	S.S	M.S.S	F	Pr<F
Replication	2	143.673	71.836	0.22	0.8050
Species	2	3375.154	1687.577	5.12	0.0094
Treatment	8	73651.080	9206.385	27.91	<.0001
Species*treatment	16	24980.401	1561.275	4.73	<.0001
Error	52	17152.161	329.849		
Total	80	119302.469			

Appendix 8: Effects of the size of explant on the percentage survival of *Rubus apatelus* *in vitro* ANOVA

SOURCE	D.F	S.S	M.S.S	F	Pr<F
Replication	2	622.222	311.111	7.00	0.0494
Treatment	2	12355.556	6177.778	139.00	0.0002
Error	4	177.778	44.444		
Total	8	13155.556			

Appendix 9: Effects of explant source on percentage response of wild blackberry species

SOURCE	D.F	S.S	M.S.S	F	Pr<F
Replication	2	11.574	5.787	0.13	0.8799
Species	2	10983.796	5491.898	122.45	<.0001
Treatment	2	13692.130	6846.068	152.65	<.0001
Species*treatment	4	3668.981	917.245	20.45	<.0001
Error	16	717.593	44.850		
Total	26	29074.074			

Appendix 10: Effects of blackberry species on percentage bud break of nodal explants of wild blackberry on hormone free full strength Ms medium *in vitro* ANOVA

SOURCE	D.F	S.S	M.S.S	F	Pr<F
Replication	2	61.790	30.895	0.14	0.8711
Species	2	3027.384	1513.692	7.00	0.0494
Error	4	864.840	216.210		
Total	8	3954.014			

Appendix 11: Effect of different concentrations of BAP with 1.0mg/l NAA 0.5mg/l and GA3 on shoot elongation of *Rubus volkensis* **at day 14 ANOVA**

SOURCE	D.F	S.S	M.S.S	F	Pr<F
Treatment	10	1.836	0.186	14.19	<.0001
Replication	2	0.008	0.004	0.30	0.7409
Error	20	0.259	0.013		
Total	32	2.102			

Appendix 12: Effect of different concentrations of BAP with 1.0mg/l NAA 0.5mg/l and GA3 on shoot elongation of *Rubus volkensis* **at day 28 ANOVA**

SOURCE	D.F	S.S	M.S.S	F	Pr<F
Treatment	10	15.207	1.521	65.26	<.0001
Replication	2	0.041	0.020	0.87	0.4337
Error	20	0.466	0.023		
Total	32	15.713			

Appendix 13: Effects of BAP and NAA on shoot multiplication of *Rubus fruiticosus* ***in vitro***

SOURCE	D.F	S.S	M.S.S	F	Pr>F
Treatment	10	157.877	3.789	6.35	0.0002
Rep	2	0.727	0.364	0.61	0.5536
Error	20	11.939	0.597		
Total	32	50.545			

Appendix 14: Effect of different concentrations of BAP with 1.0mg/l NAA 0.5mg/l and GA_3 on number of leaves of *Rubus Fruiticous* **ANOVA**

SOURCE	D.F	S.S	M.S.S	F	Pr>F
Treatment	10	286.848	28.685	5.46	0.0006
Rep	2	36.182	18.091	3.44	0.0520
Error	20	105.152	5.258		
Total	32	428.182			

Appendix 15: Effect of different concentrations of BAP with 1.0mg/l NAA 0.5mg/l and GA_3 on number of leaves of *Rubus apatelus* **ANOVA**

SOURCE	D.F	S.S	M.S.S	F	Pr>F
Treatment	10	287.636	28.764	13.29	<.0001
Rep	2	4.061	2.030	0.94	0.4078
Error	20	43.272	2.64		
Total	32	334.970			

Appendix 16: Effect of different concentrations of BAP with 1.0mg/l NAA 0.5mg/l and GA_3 on number of leaves of *Rubus volkensis* **ANOVA**

SOURCE	D.F	S.S	M.S.S	F	Pr>F
Treatment	10	199.636	19.964	7.32	<.0001
Rep	2	0.788	0.394	0.14	0.8664
Error	20	54.545	2.727		
Total	32	254.970			

Appendix 17: Effect of different concentrations of BAP with 1.0mg/l NAA 0.5mg/l and GA_3 on fresh weight of *Rubus fruiticosus* **ANOVA**

SOURCE	D.F	S.S	M.S.S	F	Pr>F
Treatment	10	6.249	0.625	15.34	<.0001
Rep	2	0.006	0.003	0.07	0.8664
Error	20	0.815	0.041		
Total	32	7.069			

Appendix 18: Effect of different concentrations of BAP with 1.0mg/l NAA 0.5mg/l and GA_3 on fresh weight of *Rubus apatelus* **ANOVA**

SOURCE	D.F	S.S	M.S.S	F	Pr>F
Treatment	10	5.989	0.599	21.99	<.0001
Rep	2	0.135	0.680	2.48	0.1091
Error	20	0.545	0.027		
Total	32	6.670			

Appendix 19: Effect of different concentrations of BAP with 1.0mg/l NAA 0.5mg/l and GA_3 on fresh weight of *Rubus volkensis* **ANOVA**

SOURCE	D.F	S.S	M.S.S	F	Pr>F
Treatment	10	6.076	0.608	27.58	<.0001
Rep	2	0.461	0.023	1.05	0.3700
Error	20	0.441	0.022		
Total	32	6.562			

Appendix 20: Effects of different concentrations of IBA on the number of roots *Rubus volkensis* ***in vitro***

Source	D.F	S.S	M.S	F	Pr > F
Treatment	4	2.913	0.728	21.31	<.0001
Error	10	0.342	0.034		
Total	14	3.255			

Appendix 21: Effects of different concentrations of IBA on the number of root length *Rubus volkensis* ***in vitro***

Source	D.F	S.S	M.S	F	Pr > F
Treatment	4	11.403	2.851	23.89	<.0001
Error	10	1.193	0.119		
Total	14	12.596			

Appendix 22: Effects of different concentrations of IBA on percentage of roots *Rubus volkensis* ***in vitro***

Source	D.F	S.S	M.S	F	Pr > F
Treatment	4	2.149	0.537	13.32	<.0005
Error	10	0.403	0.040		
Total	14	2.553			

Appendix 23: Effects of different concentrations of IBA on number of roots *Rubus volkensis* ***in vitro***

Source	D.F	S.S	M.S	F	Pr > F
Treatment	4	2.913	0.728	21.31	<.0001
Error	10	0.342	0.034		
Total	14	3.255			

Appendix 24: Effects of different concentrations of IBA on root length of roots *Rubus volkensis* ***in vitro***

Source	D.F	S.S	M.S	F	Pr > F
Treatment	4	11.403	2.851	23.89	<.0001
Error	10	1.193	0.119		
Total	14	12.596			

Appendix 25: Effects of different concentrations of IBA on percentage of roots *Rubus fruitcosus* ***in vitro***

Source	D.F	S.S	M.S	F	Pr > F
Treatment	4	10.506	2.626	32.44	<.0001
Error	10	0.900	0.081		
Total	14	11.316			

Appendix 26: Effects of different concentrations of IBA on number of roots *Rubus fruitcosus* ***in vitro***

Source	D.F	S.S	M.S	F	Pr > F
Treatment	4	2.947	0.737	19.55	0.0001
Error	10	0.377	0.037		
Total	14	3.324			

Appendix 27: Effects of different concentrations of IBA on length of roots *Rubus fruitcosus* ***in vitro***

Source	D.F	S.S	M.S	F	Pr > F
Treatment	4	12.329	3.082	20.55	<.0001
Error	10	1.500	0.150		
Total	14	13.829			

Appendix 28: Effects of different concentrations of IBA on percentage of roots *Rubus apatelus* ***in vitro***

Source	D.F	S.S	M.S	F	Pr > F
Treatment	4	6.344	1.586	52.64	<.0001
Error	10	0.031	0.030		
Total	14	6.645			

Appendix 29: Effects of different concentrations of IBA on number of roots *Rubus apatelus* ***in vitro***

Source	D.F	S.S	M.S	F	Pr > F
Treatment	4	1.964	0.491	26.40	<.0001
Error	10	0.186	0.019		
Total	14	2.149			

Appendix 30: Effects of different concentrations of IBA on length of roots *Rubus apatelus* ***in vitro***

Source	D.F	S.S	M.S	F	Pr > F
Treatment	4	9.284	2.321	47.05	<.0001
Error	10	0.493	0.049		
Total	14	9.777			

Appendix 31: Effect of different 2, 4-D concentrations with 1.0 mg/l kinetin on the fresh weight of callus of wild blackberry genotype ANOVA

Source of variation	d.f.	s.s.	m.s.	F	*p*-value.
Species	2	1707065.733	853532.867	28.04	<.0001
Treatment	4	146161.644	36540.411	6.58	0.0004
Error	38	210909.822	5550.258	55.65	
Total	44	2064137.200			

Appendix 32: Publication Abstract

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IN VITRO RESPONSES OF WILD BLACKBERRIES (Rubus spp.) TO STERILIZATION PROTOCOLS, DIFFERENT PARTS OF PLANT and SIZES OF EXPLANT USED IN MICROPATION IN KENYA

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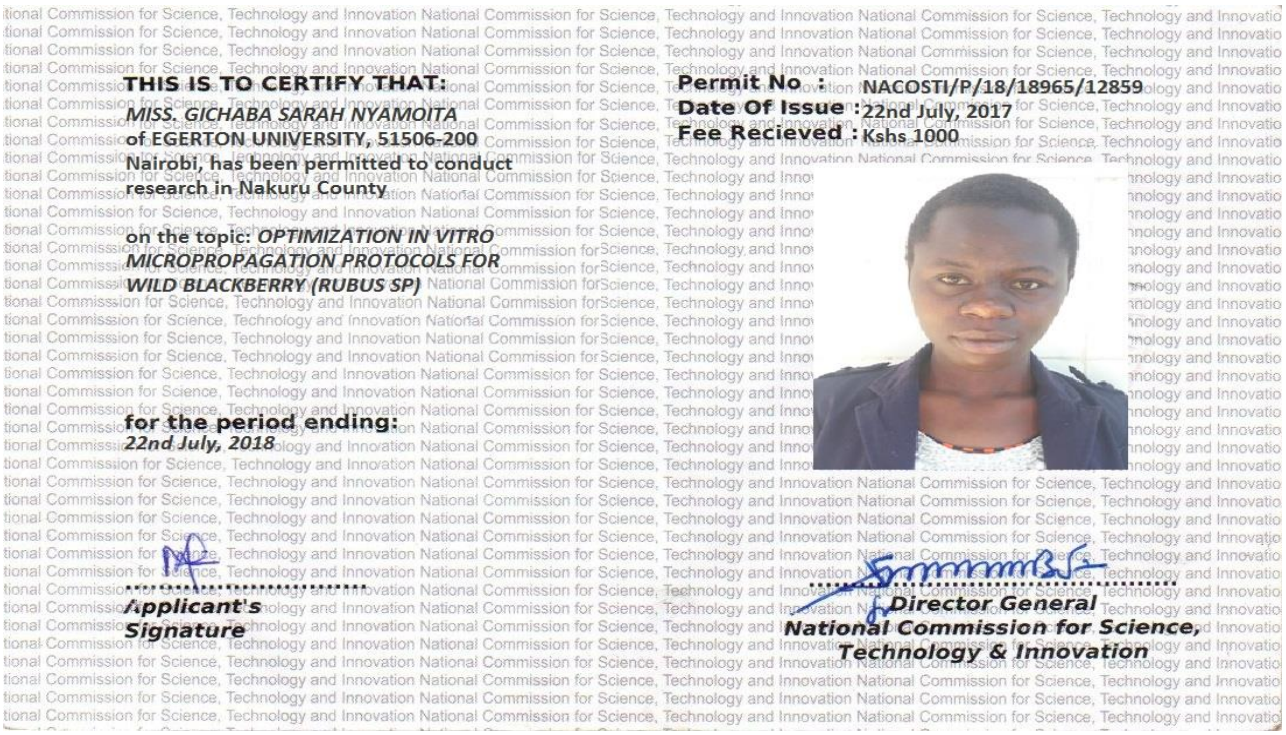
Abstract

Blackberry is a crop of great economic potential due to its health benefits to humans. Despite the economic importance, it has not been exploited in Kenya. Eighty four species have been identified in the country but there is less information on propagation about the crop. With enough research blackberry can be adopted for commercialization. To ease Commercialization a quick propagation procedure must be developed. *Rubus fruiticous, Rubus apatelus* and *Rubus volkensis* were tested on sterilization techniques, different parts of plant for source of explants and size of explant for highest survival rates *in vitro*. Complete Randomized Design composed of three replications per treatment was used. Data were subjected to analysis of variance (ANOVA) using the general linear model procedure of the statistical analysis system (SAS) program (SAS institute I.nc, 2007). Significant means were separated using Tukey's honestly significant deference (Tukey's HSD) test ($P \leq 0.05$). For sterilization experiments, a wash with tap water, Caberndezim for 10min, 5% NaOCl for 2 min and 70% ethanol for 2 min gave the least contamination of 11.11% and the highest survival rate of explants per vessel of 5.111 explants per culture vessel at 5% level of significance. Nodal sections from apical sections had the highest survival rates of explants per vessel with *Rubus fruiticosus* giving 100% survival. Between the wild species *Rubus volkensis* responded better while *Rubus apatelus* had 0.000 explants per vessel. Different explant sizes for the species *apatelus* had significant effects on survival rates in which 2.1-3.0 cm had 93.333. In conclusion, plants species respond differently to sterilization procedures. The use of carbandazim, 5% NaOCl followed 70% ethanol can be used for surface sterilization of the wild blackberry species tested for in *in vitro* culturing. Moreover, more studies on sterilization protocol, suitable source of explants and sizes best for micropropagation should be done for the over 80 different wild blackberry species in Kenya.

Appendix 33: Research Permit



THIS IS TO CERTIFY THAT:
MISS. GICHABA SARAH NYAMOITA
of EGERTON UNIVERSITY, 51506-200
Nairobi, has been permitted to conduct
research in Nakuru County

on the topic: *OPTIMIZATION IN VITRO MICROPROPAGATION PROTOCOLS FOR WILD BLACKBERRY (RUBUS SP)*

for the period ending:
22nd July, 2018

.....
Applicant's Signature

Permit No : NACOSTI/P/18/18965/12859
Date Of Issue : 22nd July, 2017
Fee Recieved : Kshs 1000

.....
Director General
National Commission for Science, Technology & Innovation