REGENERATION AND GENETIC DIVERSITY OF FRANKINCENSE TREE (*BOSWELLIA SACRA* Flueck.) ACCESSIONS FROM SOMALILAND

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

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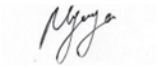

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ABSTRACT

Boswellia sacra is a frankincense-producing tree found in the Arabian Peninsula that includes Oman and southern Somaliland (Northern Somalia). In Somaliland, it is mainly distributed in the Sanaag region including Cel Afweyn where many families depend on its value chain for a living. There is a high global demand for frankincense gums and resin products due to its medicinal, cultural and cosmetic importance leading to overexploitation of existing stands, leading to failure of trees to regenerate attributed to excessive tapping on tree barks causing damage that increases pest and disease infestation. The establishment of the species is hindered by the poor seed germination rate as well as grazing of young seedlings by both wildlife and livestock. This study sought to address the decrease in *B. sacra* numbers through regeneration via tissue culture and avail information on genetic diversity of two *B. sacra* populations in Af yare Dawl-dawl and Exdad in Somaliland for conservation and germplasm enhancement. Seed viability was determined by germination rate and tetrazolium tests, which revealed 4.15 % germination rate and 3.3% viability. These values were low but comparable to previously reported percentages of less than 10%, due to lack of viable embryos attributed to self-incompatibility within the species and high tapping frequency. Clonal propagation using tissue culture applied leaf and axillary bud explants for direct and indirect *in-vitro* regeneration using plant growth regulators, such as TDZ, BAP, NAA, in efforts to develop a micropropagation protocol for mass propagation. Direct *in-vitro* regeneration did not produce any regenerants while indirect regeneration produced callus on MS media containing 5 µm TDZ. Somatic embryogenesis was initiated in MS media containing 1 µm BAP+ 0.25 µm IAA, where shoot regenerants were produced. Genetic diversity determined using morphological and molecular markers to establish diversity within and between germplasm to enhance breeding programs. Morphological characterization of *B. sacra* genotypes from Exdad and Af Yare Dawl-dawl had no significant ($p>0.05$) differences in height, height to

branching, number of stems and tree bottom swelling, however, stem type was strongly correlated to height. Hierarchical cluster analysis of principle components extracted from morphological data revealed two main clusters each with two sub-clusters with genotypes from both regions distributed within these clusters, thereby showing high similarity. Molecular characterization was done on Af Yare Dawl-dawl genotypes using SRAP markers and they revealed very low heterozygosity, H_e=0.053, indicative of very low genetic diversity. PCoA analysis of pairwise genetic distance matrix led to formation of three major clusters with 35.14%, 11.42% and 9.08% variation, indicating existence of three major groups of genotypes with different parentage. This variation indicates that genotypes from the different clusters can be used as progenitors for hybridization and domestication purposes. Low genetic diversity observed calls for urgent conservation measures of the tree species as well as introductions from other areas to enhance the germplasm found in Af Yare Dawl-dawl. Breeding programs for enriching genetic diversity in the study area should be initiated since the current population is faced with the threat of extinction should a sudden environmental change occur. Sustainable frankincense harvesting practices should be enforced to ensure sustainable exploitation of this important resource.

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LIST OF ABBREVIATIONS

2,4-D - 2,4 - Dichlorophenoxyacetic acid

AFLP - Amplified Fragment Length Polymorphism

BAP - 6-Benzylaminopurine

BSA -Bovine Serum Albumin

CRD - Completely Randomized Design

DNA - Deoxyribonucleic Acid

dNTPs – Deoxyribonucleic triphosphates

IAA – Indole Acetic Acid

IBA – Indole-3-butyric Acid

IB – Isolation buffer

ITS – Internal transcribed spacer

ISSRs - Inter-Simple Sequence Repeats

IUCN – International Union for Conservation o Nature

MAS – Marker assisted selection

MS - Murashige and Skoog

NAA- 1-Naphthaleneacetic acid

RAPD- Random Amplified Polymorphic DNA

SSRs- Simple Sequence Repeats

TDZ- Thidiazuron

SRAP - Sequence-related Amplified Polymorphism

mTR- Meta-Topolin Riboside

MemTR - 6-(3-methoxybenzylamino) purine-9-riboside (meta-methoxytopolin)

PCR - Polymerase chain reaction

PCoA- Principal Co-ordinate Analysis

PCA- Principal Component Analysis

PEG – Polyethylene glycerol

PGR – Plant growth regulator

TAE – Tris-Acetate-EDTA Buffer

TBE -Tris-Borate-EDTA Buffer

TDZ- Thidiazuron

WPM – Woody plant media

CHAPTER ONE: INTRODUCTION

1.1 Background information

Increasing demand for plants and plant products such as food, fuel, medicines, fibre, construction materials and industrial raw materials is undermining the available supplies all over the world (Giri et al., 2004; Ayensu, 1983). The changing climatic patterns and globalization of trade have made things worse for the natural plant products with high demand (Thuiller et al., 2005). Traditional plant propagation and germplasm conservation methods are playing a key role in trying to maintain a balance between demand and supply but have not been entirely successful (Engelmann and Engels, 2002). It is clear that radical measures are required if the increasing demand for plants products is to be met sustainably (Rao, 2004). One of these plants whose utilization has led to rapid decrease in its population is *Boswellia sacra* (Groenendijk et al., 2012).

Boswellia sacra is one of the olibanum producing trees in Somaliland (Farah, 1994). Olibanum or frankincense refers to dried resin exudates obtained from *Boswellia* species (Ben-Yehoshua et al., 2012). These species include *B. sacra, B. frerreana* Birdwood, *B. serrata* Roxb., *B. papyrifera* Hoscht, *B. thurifera* Roxb., *B. rivae* Engl. and *B. neglecta* S. Moore (Tisserand and Young, 2014). The frankincense is obtained through making incisions on the barks of these trees, a practice commonly known as 'tapping' (Farah, 1994). In Somaliland, two species, *Boswellia sacra* Flueck. and *Boswellia frerreana* Birdwood are used for frankincense production. Frankincense from *Boswellia sacra* is known locally as *beeyo* while that from *B. frerreana* Birdwood is known as *meydi* (Farah, 1994). Frankincense obtained from *B. sacra* is of high quality due to its high concentration of boswellic and earns a premium price (Farah, 1994). The olibanum is used in its unprocessed form for making perfumes, performing sacred religious rituals and making traditional medicines (Coppen, 1995). Processed frankincense, however, has found numerous uses throughout

the world which include manufacture of cosmetics and perfumes (Crow, 2006). Recent advances in medical research have shown that boswellic acid, a component of frankincense oil has anti-inflammatory and anti-carcinogenic properties (Suhail et al., 2011).

The numerous uses of frankincense in pharmaceutical, perfumery and cosmetic industries have created a rise in demand for frankincense (Crow, 2006). This, in turn, has translated frankincense trade in Somaliland becoming a major economic activity earning the second position after livestock as the region's leading exports (Muhumed, 2012.). Various activities from frankincense value chain, harvesting to processing, have created employment opportunities to a large number of communities in Somalia (Farah, 1994). *B. sacra* trees grow wildly in vast regions of Northern Somalia, which are owned by clans and controlled by clan elders in terms of frankincense production and management (Farah, 1994). Harvesting is normally done by men while sorting out of the gum into the various grades for marketing based on size, purity and colour is done by women (Crow, 2006).

Frankincense production in Somaliland has faced several challenges since the fall of the government in 1991, where the control over plant exploitation and governing policies have been weakening. This has led to overexploitation of the trees, thereby increasing their mortality rate (UNEP, 2005). The frankincense trade is currently unregulated, leaving harvesters at the mercy of exporters and brokers (Crow, 2006). Lack of local processing facilities has locked out local communities from the benefits of value addition (ICPALD, 2011). The increasing demand amid low productivity levels calls for interventions that will seek to support protection of the existing trees while seeking to develop appropriate propagation mechanisms if sustainability of the resource is to be assured. This forms the basis for the present study, to seek to employ propagation technologies and characterisation of the available species to inform conservation efforts.

1.2 Statement of the problem

High demand for frankincense globally has led to over-exploitation of frankincense trees through over-tapping of already declining tree populations. The present practice has been creating too many incisions in one plant which makes the trees fail to regenerate and eventually die. Too many wounds have also exposed the trees to infections and pests, which affect their normal physiology. These factors have led to the decline in population of frankincense trees (Groenendijk et al., 2012). As a result, *Boswellia sacra* is now considered a threatened species in Somalia (IUCN, 2021).

Another key factor undermining the population of *Boswellia* forests is the low seed germination rate. This coupled with the harsh environmental conditions where the trees grow and feeding of young seedlings by animals reduces the likelihood of success in propagation from seed under both natural and modified environments (Raffaelli et al., 2003, Swartout and Solowey, 2018).

Information on genetic variability within *B. sacra* populations in Somaliland is also missing, which could provide information in support to the existing population conservation and propagation. Therefore, the morphological differences within these populations cannot be attributed accurately to environmental or genetic factors (Thulin and Warfa, 1987). Further, this information gap also greatly affects the choice of genotypes to be used as progenitors in conservation and domestication efforts as well as establishment of breeding programs for the species.

1.3 Justification

Most of the communities in Somaliland, over 70%, earn their livelihood along the frankincense value chains (Farah 1994), making the trees very crucial to the economy with an annual export value estimated at $7.3 million. *Boswellia sacra* is also considered sacred by the Somali people and is an important part of their culture, However, its overexploitation has threatened the tree population and therefore much effort is needed to address the propagation challenges if sustainable

production is to be achieved. Efforts to propagate the tree through seed have led to frustrations from failures due to the poor germination rates of less than 10% (Swartout and Solowey, 2018). Mass propagation through cuttings has also been attempted but poor rooting and high mortality rates have been reported (Raffaelli et al., 2008). Therefore, there is a need to investigate the possibility of mass propagation of *B. sacra* through tissue culture technology.

It is also very crucial that the genetic variability within *Boswellia sacra* populations from different regions in Somaliland should be determined to support ongoing conservation and propagation efforts. Frankincense from *B. sacra* populations from different regions shows visual differences in colour and size (Svoboda et al. 2001). This variation has been attributed to environment, tree age, post-harvest storage or annual rainfall (Svoboda et al. 2001; Crow, 2006). Many morphological differences between *B. sacra* trees have been described (Thulin and Warfa, 1987) but the effect of genetic variation between the populations has not been investigated, hence the importance of this study. Previous studies in Oman used ISSR and ITS markers on *B. sacra* (Coppi et al., 2010), but this has not been done in Somaliland. There is no information available regarding use of SRAP markers on *B. sacra* population studies, in which this study seeks to explore.

Development of an efficient micropropagation protocol and the determination of the genetic diversity between and within these populations will greatly aid conservation efforts. This in turn safeguard the income of communities dependent on this important resource as well as earning the country revenue through foreign exchange.

1.4 Objectives

1.4.1 General Objective

To determine seed viability, genetic diversity and micropropagation of *B. sacra* populations to support conservation and mass propagation efforts in Somaliland

1.4.2 Specific objectives

1. To determine seed viability and explant micro-propagation using different plant growth regulators (PGRs) to enhance propagation in genetic improvement of *B. sacra.*
2. To assess genetic diversity using morphological and molecular markers of select *B. sacra* genotypes from Af Yare Dawl-dawl and Exdad regions of Somaliland to enhance breeding programs.

1.5 Hypothesis

1. There is no effect of plant growth regulators on direct organogenesis or somatic embryogenesis on *B. sacra* leaf and axillary bud explants.
2. There is no genetic diversity between and within populations of *B. sacra* from Exdad and Af Yare Dawl-dawl regions of Somaliland that can be revealed by morphological and molecular markers

CHAPTER TWO: LITERATURE REVIEW

2.1 Distribution of *Boswellia sacra*

Boswellia together with other 16 genera belong to the family *Burseraceace.* The genus *Boswellia* consists of about 20 species geographically distributed from West Africa to the Middle East with other species located in Eastern Africa and North of Madagascar (Weeks et al., 2005; Tadesse et al., 2007). The genus is characterized by tree and shrub species having papery outer bark, a greenish inner bark, watery aromatic resins and wood with milky latex (Hussain et al., 2013). Species belonging to this genus are generally found in lowlands with altitudes of 950- 1800m, a temperature range between 20- 25^0 C and annual rainfall of less than 950mm (Tadesse et al., 2007). Several of these species are used in various parts of the world for frankincense production. In Ethiopia, *B. papyrifera* and *B. rivae* are used for frankincense production while in India, *B. serrata* and *B. ovalifoliolata* are the primary frankincense producing species. In the northern parts of Kenya, *B. neglecta* is used for frankincense production (Thulin, 2020).

Boswellia sacra is native to the Arabian Peninsula, which includes Yemen, Oman, Somaliland and Northern Somalia (Thulin and Warfa, 1987). In Somaliland, most *Boswellia sacra* forests are found in the Sanaag region, particularly in the Cal Madow mountains and El Afweyn in the southern part of the country (DeCarlo and Ali, 2014). This region is characterized by an annual rainfall of 750- 850mm and an altitude of 700- 800m above sea level.

2.2 Biology of *Boswellia sacra*

Boswellia sacra grows up to 8m in height usually with many stems although trees with a single stem have been observed. The leaves are pinnately clustered at the tips of the branches. Flowers are born at the tips of the twigs appearing as loosely grouped, slender spikes that develop into a capsule that releases one seed after maturity (Thulin and Warfa, 1987). No literature is available

on the reproductive biology and pollination systems, although personal observations by Coppi et al. (2010) revealed that frequent visits of the flowers by insects may promote cross-pollination, self-pollination may also take place due to the bisexual nature of the flowers. The chromosome number of *B. sacra* is 2n=22 (Thulin and Warfa, 1987).

2.3 Importance and utilization of *Boswellia sacra*

Boswellia sacra is primarily used for frankincense production. Frankincense is obtained from the tree by making incisions on the bark. White milky resin is produced and allowed to dry. It is then harvested by scrapping it off the bark and further drying is done (Crow, 2006). The dried resin is then sought into three grades according to colour and size. Grade I, tears, is whitish to clear, grade II is reddish and grade III which is simply dust and sifting, and a mixture of both white and red colours (Svoboda et al., 2000; Badria, 2015) Whitish to clear dried resins with the largest particles are of the highest quality and will fetch the highest price (Svoboda et al., 2001).

Harvesting of frankincense starts after the trees are 5 to 7 years old, have attained a height of 4 to 5 m and a diameter of at least 15 cm (Svoboda, 2001). Harvesting is mainly done during the dry season as rainfall affects the proper drying of the resin tears.

2.3.1 Utilization of frankincense in the ancient world

For centuries, frankincense has had numerous uses particularly in religious rituals and medicinal uses (Van Beek, 1960). This made it very valuable and was worth more than its weight in gold (Badria, 2015). The Arab traders who actively engaged in frankincense trade were the wealthiest men at the time (Van Beek, 1960). In ancient Israel, frankincense was used to make a blend of incense that was considered holy and reserved for Yahweh in the tent of meetings (Bible, Exod. 3:34), placed on the offerings of the first fruits (Bible, Lev. 2: 14-16) and was offered together with the bread of sanctuary to Yahweh every Sabbath (Bible, Lev. 24:7). Queen Sheba is said to

have presented large amounts of frankincense from Yemen to King Solomon in 1950 BC (Badria, 2015). In the first century CE, frankincense was one of the gifts that were presented to Jesus by the three magi (Bible, Matthew 2:11).

In ancient Egypt, frankincense was burned in the morning, at sunrise to please the gods (Sipos et al., 2004). Frankincense was used to treat throat infections, asthma and to stop bleeding (Aboelsoud, 2010). Frankincense was also used in the mummification and embalming of dead bodies for slowing down decomposition and odour (Badria, 2015).

2.3.2 Present Utilization of Frankincense

Frankincense in modern times is not as valuable as it once was. Literature indicates that one kilogram of raw frankincense costs between $2 and $10 in Somaliland, between $25 and $50 when it reaches the Middle East markets and finally 30 ml of frankincense oil which is obtained from less than one kilogram of raw frankincense costs on average over $70 in the West (DeCarlo and Ali, 2014). However, it has as many uses as it had then. Today, unlike ancient times, frankincense is used in two forms namely raw frankincense and frankincense essential oil. Frankincense oil is prepared by distillation of raw frankincense (Svoboda et al., 2001).

Raw frankincense is still used in religious rituals, making perfumes and traditional medicine. Frankincense smoke is believed to keep away evil spirits (Farah, 2008). It is also burned as incense in the Roman Catholic Church, Orthodox church, Buddhist religion as well as in many mosques around the world (Coder, 2011).

Medicinal uses of raw frankincense are also numerous. Somalis use frankincense to treat inflammatory complications, chest congestions, treating wounds and psychic disorders (Farah,

2008). Frankincense oil from *Boswellia sacra* has a balsamic and sweet smell that makes it ideal for use in the manufacture of perfumes and cosmetics (Crow, 2006).

2.4 Propagation of *Boswellia sacra*

Boswellia sacra can be propagated through seeds. The seeds are known to have a very low germination rate (Swartout and Solowey, 2018) and thus are not ideal for mass propagation. Propagation through cuttings is also possible. However, cuttings take long to root and their mortality rate upon transfer to the field is quite high as indicated by Raffaelli et al. (2008). In Somaliland, the existing *B. sacra* populations are not domesticated, and thus the few seedlings that manage to grow are browsed on by both livestock and wildlife further diminishing chances of establishment(A. Y. Farah, 1994). There is very limited information concerning micropropagation of *B. sacra.*

2.5 Tissue culture and its applications

Plant tissue culture refers to the science of establishing and maintaining plant organs such as shoots, roots, flowers and embryos or plant tissues in artificial media under aseptic conditions (Hartmann et al., 1997). It has proven to be a breakthrough in plant propagation as it allows mass propagation of genetically uniform plants (Farahani et al., 2008).

Tissue culture techniques rely on the fact that individual plant cells contain the whole genome of the plant. The plant cells, whether highly mature and differentiated, when grown in a suitable nutrient media can revert to their meristematic state (De Veylder et al., 2007). Therefore, it is possible to regenerate the whole plant from a single cell, or tissue. This, however, is only possible if their cell membranes and nucleus are intact (Bhojwani and Razdan, 1996), although recent

advances have enabled regeneration from protoplast (Gamborg et al., 1974). This is contrary to animal cells which cannot revert to their undifferentiated state (Birnbaum and Alvarado, 2008).

Micro-propagation is the use of minute plant parts for rapid multiplication of the mother plant, where the plant parts are obtained (Hartmann et al., 1997; Singh, 2015). The general process of micro-propagation involves culturing plant parts in nutrient media, inducing shoot formation, inducing root formation and acclimatization of the plantlets followed by planting out in the field (Hartmann et al., 1997). However, a mass of undifferentiated cells called callus can be obtained, followed by somatic embryogenesis or organogenesis to obtain plantlets that can then be acclimatized.

Tissue culture has many applications today. Rapid mass propagation of plants such as forest trees that would otherwise take long to grow via conventional propagation methods such as seed and cuttings is one of the most celebrated applications (Thorpe, 1983). Mass propagation of high-yielding genotypes of asexually propagated important food crops has been another key successful application. Mass clonal propagation of disease-free superior genotypes of bananas has been very successful (Farahani et al., 2008). Mass propagation of potatoes has also been reported (Estrada et al., 1986)

Tissue culture has been a very important tool to breeders in plant improvement. It is now possible to make intra-specific crosses, in a process known as somatic hybridization (Gamborg et al., 1974). Embryos following somatic hybridization are normally aborted but plant hybrids can still be obtained through embryo rescue techniques (Van Tuyl et al., 1991; Sharma et al., 1996). Somaclonal variation is another area of tissue culture that can be utilized by breeders (Larkin and Scowcroft, 1981). It has been observed that in a somaclonal breeding program, new varieties can be obtained. Variation generated from tissue culture depends on explant type, growth regulators

used, genotype and degree of departure from organized growth (Karp, 1995). Anther culture is another important tool to breeders that leads to formation of haploid plants. Developing anthers are isolated from unopened flowers and cultured in nutrient media where the microspores in the anther tissues develop into callus from where the haploid plants can be obtained (Junzhi, 1983; Nitsch and Nitsch, 1969). Haploid plants are very important in a breeding program especially when a large number of homozygous lines are required (Bajaj, 1990).

2.5.1 Choice of explant

The choice of plant material is very crucial if the tissue culture is to be successful (Pierik, 1997). Some of the factors that affect the explant's tissue culture include the genotype, age, size, source and physiological stage of the donor plant (Yildiz, 2012). The genotype of the explant has a major effect on its regeneration capacity. Regeneration capacity varies among families, species and even genotypes within the same species (Yildiz, 2012). Herbaceous species have a higher regeneration capacity compared to woody species (Pierik, 1997)

2.5.2 Growth regulators

The proliferation and general differentiation of cultured plants will depend on the growth regulators, either actively produced by the growing plant in the culture or purposely induced in the growing media (Gamborg et al., 1976). Of the commonly recognized plant growth regulators, which include Auxins, cytokinins, gibberellins, ethylene and abscisic acid. Auxins and cytokinins and their interaction are of the most importance in plant micropropagation (Gamborg et al., 1976).

2.5.2.1 Auxins

The most common auxins used include IAA, IBA and NAA. These are synthetic and are added into plant growth media to control callus formation and root growth. Derivatives of IAA and IBA have been used successfully in callus formation of tobacco culture (Maeda and Thorpe, 1979). Other auxins such as 2,4 D have also been used in callus formation and suspension cultures.

2.5.2.2 Cytokinins

Together with auxins, cytokinins are important in cell division during culture growth. This is because they both affect the cell cycle (Gaspar et al., 1996). A balance between these two plant growth regulators has to be maintained. Some of the commonly available cytokinins include kinetin and BAP. BAP has been used successfully for shoot proliferation in *B. papyrifera* (Abrham, 2011) and *B. ovalifoliolata* (Chandrasekhar et al., 2005).

2.5.2.3 Gibberellins

The main effect of gibberellins in plant cultures include is normal callus growth although retardation of roots and shoot and the opposite has been observed (Lance et al., 1976). Gibberellic acids have been known to reduce meristematic initiation but are essential in development of already formed organs (Gaspar et al., 1996). The knowledge of the optimum combinations of the above plant growth regulators is very crucial in obtaining vigorous cultures.

2.6 Importance of genetic diversity studies

The increase in human population over the past few centuries has led to overutilization of plant resources. Urbanization has also led to a sharp decrease in undisturbed plant ecosystems (McKinney, 2002). Plant genetic resources are rapidly disappearing (Harlan, 1975). This has made

scientists around the world realize the importance of plant genetic diversity (Arora, 1997). It will not only play a role in the conservation of endangered species but also present an opportunity for plant improvement (Ramanatha and Hodgkin, 2002). This is particularly important if the rising global demands for plant products are to be met (Holl, 1975).

Genetic diversity arises as a result of gene flow, mutations or sexual reproduction. Gene flow leads to changes in allelic frequencies when genotypes arrive or leave and interact with the resident genotypes (Frankham et al., 2002). Mutations are recurrent heritable changes from one allelic state to another as a result of large or small alterations in the DNA (Darnell et al., 1990). Sexual reproduction leads to variation as a result of genetic recombination especially during crossing over in gametogenesis and through hybridization (Hartwell et al., 2008).

2.6.1 Assessing genetic variation in plants

Several methods have been used to assess the genetic variation in plants. They can be classified into three categories namely morphological, biochemical and molecular (Weber and Wricke, 1994).

Morphological methods assess the physical observable differences between plants to determine the genetic diversity. Features such as leaf size, pod colour, flower colour, plant height, fruit shape can be used to represent genetic polymorphism and are easily identified and manipulated (Bretting and Widrlechner, 1995). However, they are limited in number and are highly influenced by environment and development stage of the plant (Staub et al. 1996).

Biochemical methods make use of isozymes which are alternate structural forms of an enzyme (Markert and Møller, 1959). Isozymes are analysed by their molecular weight or electrophoresis

(Bretting and Widrlechner, 1995). Differences in the isozymes reflect the products of different alleles rather than genes (Staub et al., 1996).

Molecular methods, also known as DNA markers are fragments of DNA that show variation and they can be used to detect polymorphism between genotypes in a population (Staub et al., 1996). Several types of DNA markers are available and can be used successfully for diversity studies. Such markers include restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLPs), simple sequence repeats (SSRs), single nucleotide polymorphisms (SNPs), inter simple sequence repeats (ISSRs) and random amplified polymorphic DNA (RAPD) (Nadeem et al., 2018).

2.6.1.1 Restriction Fragment Length Polymorphism (RFLP)

This is the earliest genetic marker developed and is based on southern blot. Southern blot is a hybridization technique that allows identification of a particular size of DNA from a mixture (Southern, 1975). Genomic DNA is digested using restriction enzymes and fragments of varying lengths obtained (Staub et al., 1996). These fragments are separated using gel electrophoresis according to their molecular weight. The gel is transferred to a nitrocellulose membrane filter with radiolabelled oligonucleotide sequences that anneal to the fragment sequences, a process referred to as southern blotting (Southern, 1975). The main advantage of RLFP is that the results are highly reproducible and requires no specialized equipment. The probe that is used does not have to be sequenced (Staub et al., 1996). RFLP has several disadvantages. It requires large amounts of high-quality DNA. It is also very difficult to automate. The fact that it uses radioactivity for visualization makes it unsafe. It is very labour-intensive especially if a large population is involved (Saraswathy and Ramalingam, 2011).

2.6.1.2 Random Amplified Polymorphic DNA (RAPD)

This is a Polymerase Chain Reaction based marker system. PCR using 10 nucleotides long arbitrary primer amplifies the total genomic DNA of an individual plant. This primer binds to many different loci and is used to amplify random sequences that are complementary to it from a complete DNA sequence. PCR products are then visualized on agarose gel stained with ethidium bromide (Williams et al., 1990). Advantages of RAPD include high frequency of polymorphism, uses small amounts of DNA, the primer sequence does not have to be known and the process can be automated (Kumar and Gurusubramanian, 2011). The main shortcoming of RAPD is that a random primer is used, which depending on laboratory conditions can bind elsewhere and thus cannot be standard (Staub et al., 1996).

2.6.1.3 Amplified Fragment Length Polymorphism (AFLP)

This is a highly sensitive method based on RAPD and RFLP. The target DNA is cut using two restriction enzymes, a rare cutter and a frequent cutter (Vos et al., 1995). Oligonucleotide "adapters" are ligated to the ends of each fragment, one end with a complementary sequence for the rare cutter and the other with the complementary sequence for the frequent cutter. This way only fragments that have been cut by the frequent cutter and rare cutter will be amplified (Vos et al., 1995). Primers are designed from the known sequence of the adapter, plus 1-3 selective nucleotides which extend into the fragment sequence. Sequences not matching these selective nucleotides in the primer will not be amplified. PCR is carried out and the results visualized in a high resolution gel electrophoresis system (Nadeem et al., 2018; Vos et al., 1995).

AFLP can be conducted using small amounts of DNA. It has a very high genotyping throughput and does not require sequence information of the probes to generate the fingerprint (Vos et al., 1995). However, its limitations include the fact that it requires high-quality DNA for complete restriction digestion and it is very expensive (Nadeem et al., 2018).

2.6.1.4 Simple Sequence Repeats (SSR)

These are DNA sequences of repeats lengths of a few base pairs. They are also known as microsatellites. They are tandem repeats of 1-6 nucleotides that occur throughout the plant's genome. They are highly polymorphic and this polymorphism is easily detected by PCR (Kalia et al., 2011). The development of SSR fingerprints involves the development of an SSR library and then detection of specific microsatellites. After this, the detection of favourable regions for primer designing is done and then PCR is performed (Kalia et al., 2011). Interpretation and evaluation of banding patterns are performed and assessment of PCR products is performed for determination of polymorphism by electrophoresis (Nadeem et al., 2018). SSRs have a high reproducibility capacity, they are abundant, they can be automated and have a high throughput genotyping capacity (Nadeem et al., 2018). However, nucleotide sequence information is required and they require high initial cost (Kalia et al., 2011).

2.6.1.5 Inter Simple Sequence Repeats (ISSR)

DNA segments between two identical microsatellite regions but oriented in opposite directions are amplified. Primers (15-30 bases long) are used thus permitting use of high annealing temperatures ensuring high stringency (Zietkiewicz et al., 1994). The primers used in ISSRs are anchored at the 3′ or 5′ end having 1 to 4 degenerate bases, which are extended into the flanking sequences (Nadeem et al., 2018). Detection is done using polyacrylamide gel electrophoresis in combination

with radioactive probing or agarose gel electrophoresis (Reddy et al., 2002). ISSRs are highly specific and do not require prior knowledge of sequence information of the primers. They are highly polymorphic, although the level of polymorphism depends on the method of visualization used. They are also very quick and simple to use (Reddy et al., 2002). Their limitation is that they are dominant (Nadeem et al., 2018). ISSR analyses of six populations of *B. sacra* Flueck. from three regions of Oman revealed a relatively high variation of 38.1% among populations (Coppi et al., 2010).

2.7 Biotechnology in tree breeding

Tree breeding, unlike crop breeding, poses many challenges to breeders including their large size that require large areas for experimental set up thereby increasing costs. Trees take a long time to reach sexual maturity implying that crosses among selected parents need years to complete. Trees within a species also show reproductive cycle differences creating problems during making crosses further delaying and complicating the production of offspring needed for testing. Delayed expression of desired traits such as wood quality whose evaluation takes decades for reliable assessment further increases the duration (El-Kassaby et al., 2014; Namkoong et al., 1988). Forest trees species cover extensive geographical locations within which they express various levels of adaptation therefore testing should be extensive, covering extended geographical locations to avoid possible maladaptation that could be caused by any mismatch between planting stock and its ecological niche (Namkoong et al., 1988). To overcome these challenges, tree breeding programs utilise biotechnology tools. In most programs, superior genotypes are selected in the natural populations also known as mass selection. These genotypes may consist important traits such as architectural patterns (stem form and branching patterns), internal wood properties (grain

angle and fiber sizes) and resin quality as per the industry requirements. The genotypes selected maybe propagated clonally through tissue culture, tested over different locations and the best clones distributed as a variety (White, 2001). However, in most instances, there is need for genetic improvement and tools such as marker assisted selection (MAS) have been incorporated into tree breeding programs to aid in this.

CHAPTER THREE

IN-VITRO REGENERATION OF *BOSWELLIA SACRA* FLUECK. GENOTYPES FROM AF YARE-DAWL-DAWL AND EXDAD REGIONS, SOMALILAND

Abstract

Boswellia sacra is a frankincense-producing whose frankincense is of high quality and has numerous cultural, medicinal and cosmetic uses. High demand for frankincense has led to unsustainable utilization leading to increased tree mortality rates thereby listed as near threatened. Natural regeneration in their natural habitat is very low owing to their low seed viability and browsing on seedlings by wild and domestic animals. This study investigated seed regeneration and clonal propagation of *B. sacra* via tissue culture using direct and indirect organogenesis and somatic embryogenesis for regeneration to enhance breeding programs and conservation. Due to unavailability of seeds from the trees at Af Yare and Exdad in Somaliland, 60 seeds were used for germination rates, viability and regeneration. Seed viability was determined using tetrazolium and germination tests and preceded by a five-minute buoyancy test in water. For tetrazolium tests, dissected seeds were soaked in 0.1% tetrazolium solution for 15 mins and observed for colour change while germination tests were carried out in propagation trays on 1:1 peat moss: coco peat media. In tissue culture, direct regeneration, leaf and axillary bud explants were cultured in MS media consisting various levels of BAP, TDZ and mTR while for indirect regeneration, leaf explants were used for callus induction and subsequent somatic embryogenesis and shoot regeneration.

Tetrazolium screening revealed 3.3% viability while germination test was 4.15%. No shoots were produced via direct regeneration while indirect regeneration via callus was achieved at 5 µm TDZ. Somatic embryogenesis was achieved on MS media containing 1 µm BAP+ 0.25 µm IAA resulting in 15% shoot regeneration. It is concluded that a 5-minute sink test is sufficient to be used as a

quick method to determine seed viability in the field. No regeneration has been reported for previously *B. sacra*. However, the regeneration obtained here is quite low and more research on somatic embryo conversion as well as elimination of hyperhydricity during shoot development should be done.

3.1 Introduction

Boswellia sacra is a frankincense-producing tree endemic in the arid and semi-arid lands (ASALs) of Somaliland (Farah, 1994). *Boswellia sacra* forests are found in El Afweyn and Sanaag regions, particularly in the Cal Madow mountains, in the southern part of the country near its border with Puntland (DeCarlo and Ali, 2014). *B. sacra* trees grow up to 8 m, with single or multiple stems which have a characteristic peeling papery bark. The species is deciduous, shedding its pinnately clustered leaves during the summer (Thulin and Warfa, 1987; Daly et al., 2010). Flowers normally emerge in summer when the trees have already shed their leaves (Lippi et al., 2011).

The natural regeneration of *B. sacra* is hampered by its biology, among other factors such as browsing of young seedlings by livestock and wild animals (Raffaelli et al., 2003). Germination frequency of the seeds has been reported to be less than 10% (Swartout and Solowey, 2018). Al-Harrasi et al. (2019) describe *B. sacra* as having the lowest seed germination rate in the genus. Propagation through cuttings has not been successful as the cuttings rarely root upon transplant to the field (Raffaelli et al., 2008). However, *in-vitro* propagation attempts on the species have not been reported prior to this study.

Micro-propagation has been celebrated for its high fecundity making it ideal for mass propagation of species that produce few seeds, sterile seeds or no seeds at all (Neumann et al., 2009; George et al., 2008) but this has not been done for *B. sacra*. It is also an important tool in conservation of

endangered species like *B. sacra* (Fay, 1992). Two main methods of micro-propagation are used, namely organogenesis and somatic embryogenesis. Organogenesis involves development of adventitious shoots from explants upon inoculation in appropriate media and culture conditions and it has direct and indirect methods. Direct organogenesis has been reported in many woody species including *B. ovalifoliolata* (Chandrasekha et al., 2005) and *B. serrata* (Purohit et al., 1995). Indirect organogenesis is somatic embryogenesis. Somatic embryogenesis comprises development of embryos from meristematic somatic cells which can then be grown into full plants (Phillips and Garda, 2019; Thorpe, 1983). Two methods of somatic embryogenesis are described, direct and indirect (Gaj, 2004). The difference between the two is that indirect somatic embryogenesis is preceded by a callus phase. Somatic embryogenesis is preferred to direct organogenesis because the latter presents difficulty and time required in rooting of the shoots in some species (Lu and Thorpe, 1987). Since none of the methods has been applied to *B. sacra* regeneration, this study sought to explore both methods of invitro regeneration.

3.2 Materials and methods

3.2.1. Seed collection sites

Boswellia sacra fruits were collected from two valleys: Exdad and Af Yare Dawl-dawl in Somaliland. These regions were the only areas accessible due to the security situation in the region at the time of site visit. The number of fruits collected ranged from 4 to 72 per tree and these were collected from 13 trees in Exdad and 16 from Af Yare Dawl-dawl. The fruits were collected from trees that were at least 10 m apart to avoid sampling bias and were then stored separately in envelopes. The fruits were transported to the University of Nairobi, Upper Kabete campus for seed tests.

3.2.2 Seed extraction

All the collected fruits were dissected using a scalpel along the dehiscence lines to release the seeds. The number of seeds obtained from fruits per tree was noted. Seeds from each tree were then weighed on a bench weighing balance (0.0001g tolerance). The 1000 seed weight for individual trees was then determined using the formula: $\frac{w(g)}{n} x\ 1000$, where *n*= no of seeds and *w*= weight of the seeds. The mean 1000 seed weight from each site was determined. After the 1000 seed determination, the seeds from both sites were bulked.

3.2.3 *Boswellia sacra* seed viability tests

Boswellia sacra fruit capsules were collected from trees in Exdad and Af Yare Dawl-dawl and stored separately for individual trees (Table 3.1). Capsules were collected on trees at least 10 m apart. Most trees in the sampling areas had no fruits. However, 220 capsules from 13 trees in Exdad and 413 from 16 trees in Af Yare Dawl-dawl were collected. Seeds were later extracted (Image 3.1) and weighed using a table weigh balance to determine 1000 seed weight. Seed viability tests were also carried out.

Table 3.1: Fruit capsules of *B. sacra* collected from trees in Exdad and Af Yare Dawl-dawl regions, Somaliland

Exdad		Af Yare Dawl-dawl	
Tree no.	No of capsules	Tree no.	No of capsules
AD 17	6	BD 25	20
AD 30	10	BD 6	4
AD 24	34	BD 7	73
AD 16	9	BD 3	10
AD 27	44	BD 15	11
AD 20	5	BD 21	72
AD 19	8	BD 12	9
AD 21	13	BD 11	41
AD 29	37	BD 13	43
AD 28	4	BD 18	21
AD 2	6	BD 20	9
AD 12	24	BD 23	17
AD 13	20	BD 4	9
		BD 7	33
		BD 24	23
		BD 11	18
Total	220		413

Two types of viability tests were done namely tetrazolium and germination tests according to MacKay (1972) with a total of 60 seeds. Each test was carried out using 30 seeds. Tetrazolium test was conducted by first carrying out a five-minute sink test. Seeds were immersed in water and allowed to settle for 5 minutes. After 5 minutes, floating seeds were collected on a sieve and placed on a petri dish while seeds that sank were placed on another petri dish. All seeds, floating and sinking lots, were dissected longitudinally using a scalpel into two halves and one half discarded. The halves were immersed into 15% Tetrazolium solution separately in petri dishes for 15 minutes. Colour change was observed for some seeds. Seeds that changed from white/cream to pink were considered viable while those that did not were deemed not viable according to França-Neto and Krzyzanowski (2019).

In the germination screening test, 30 seeds were subjected to a 5-minute sink test and then separated into sinking and floating seeds just like for tetrazolium test. The separated seeds were soaked for 24 hours. On the following day, the seeds were sown in propagation trays containing 1:1 cocopeat to peat moss media. Each seed was sown individually into a single tray cell. The trays were placed in a greenhouse, watered daily and germination observed. Germination rate was considered for both sinking and floating seeds.

3.2.4 Micro-propagation of *B. sacra*

3.2.4.1 Source of explants

Seeds were collected before this study from Exdad area in El Afweyn region, Somaliland. The seeds were sown in a cocopeat-peat moss mixture and germinating seedlings potted in soil. The potted *B. sacra* plants were raised in a greenhouse at the field station of the University of Nairobi, Upper Kabete Campus and used as sources of explants.

3.2.4.2 Media preparation

MS media was prepared by dissolving 4.4 g of commercial MS (Duchefa©) media in 800ml of water. Sucrose was added at the rate of 30g/L and volume topped up to 1 L. The pH was adjusted to 5.8 using NaOH or HCL. Where PVP was used, it was added at this stage. Gelrite, 2g/L, was then added. The media was heated until boiling in a microwave oven and dispensed into glass vials before autoclaving, or dispensed into 1L glass bottles and dispensed into sterile single-use petri dishes after autoclaving. Media was sterilized in an autoclave at 121°C for 15 minutes. The efficacy of sterilization was confirmed by the use of autoclaving tape. PGRs were added from pre-prepared stock solutions either before autoclaving or after autoclaving.

3.2.4.3 Explant preparation and sterilization

Axillary buds and young leaves were harvested from mother plants in the greenhouse and taken to the tissue culture laboratory at the University of Nairobi, College of Agriculture and Veterinary Sciences. Sterilization was done by washing the explants in 70% ethanol solution for two minutes and then soaking them in 15% Jik solution with a few drops of tween twenty for 15 minutes. The explants were subjected to three rinses of three minutes each, using distilled sterile water. Explants that appeared burnt or bleached were discarded.

3.2.4.4 Inoculation of *B. sacra* explants in media

Inoculation was done under a sterile laminar flow hood. The leaf explants were inoculated with the adaxial side touching the media. Axillary buds were placed such that the base was embedded into the media.

3.2.4.5 Growth culture conditions

Cultures were incubated in a growth room with temperatures set at 25±2 °C. Lighting was maintained at 16/8-hour cycles of light and darkness. However, for some experiments, total darkness was maintained.

3.2.4.6 Experimental design, data collection and analysis

All experiments were set in a completely randomized design (CRD) with four replicates. The sample size ranged from 40 to 280 for *in-vitro* regeneration and 30 for seed viability tests. Data on morphological response was collected weekly. Data analysis was carried out using GenStat© software 15th edition. Significance was tested at 5% and means separation done using LSD at 5%.

3.3 Results

3.3.1 *Boswellia sacra* seed tests

3.3.1.1 Determination of *B. sacra* 1000 seed weight

After seeds were extracted from the capsules (Image 3.1), the percentage number of capsules with seeds per site was determined. Only 21.6% of the capsules from Af Yare Dawl-dawl and 38.6% from Exdad had seeds (Fig. 3.1). This difference was not significant ($p>0.05$, Appendix 14).

Image 3.1: *Boswellia sacra* fruit capsules. 2. Single capsule with a) dehisced pericarps b) single seed 3. *B. sacra* seeds after extraction from fruit capsules

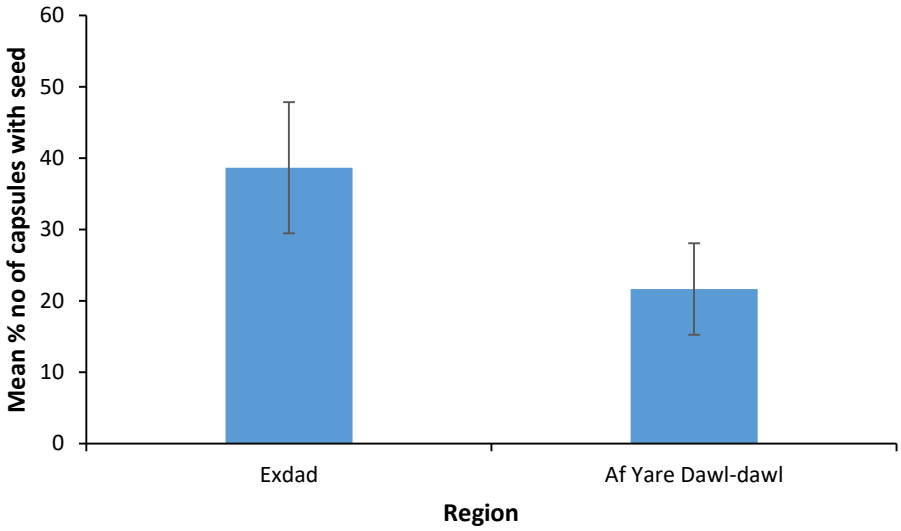


Figure 3.1 Percentage of *B. sacra* capsules with seed collected from Af Yare Dawl-dawl and Exdad.

The mean 1000 seed weight on seeds from Exdad was 2.4 g compared to seeds collected from Af Yare Dawl-dawl at 1.9 g (Fig. 3.2). There were no significant differences ($p>0.05$, Appendix 15)

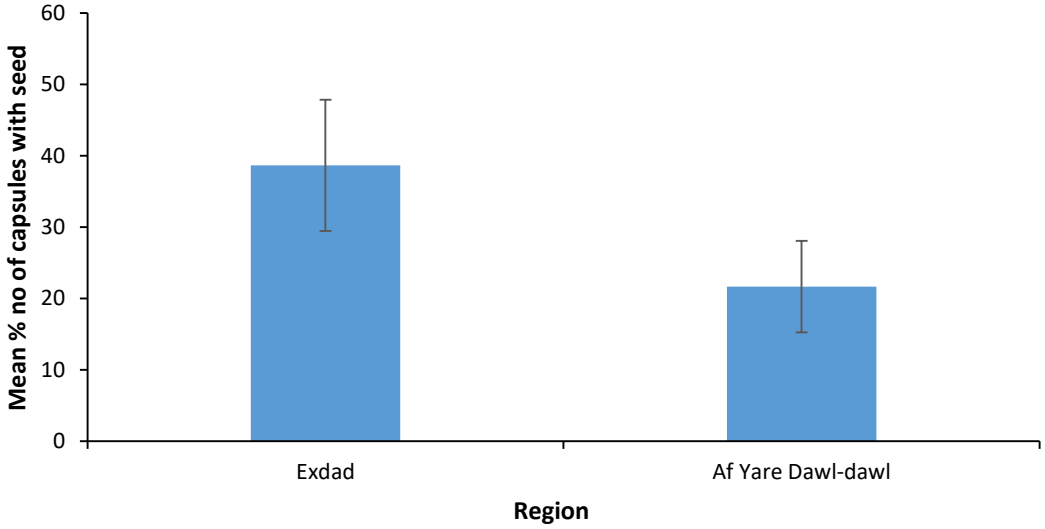


Figure 3.2: 1000 seed weight of seeds collected from Af Yare Dawl-dawl and Exdad

3.3.1.2 Tetrazolium test on *B. sacra* seeds

After a five-minute sink test, 36.36% *B. sacra* seeds sank while 63.64% of seeds floated. Both lots were soaked in 15% tetrazolium solution separately for 15 minutes and observed for colour change. There was a 25% colour change on the seeds that sank. There was no colour change on floating seeds (Image 3.2). The overall viability revealed by the tetrazolium test was 3.3%.

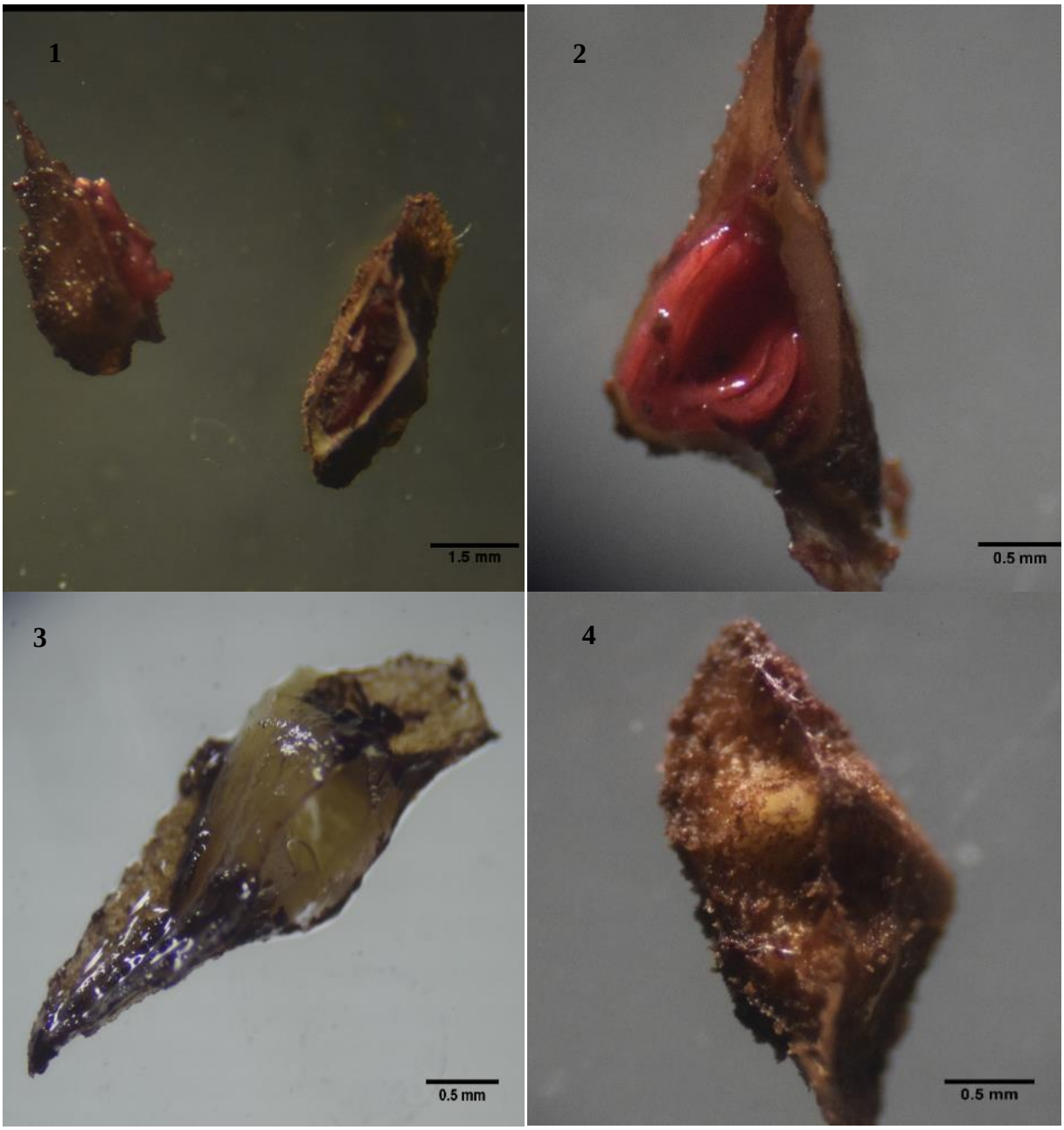


Image 3.2: Dissected *B. sacra* seeds after tetrazolium tests 1, 2) colour change to pink, 3,4) no colour change after tetrazolium test.

3.3.1.3 Germination test on *B. sacra* seeds

Another five-minute sink test was carried out before *B. sacra* seed germination test. Only 16.6% sank after five minutes while 83.4% of the seeds floated. After 12 days on the peat moss coco peat media, 40% of seeds geminated in the sinking lot but none germinated in the floating lot (Image 3.3), with a 4.15% overall germination rate.

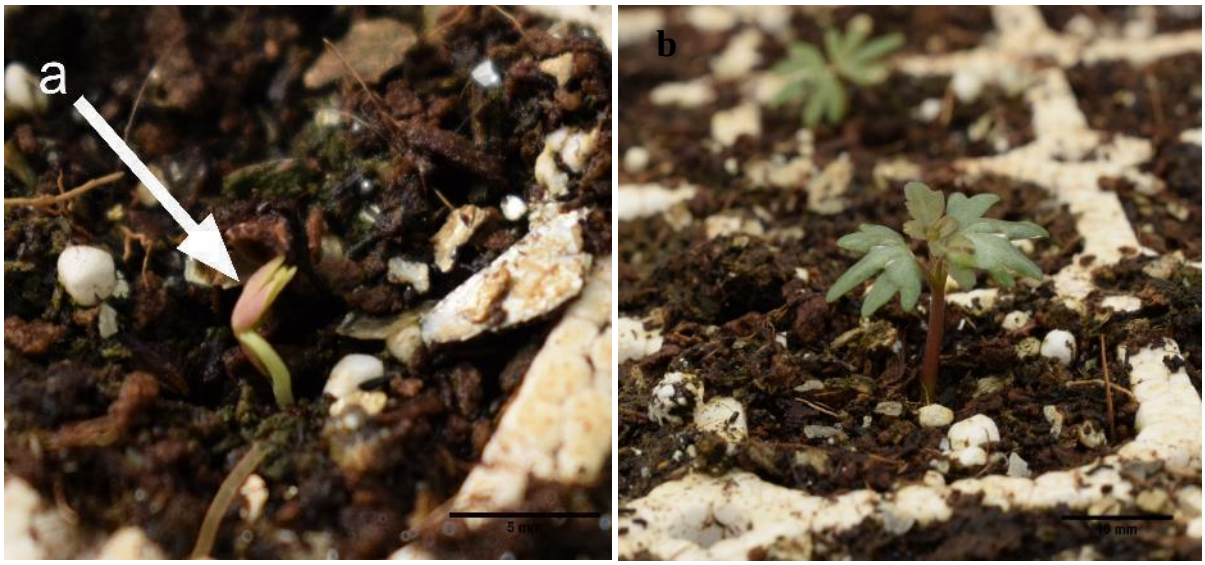


Image 3.3: a) *B. sacra* seed germinating after 12 days b) *B. sacra* seedling, 24 days after sowing

Due to the low germination rates of the seeds, tissue culture using different explants was used to resolve propagation issues.

3.3.2 Effect of different hormones, combinations and explants on shoot production via direct organogenesis

3.3.2.1 Effect of BAP, meta-topolin riboside and thidiazuron on leaf explants

The experiments were established to find out which hormones would lead to the production of regenerants directly via direct organogenesis or indirectly from calli via indirect organogenesis.

a) Effect of 6-Benzylaminopurine on *B. sacra* leaf explants

Leaf explants cultured in MS media consisting 0, 8.9 and 13.3 µm BAP in the quest to produce regenerants via direct organogenesis showed variations in explant survival. In the first week, although there was 100% explant survival for the media consisting 0 and 13.3 µm BAP and 90% for 8.9 µm BAP (Fig. 3.3), there were no significant ($p>0.05$) differences between treatments (Appendix 1). In weeks 2, 3 and 4, a consistent trend was observed, where the media containing 13.3 µm BAP had the highest survival rate, followed by 8.88 µm BAP and finally where no BAP was used. In week two, the explant survival rate was 65%, 80% and 90% for 0, 8.9 µm and 13.3 µm BAP respectively (Fig. 3.3) but there were no significant differences (appendix 3). The observed explant survival rate in weeks 3 and 4 was 55% and 20% for 13.3 µm BAP, 45% and 10% for 8.88 µm and 15% and 5% for 0 µm BAP (Fig. 3.3) but there were no significant differences ($p>0.05$, appendix 3). At the end of the fifth week in culture, all the explants had died. The media had also turned brown (Image 3.4). There was no shoot or callus development for the entire period the explants were on the media.

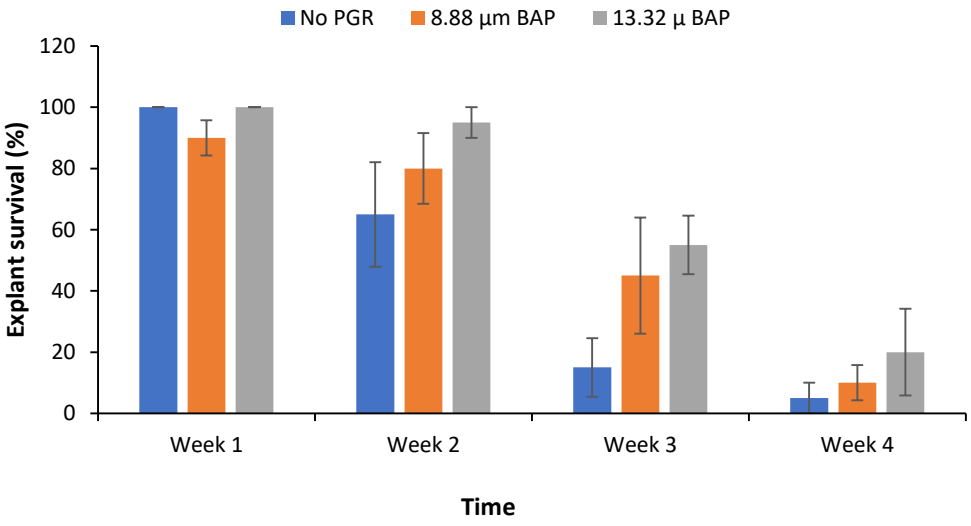


Figure 3.3: Effect of BAP on leaf explant survival for direct shoot organogenesis (n=60).

Image 3.4: Media browning and death of leaf explants after 5 weeks of culture on MS media containing 8.88 µm BAP**.**

b) Effect of Meta-topolin riboside on *B.* sacra leaf explants

Since there were no regenerants on media containing BAP, media consisting mTR (9.1 and 13.7 µm) were used to determine its ability to produce shoots. After one week of culture, there was 100% explant survival in both treatments (Fig. 3.4). There were no significant differences between the treatments ($p>0.05$). There was a decrease in explant survival rate in weeks 2, 3 and 4 with 9.12 µm mTR having the highest explant survival rate all through but no regenerants. In week two, 90% and 65% explant survival rates were recorded in 9.12 µm and 13.68 µm mTR respectively (Fig. 3.4). There were no significant differences between the treatments ($p> 0.05$, Appendix 3). In week 3, explant survival rate observed was 45% and 35% which reduced further to 25% and 20% for 9.1 and 13.7 µm mTR, respectively (Fig. 3.4) but there were no significant differences between the treatments ($p>0.05$, Appendix 3). There was no survival of the explants after 5 weeks in culture, thereby indicating that direct organogenesis was not viable using mTR.

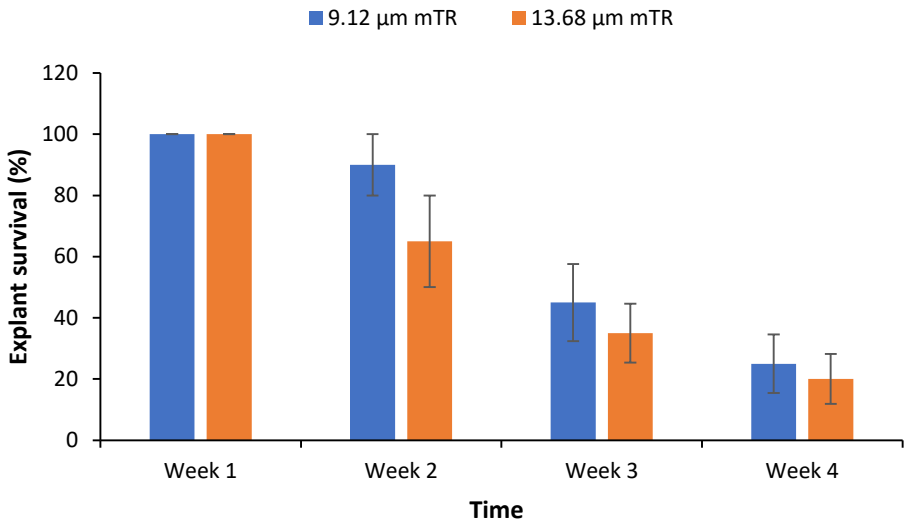


Figure 3.4: Effect of BAP and mTR on explant survival for direct organogenesis (n=40).

c) Effect of Thidiazuron on leaf explants

Since the media containing BAP or mTR did not produce any regenerants or calli, leaf explants were cultured on MS media containing TDZ at 2.3, 4.5 and 6.8 µm. At one week, the explant survival rate was 100% in both 2.3 and 4.5 µm TDZ and 80% in 6.81 µm TDZ but there were no significant differences between the treatments (Fig. 3.5, Appendix 4). In week 2, there were variations in survival of the explants within the range of 60-95% for the three treatments but there were no significant differences ($p>0.05$, Appendix 2). A similar trend of survival was observed for weeks 3 and 4 within the range of 25-50% (Fig. 3.5) for the treatments but there were no significant differences ($p>0.05$, Appendix 2). In the fifth week, all the explants died and all the media had turned brown (Image 3.5). There were no calli or shoots formed.

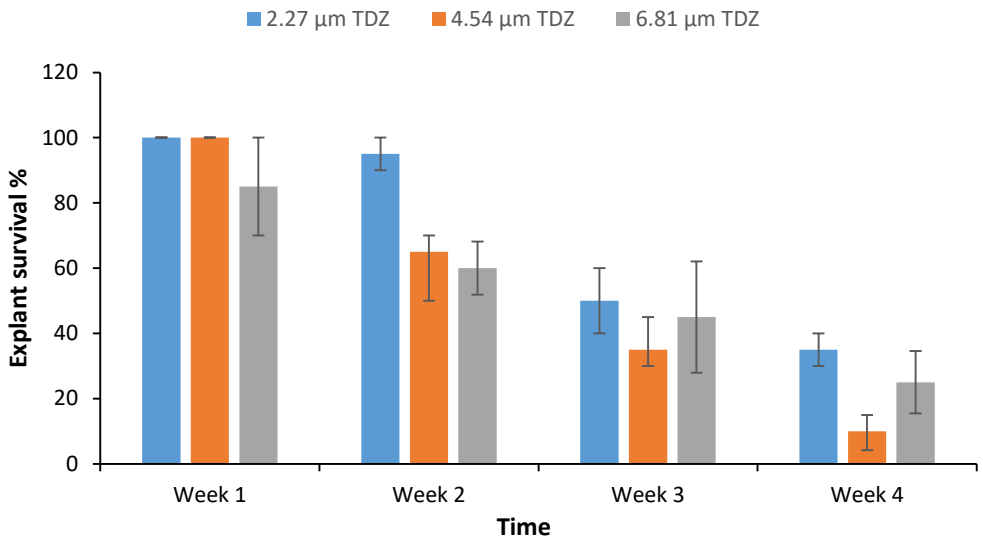


Figure 3.5: Effect of TDZ on leaf explant survival for direct organogenesis (n=60)

Image 3.5: Browning of media and death of leaf explants after 5 weeks of culture on media containing TDZ

3.3.2.2 Effect of PVP on media non-browning

In the previous experiments, there was consistent browning of media when the explants were cultured on any media, which may have affected calli or shoot production hence the screening done using different concentrations of PVP known to reduce such effects.

Leaf explants were cultured in MS media containing BAP (22.2 µm) and different levels of PVP at 0, 0.5, 1 and 1.5 g/L and variations were observed over time. At the end of the first week, 100% media non-browning was recorded in treatments with 0, 1 and 1.5 mg/L PVP while a 90% media non-browning rate was recorded in PVP with 0.5 g/L PVP (Fig. 3.4). There were no significant differences ($p>0.05$) between these treatments (Appendix 3). In the second week, there was an increase in browning with high variation between treatments. The media containing 0 g/L PVP had the lowest level of non-browning at 30%, followed by 85% for both 0.5 and 1 g/L levels (Fig. 3.4.). The highest levels of non-browning at 90% were observed in 1.5 g/L PVP. There were significant ($p<0.05$) differences between treatments (Appendix 3). The third week saw a reduction to 0% media non-browning in 0 g/L PVP. 1 g/L had the highest media non-browning rate of 75% followed by 1.5 and 0.5 g/L at 60% and 45% respectively (Fig. 3.4.). There were significant ($p\leq0.05$) differences between treatments (Appendix 3). Media non-browning reduced for all the treatments in weeks 4, 5 and 6 with the highest non-browning in 1 g/L, followed by 0.5 g/L and 1.5 g/L having the lowest media non-browning rate in that fashion. In the 4^{th} week, media non-browning was 0%, 45%, 55% and 30% for 0, 0.5, 1 and 1.5 g/L PVP, respectively (Fig. 3.4). There were significant differences ($p\leq0.05$) between the treatments (Appendix 4). In week 5, 0%, 35%, 40% and 20% media non-browning rate was observed in 0, 0.5, 1 and 1.5 g/L PVP respectively. The treatments were significantly ($p\leq0.05$) different (Appendix 4). Week six had the lowest media non-browning rate at 0%, 10% 25% and 5% for the treatments 0, 0.5, 1 and 1.5 g/L PVP,

respectively (Fig. 3.4). The treatment values were not significantly different (p>0.05, Appendix 4). In the seventh week, all the media turned brown. No explant survived beyond the sixth week. No growth or development was observed in all the treatments all through.

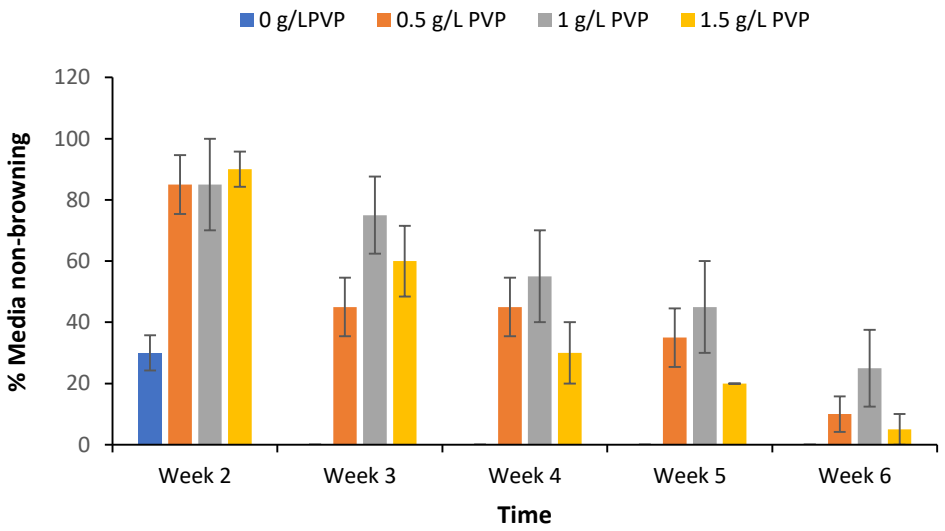


Figure 3.6: Effect of PVP on reduction of phenolic compounds on media of *B. sacra* explants (n=80)

3.3.2.3 Effect of BAP and TDZ on survival of axillary bud explants

Experiments with leaf explants and media with BAP, TDZ and mTR did not give any regenerants or calli. This experiment was set with the aim of direct shoot organogenesis from axillary buds.

a) Effect of BAP on axillary bud explants

Axillary bud explants were cultured in MS media consisting of 0, 2.2, 22.2 and 44.49 µm BAP alongside 1 g/L PVP. Explant survival of 100% was observed on all the treatments after one week (Fig. 3.7). In week 2, the explant survival reduced to 20% in 0 µm and 80% in 2.2 µm but remained at 100% in both 22.2 and 44.39 µm BAP. After the second week, 22.2 µm BAP had the highest survival rate of all the other treatments. All the explants in 0 µm died in the third week, but there was 40%, 60% and 80% survival observed in 2.2, 22.2 and 44.39 µm respectively. In week 4, the

explant survival remained at 40 % in 2.22 µm BAP but reduced to 30% and 60% in 22.2 and 44.39 µm respectively (Fig. 3.7). Explant survival remained at 10% in 22.2 µm BAP in weeks 5 and 6 but reduced from 20% to % in 0 µm and from 30% to 10% in 22.2 µm (Fig. 3.7). After week 6, all remaining explants died.

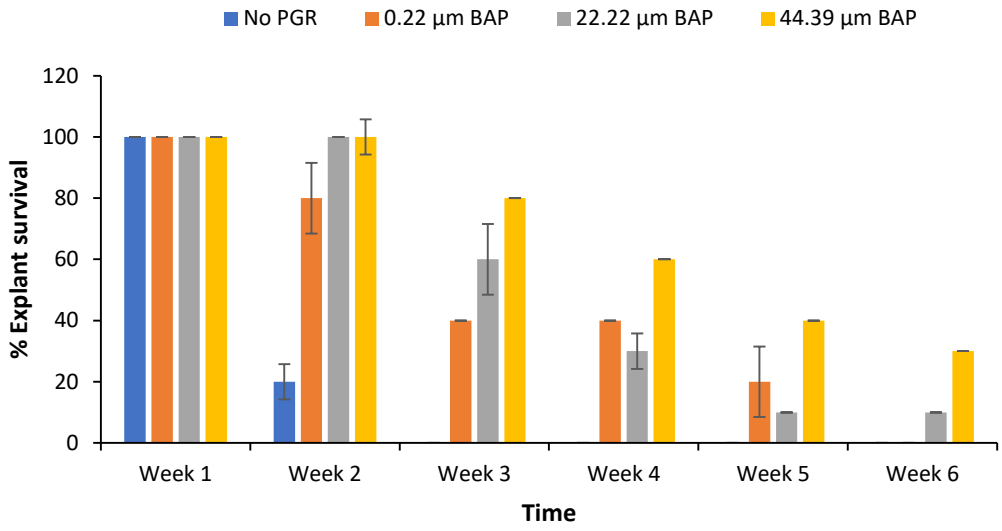


Figure 3.7: Effect of BAP on axillary bud explant survival for direct shoot organogenesis (n=40).

b) Effect of TDZ on axillary bud

Thidiazuron was added to MS media at the rates of 22.7 µm and 45.4 µm. Axillary buds were then cultured in this media and observations recorded weekly. In weeks 1 and 2, the explant survival rate was 60% in both treatments. In the third week, explant survival reduced to 30% in 22.7 µm and 40% in 45.4 µm. Explant survival remained unchanged in weeks 4 and 5 at 20% and 40% in 5 and 22.2 µm TDZ (Fig. 3.8). All the explants died after the fifth week.

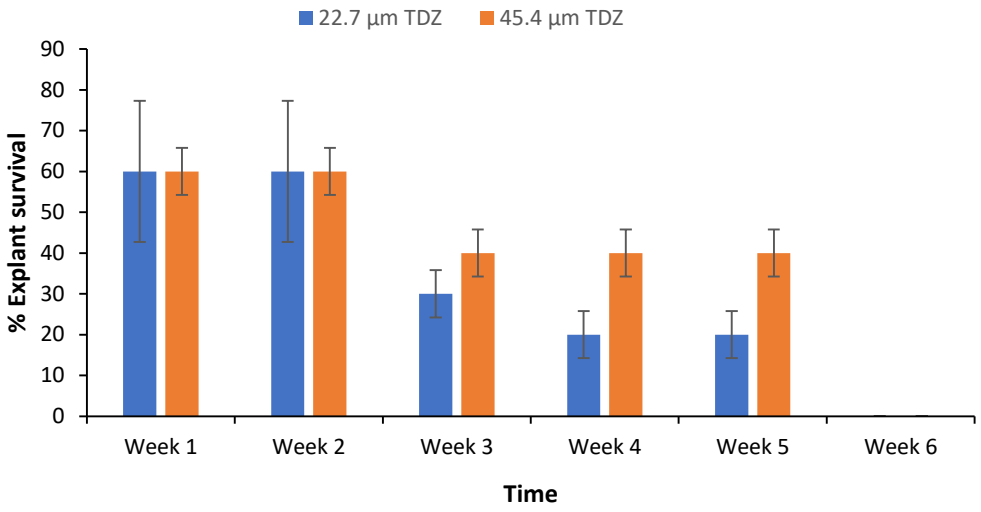


Figure 3.8: Effect of TDZ on axillary bud explant survival for shoot organogenesis (n=20)

3.3.2.4 Effect of different hormones on axillary bud breaking

Bud breaking has been known to lead to shoot organogenesis and shoot multiplication in some woody plant species. Axillary buds of *B. sacra* were cultured in BAP and TDZ in several levels to induce bud breaking followed by shoot organogenesis. Some treatments exhibited bud breaking but no shoot organogenesis was observed as discussed below.

a) Effect of BAP on axillary bud breaking

Axillary buds were cultured in MS media containing 0, 2.2, 22.2 and 44.4 µm BAP and PVP (1 g/L). Bud breaking was observed on all the treatments except in the control (Fig. 3.9). In the first week, 50% bud breaking was observed on 44.39 µm BAP and 40% in 22.2 µm BAP. No bud breaking was observed in 2.22 µm BAP (Fig 3.9). The highest bud breaking frequency was observed in week 2 in all the treatments. Bud-breaking rate of 90% was observed in 22.2 µm while 80% and 20% bud breaking were observed in 22.2 µm and 2.22 µm BAP respectively (Fig 3.9).

There was no further bud breaking observed after the second week. The leaflets that had formed became necrotic (Image 3.6). Eventually, all the explants died.

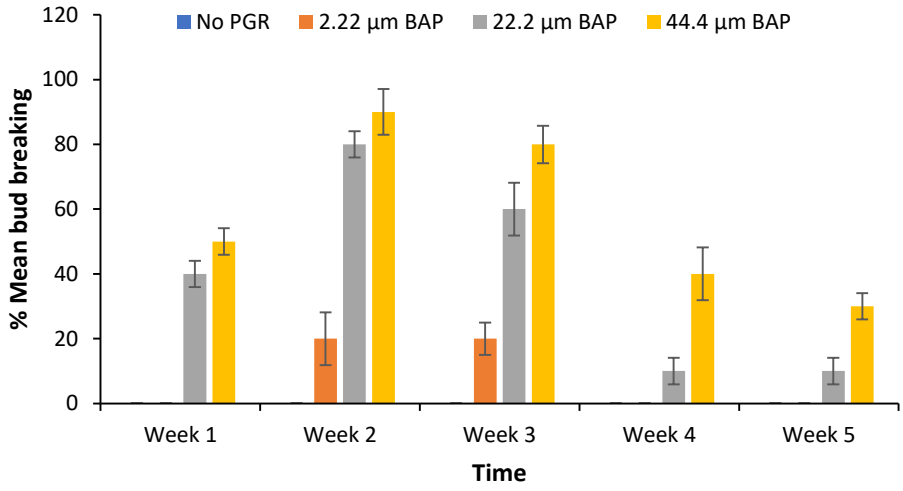


Figure 3.9: Effect of BAP on axillary bud breaking (n=40).

b) Effect TDZ on axillary bud breaking

Axillary buds were cultured in MS media containing TDZ at the rates of 22.7 µm and 45.4 µm. The media was also supplemented with 1 g/L PVP. In week 1, 10% bud breaking was observed in both treatments. In week 2, there was an increase to 40% in 22.7 µm TDZ and 20% in 45.4 µm TDZ (Fig. 3.8). There was no further increase in bud breaking. In the following weeks, the explants started dying and in week 5, all explants had died.

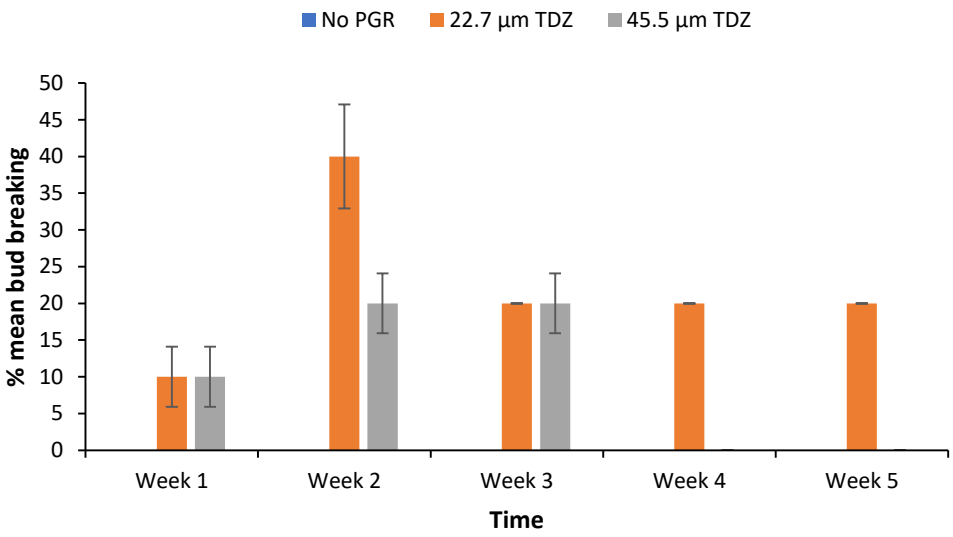


Figure 3.10: Effect of TDZ on axillary bud breaking (n=30)

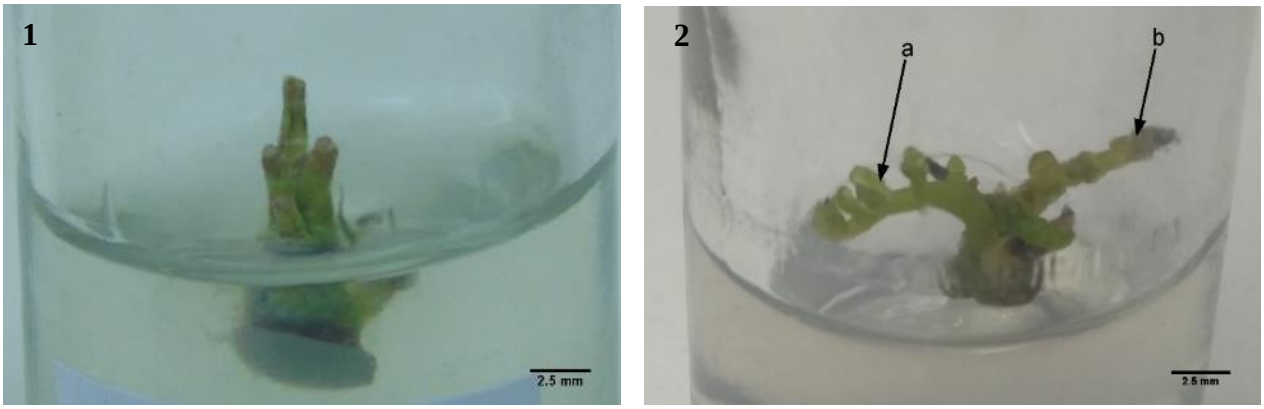


Image 3.6: Axillary buds performance (1) explants cultured in MS media with no PGR + 1 g/L PVP showing no signs of bud breaking after 3 weeks. (2) explants cultured on MS media with 44.39 µm BAP having two leaves after 2 weeks (a) vigorous growth (b) signs of necrosis

3.3.3 Effect of different hormones and combinations on calli production for indirect organogenesis

This experiment was established because the *B. sacra* explants did not produce any regenerants via direct organogenesis. Experiments were therefore conducted to induce shoots indirectly through callus induction followed by somatic embryogenesis and shoot regeneration.

3.3.3.1 Effect of TDZ on callus induction from leaf explants

Leaf explants were cultured on MS media consisting 0, 2, 5 and 8 µm TDZ with 1 g/L PVP for callus induction. The cultures were incubated in total darkness and observations made weekly for five weeks. There was calli formation on all treatments except the control. Calli started forming in the second week of culture. Calli formed on both the petioles and midribs. After four weeks of culture, there was no further calli formation observed and the explants with no calli became necrotic (Image 3.7).

In the second week, the highest callus induction frequency of 32.5% was observed in treatment consisting 5 µm TDZ. In the other treatments, 2 µm and 8 µm TDZ, only 10% of the explants had formed callus. Explants had most calli developing on petioles compared to the midribs in all treatments. Explants with calli on petioles were 10%, 32.5% and 10% while on the midribs they were 2.5%, 12.5% and 2.5% for 2 µm, 5 µm and 8 µm TDZ respectively (Table 3.2). All the treatments were significantly different ($p<0.05$, appendix 4).

The callus induction rate increased in week 3 for all the treatments. The highest callus induction rate was recorded on explants cultured in 2 µm TDZ at 90% followed by 5 µm TDZ at 87.5% while the 8 µm TDZ had the least callus at 37.5% (Table 3.1). All the treatment differences were significant ($p<0.05$, appendix 4). There was 90% callus formation on petioles of explants cultured in 5 µm TDZ, 87.5% in 2 µm TDZ and 37.5% in 8 µm TDZ. Observed calli on midrib were 87.5%, 80% and 35 % on explants in 2, 5 and 8 µm TDZ respectively (Table 3.2). All treatments were significantly different ($p<0.05$, appendix 4)

In weeks four and five, all explants cultured in 5 µm TDZ formed callus with 97.5% of these explants developing calli on petioles and 72.5% on the midribs. There was a 97.5% callus induction frequency in 2 µm TDZ with 95% of the explants forming callus on petioles and 72% on midribs.

The least callus induction frequency was 60% recorded in the treatment consisting 8 µm TDZ with 60% of the explants forming callus on petioles and 30% on midribs (Table 3.2). All the treatment differences were significant ($p \leq 0.05$, Appendix 4).

Table 3.2: Callus induction frequency on leaf explants of *B. sacra* under varying TDZ levels of TDZ over time (n=160).

	Mean % callus observed											
	Week 2			Week 3			Week 4			Week 5		
Treatment	Total	Petiole	Midrib	Total	Petiole	Midrib	Total	Petiole	Midrib	Total	Petiole	Midrib
No PGR	0	0	0	0	0	0	0	0	0	0	0	0
2 µm TDZ	10	10	2.5	90	87.5	62.5	97.5	95	72.5	97.5	95	72.5
5 µm TDZ	32.5	32.5	12.5	87.5	80	72.5	100	97.5	77.5	100	97.5	77.5
8 µm TDZ	10	10	2.5	37.5	35	25	50	50	30	50	50	30
Mean	13.1	13.1	4.3	53.7	50.6	40	61.8	60.6	45	61.8	60.6	45
CV (%)	6.09*	6.09*	4.78*	3.15*	3.31*	3.94*	2.75*	2.70*	3.62*	2.75*	2.70*	3.62*
LSD (5%)	9.69	9.69	6.67	10.43	13.09	14.41	14.58	15.25	15.08	14.58	15.25	15.08
P value (5%)	<0.001	<0.001	0.007	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

*%CV obtained after data transformation using Log_{10}

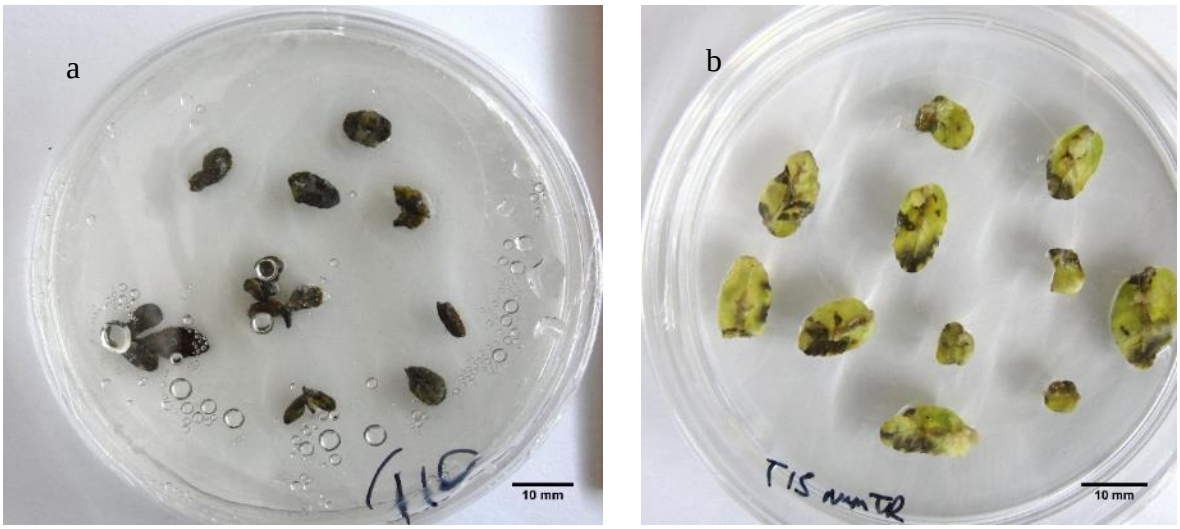


Image 3.7: Dead explants (a) on media with no PGRs and explants forming callus (b) on media with 5µm MemTR after four weeks of culture.

3.3.3.2 Effect of Meta-methoxytopolin on callus induction from leaf explants

Leaf explants were cultured in MS consisting 5, 8 and 10 µm MemTR and 1g/L PVP. These were incubated in total darkness. In the second week of culture, calli had started forming on the petioles and midribs. After four weeks of culture, there was no increase in callus induction frequency.

At the end of week 2, the mean percentage number of explants with calli was 10%, 15 % and 17.5% on explants cultured in 5, 8 and 10 µm MemTR respectively (Table 3.3) with no significant differences ($p>0.05$, appendix 5) between treatments. Observed calli on petioles were 10%, 15% and 17.5% on 5, 8 and 10 MemTR respectively. Calli on midribs was lower, 2.5% in both 5 and 10 µm MemTR and 10% for 8 µm MemTR (Table 3.3). Treatment differences were not significant ($p>0.05$)

Three weeks of culture led to an increase in the total explants with calli to 50% in 8 and 10 µm MemTR and 32.5% in 8 µm MemTR (Table 33). There were no significant differences between the treatments ($p>0.05$, Appendix 5). The number of explants with calli on petioles was 50% in 8 and 10 µm MemTR and 27.5% in 5 µm MemTR. These differences were significant ($p<0.05$, Appendix 5). Explants with calli on midribs were 22.5% in 8 and 10 µm MemTR but were lower in 5 µm MemTR at 17.5% (Table 3.3) but there were no significant differences ($p>0.05$, Appendix 5).

In the 4^{th} and 5^{th} weeks of culture, both 8 and 10 µm MemTR had a 60% calli induction frequency but differed on the number of explants forming calli on petioles and midribs. In 8 µm, 57.5% of the explants developed calli on the petioles and 30% on the midribs while in 10 µm 60% of the explants developed calli on petioles and 32.5% on midribs. The lowest calli induction frequency was observed on 5 µm MemTR at 40% with 32.5% explants forming calli on the petioles and 17.5% on the petioles (Table 3.3). All the treatment values were significantly different ($p\leq0.05$, Appendix 5).

Table 3.3: Performance of leaf explants on different concentrations of MemTR over time (n=120)

	Mean % callus observed											
	Week 2			Week 3			Week 4			Week 5		
Treatment	Total	Petiole	Midrib	Total	Petiole	Midrib	Total	Petiole	Midrib	Total	Petiole	Midrib
10 µm MemTR	15	15	2.5	50	50	22.5	60	60	32.5	60	60	32.5
5 µm MemTR	10	10	2.5	32.5	27.5	17.5	40	35	17.5	40	35	17.5
8 µm MemTR	17.5	17.5	10	50	50	22.5	60	57.7	35	60	57.5	35
Mean	14.1	14.1	5	44.16	42.5	20.8	53.3	50.8	28.3	53.33	50.8	28.3
LSD (5%)	12.79	12.79	9.98	19.04	19.04	18.66	22.62	22.4	18.85	22.62	23.55	18.85
P value (5%)	<0.05	<0.05	<0.05	<0.05	>0.05	<0.05	<0.05	<0.05	<0.05	<0.05	>0.05	<0.05

*%C.V. values calculated after data transformation using Log_{10}

3.3.3.3 Effect of MemTR and TDZ combinations on callus induction from leaf explants

MS media was supplemented with a combination of the PGRs MemTR and TDZ at 1 µm TDZ and 10 µm MemTR, 2 µm TDZ and 10 µm MemTR, 1 µm TDZ and 15 µm MemTR and finally 2 µm TDZ and 15 µm MemTR. PVP was also included at 1 g/L. Leaf explants were cultured in these media and incubated in total darkness.

After two weeks of culture, explants had started developing calli. The highest calli induction frequency was observed on 2 µm TDZ+ 10 µm MemTR with 27.5% forming on petioles and 10% on midribs followed by 1 µm TDZ + 15 µm MemTR at 20% with 20% forming on petioles and 5% on midribs, 2 µm TDZ + 15 µm MemTR had 17.5 % with 15% forming on petioles and 10% on midribs and the least density observed on 1 µm TDZ + 10 µm MemTR at 12.5 % with 12.5% on petioles and 2.5% on midribs (Table 3.4). All the treatment differences in week 2 were not significantly different ($p>0.05$, Appendix 6).

There was an observed increase in the callus induction frequency in all treatments in week 3. The highest callus induction frequency was observed on 2 µm TDZ + 10 µm MemTR at 70%. Callus induction frequency in 1 µm TDZ + 15 µm MemTR was 55% while in 2 µm TDZ + 15 µm MemTR and 1 µm TDZ + 10 µm MemTR was 45% and 40% respectively (Table 3.4). The treatment differences were significant ($p<0.05$, Appendix 6). Explants with calli had developed them on

petioles but 25%, 22.5%, 42.5% and 30% of the explants in 1 µm TDZ + 10 µm MemTR, 1 µm TDZ + 15 µm MemTR, 2 µm TDZ + 10 µm MemTR and 2 µm TDZ + 15 µm MemTR respectively had also developed callus on midribs (Table 3.4). The treatment differences between explants with calli on midribs were not significant ($p>0.05$, Appendix 6)

The highest calli induction frequency was observed on 2 µm TDZ and 10 µm MemTR combo at 85%, with 85% of the explants developing callus on the petioles and 65% on the midribs. This was followed by 2 µm TDZ and 15 µm MemTR combo with 75% total calli induction frequency and 62.5% and 60% of the explants forming calli on petioles and midribs respectively. The 1 µm TDZ and 15 µm MemTR treatments had 70% calli induction frequency with 70% and 42.5% for petioles and midribs, respectively. Least calli frequency was observed on 1 µm TDZ and 10 µm MemTR combination at 57.5%, 55% of the explants forming calli on petioles and 52.5% on the midribs (Table 3.4). All treatments were significantly different ($p\leq0.05$, Appendix 6).

Table 3.4: Performance of leaf explants on different combinations of MemTR and TDZ over time (n=120)

	Mean % callus observed											
	Week 2			Week 3			Week 4			Week 5		
Treatment	Total	Petiole	Midrib	Total	Petiole	Midrib	Total	Petiole	Midrib	Total	Petiole	Midrib
1 µm TDZ + 10 µm MemTR	12.5	12.5	2.5	40	40	25	57.5	55	52.5	57.5	57.5	52.5
1 µm TDZ + 15 µm MemTR	20	20	5	55	55	22.5	70	70	42.5	70	70	42.5
2 µm TDZ + 10 µm MemTR	27.5	27.5	10	70	70	42.5	85	85	65	85	85	65
2 µm TDZ + 15 µm MemTR	17.5	15	7	45	45	30	62.5	62.5	60	75	75	60
Total	19.3	18.75	6.25	52.5	52.5	30	68.75	68.1	55	71.8	71.8	55
C.V (%)	17.07*	18.43*	17.69*	7.62*	7.62*	15.83*	6.73*	7.75*	7.99*	6.60*	6.60*	7.99*
LSD (5%)	19	19.13	14.41	19.89	19.89	24.56	23.32	26.96	27.6	25.45	24.5	27.6
P value (5%)	<0.05	>0.05	>0.05	<0.05	<0.05	<0.05	<0.05	>0.05	>0.05	<0.05	<0.05	>0.05

*%C.V. values calculated after data transformation using Log_{10}

3.3.3.4 Effect of BAP on callus induction from leaf explants

Leaf explants were cultured in MS media containing 2, 5 and 8 µm BAP and 1 g/L PVP. The cultures were incubated in total darkness. After two weeks of culture, calli had started forming on the explants.

After two weeks of culture, total explants with calli ranged from 12.5% to 22.5 % in all treatments. There was 15%, 12.5% and 22.5% callus formation on petioles of explants in 2, 5 and 8 µm BAP. Explants with calli on midribs were 5% and 2.5% in 2 and 8 µm BAP. Explants in 5 µm BAP did not form any callus on midribs (Table 3.5). Treatment differences were not significant ($p>0.05$, Appendix 7).

In week 4, there was 40% callus formation on 2 and 8 µm BAP, while only 30% of explants formed callus on 5 µm BAP. There was also 40% callus formation on petioles of explants in both 2 and 8 µm BAP and 30% in 5 µm BAP. Callus formation on midribs was 20% for explants in both 5 and 8 µm BAP and 10% for explants in 2 µm BAP. The highest callus induction frequency was 55% observed on 8 µm BAP, with 50% of the explants forming callus on the petioles and 30% on the midribs in weeks 4 and 5 (Table 3.5). An equal callus induction frequency was observed on 2 and 5 µm BAP but there were differences in the number of explants forming callus from midribs and petioles (Table 3.5). Despite this, all the callus induction rates were not significantly different ($p>0.05$, Appendix 7).

Table 3.5: Performance of calli under different concentration levels of BAP over 5 weeks of culture (n=120)

	Mean % callus observed											
	Week 2			Week 3			Week 4			Week 5		
Treatment	Total	Petiole	Midrib	Total	Petiole	Midrib	Total	Petiole	Midrib	Total	Petiole	Midrib
2 µm BAP	17.5	15	5	40	40	10	45	40	15	45	40	15
5 µm BAP	12.5	12.5	0	30	30	20	45	37.5	25	45	37.5	25
8 µm BAP	22.5	12.5	2.5	40	40	20	55	50	30	55	50	30
Mean	17.5	13.3	2.5	36.6	36.6	16.6	48.3	42.5	23.3	48.3	42.5	23.3
C.V %	4.22*	4.17*	0*	3.22*	3.22*	3.90*	2.58*	3.08*	3.56*	2.58*	3.08*	3.56*
LSD (5%)	13.33	11.3	7.05	22.62	22.62	10.56	21.98	21.98	15.08	21.98	23.09	40.4
P value (5%)	0.287	0.849	0.323	0.537	0.537	0.1	0.1	0.519	0.126	0.519	0.463	0.126

*%C.V. values calculated after data transformation using Log_{10}

3.3.3.5 BAP and IAA combinations on callus induction from leaf explants

MS media was supplemented 2 µm IAA in combination with BAP at 2, 5 and 8 µm. PVP was also added to the media at 1 g/L. Leaf explants were cultured in these media for calli induction. Incubation of the cultures was done in total darkness. Callus started forming on the petioles and midribs in the second week of culture.

The highest calli induction frequency in week two was observed on 2 µm IAA + 2 µm BAP at 25% with 22.5% of the callus on petioles and 2.5 % on midribs. The explants in 2 µm IAA + 5 µm BAP and 2 µm IAA + 8 µm BAP had 7.5% callus formation but differed in calli on petiole and midribs. There was no callus formed on midribs of explants cultured on 2 µm IAA + 5 µm BAP (Table 3.6). Total calli formation values were not significant ($p > 0.05$, Appendix) but both calli on midribs and petioles values were significantly different ($p<0.05$, Appendix 8).

In the third week total explants with calli increased to 47.5% in 2 µm IAA + 2 µm BAP and to 25% in both 2 µm IAA + 5 µm BAP and 2 µm IAA + 8 µm BAP. These differences were not significant ($p>0.05$, Appendix). Explants with calli on petioles were 40% in 2 µm IAA + 2 µm BAP, 25 % in 2 µm IAA + 5 µm BAP and 20% in 2 µm IAA + 8 µm BAP. There was 15% callus formation on midribs of explants in both 2 µm IAA + 2 µm BAP and 2 µm IAA + 8 µm BAP and

7.5 % in 2 µm IAA + 5 µm BAP (Table 3.6). All the treatment differences were not significant ($p>0.05$, Appendix 8).

In weeks four and five, 60% calli formation was observed on the treatment with 2µm IAA + 2 µm BAP. Explants in 2 µm IAA + 5 µm BAP and 2 µm IAA + 8 µm BAP had 25% and 30% callus formation density. There were significant differences between the treatments ($p<0.05$, Appendix 8). In both weeks callus formation on observed on petioles was 52.5%, 25% and 20% on 2 µm IAA + 2 µm BAP, 2 µm IAA + 5 µm BAP and 2 µm IAA + 8 µm BAP respectively. These differences were significant ($p< 0.05$, Appendix 8). Callus formation on midribs was 20% for both 2 µm IAA + 2 µm BAP and 2 µm IAA + 8 µm BAP and 7.5 % on 5 µm BAP and 2 µm IAA but with no significant differences ($p>0.05$, Appendix 8).

Table 3.6: Effect of BAP and IAA on callus induction on leaf explants (n=120).

	Mean % somatic embryo formation											
	Week 2			Week 3			Week 4			Week 5		
	Total	Petiole	Midrib	Total	Petiole	Midrib	Total	Petiole	Midrib	Total	Petiole	Midrib
2µm IAA+2 µm BAP	25	22.5	2.5	47.5	40	15	60	52.5	20	60	52.5	20
2µmIAA+5 µm BAP	7.5	7.5	0	25	25	7.5	25	25	7.5	25	25	7.5
2µmIAA+8 µm BAP	7.5	2.5	7.5	25	20	15	30	20	20	30	20	20
Mean	13.3	10.8	3.3	32.5	28.3	12.5	38.3	32.5	15	38.3	32.5	15
C.V %	5.11*	5.37*	0*	4.23*	2.60*	5.90*	4.75*	3.95*	6.33*	4.75*	3.95*	6.33*
LSD (5%)	13.5	10.9	6.53	21.9	21.9	19	24.4	27	19	24.4	27.6	19
P (5%)	<0.05	<0.05	<0.05	<0.05	>0.05	>0.05	<0.05	>0.05	>0.05	<0.05	<0.05	>0.05

*%C.V. values calculated after data transformation using Log_{10}

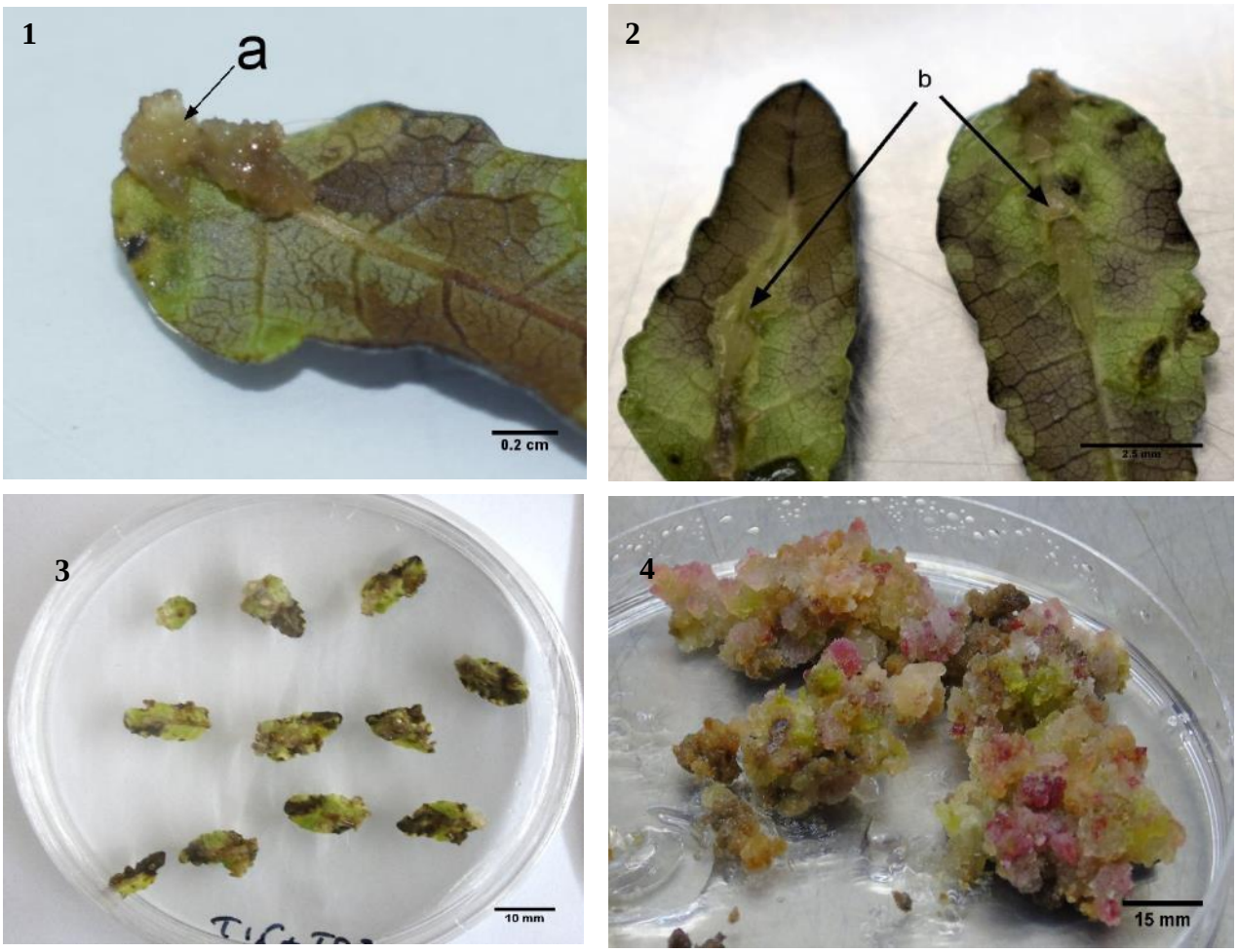


Image 3.8: Calli on 1. petiole 2. midribs 3. After 4 weeks of culture 4. After 8 weeks of culture

3.3.4 Effect of BAP and NAA on somatic embryogenesis from calli.

3.3.4.1 Effect of BAP on somatic embryogenesis from calli

Calli were cultured in MS media containing 1, 2 and 3 µm BAP. PVP was added at 1g/L. After one week, globular stage of somatic embryogenesis could be observed and in week 5, the heart and cotyledonary stages of somatic embryogenesis could be observed (Image 3.9).

There was 17.5 % somatic embryogenesis on calli cultured in media with no PGR in the first week, 45 % in 1 µm BAP, 25% in 2 µm BAP and 12.5 % in 3 µm BAP (Table 3.7). In week 2, somatic embryo formation rate increased to 37.5% in media with no PGR, 57.5 % in 1 µm BAP, 30% in 2 µm BAP and 25% in 3 µm BAP (Table 3.7).

Highest somatic embryogenesis rate was recorded in week three and there was no further somatic embryo formation in the subsequent weeks. Of the calli cultured in 1 µm BAP, no PGR, 2 µm BAP and 3 µm BAP treatments, 60%, 40%, 35% and 25% formed somatic embryos respectively (Table 3.7).

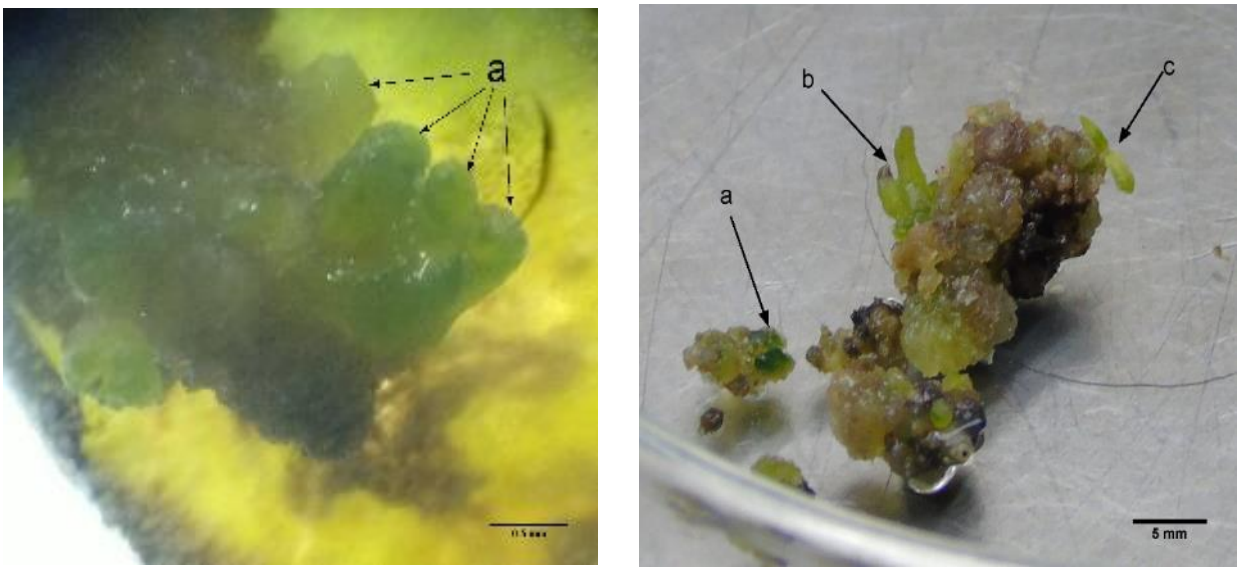


Image 3.9: Stages of somatic embryogenesis on *B. sacra* (a) globular stage, (b) heart stage and (c) cotyledonary stage.

3.3.4.2 Effect of BAP and NAA on somatic embryogenesis from calli

Calli were cultured MS media containing a combination of BAP and NAA at 1 µm BAP + 0.5 µm NAA, 2 µm BAP + 1 µm NAA and 3 µm BAP + 1.5 µm NAA. At the end of the first week, there was 45%, 60% and 70% somatic embryo formation on calli cultured in 1 µm BAP + 0.5 µm NAA, 2 µm BAP + 1 µm NAA and 3 µm BAP + 1.5 µm NAA respectively. The treatment differences were significant ($p<0.05$, Appendix 8). Somatic embryo formation rate increased to 100% in µm BAP + 1 µm NAA in week 2, 77.5 % in 3 µm BAP + 1.5 µm NAA and to 52.5% in 1 µm BAP + 0.5 µm NAA (Table 3.7). There were significant differences between the treatments ($p<0.05$, Appendix 8).

In weeks 3 and 4, somatic embryogenesis rate remained at 60% in 1 µm BAP + 0.5 µm NAA, but increased from 87.5% to 92.5% in 3 µm BAP + 1.5 µm NAA (Table 3.7). The treatment differences were significant ($p<0.05$, Appendix 8). No further increase in somatic embryogenesis was observed after the fourth week.

Table 3.7: Development of somatic embryos on calli cultured in various levels of BAP and NAA over time (n=280)

	Mean % calli with somatic embryo development			
Treatment	Week 1	Week 2	Week 3	Week 4
No PGR	17.5	37.5	40	40
1 µm BAP	45	57.5	60	60
2 µm BAP	25	30	35	35
3 µm BAP	12.5	25	25	25
1 µm BAP + 0.5 µm NAA	45	52.5	60	60
2 µm BAP + 1 µm NAA	60	100	100	100
3 µm BAP + 1.5 µm NAA	70	77.5	87.5	87.5
Mean	39.3	54.2	58.2	58.2
C.V%	29.7	18	16.2	16.2
LSD (5%)	17.13	14.35	13.9	13.9
P (5%)	<0.001	<0.001	<0.001	<0.001

3.3.5 Effect of different hormones and combinations on shoot production via somatic embryogenesis

3.3.5.1 Effect of BAP and IAA on shoot development from somatic embryos

Micro-shoots that developed in the somatic embryogenesis stage were excised and cultured in MS consisting 0, 1 µm BAP + 0.25 µm IAA, 2 µm BAP + 0.25 µm IAA and 3 µm BAP + 0.25 µm IAA. Most micro-shoots died during the 1st week of culture. Only 10 % regeneration was recorded in media with no PGR and 15 % in 1 µm BAP + 0.25 µm IAA (Table 3.8). There were significant differences between the treatments ($p<0.05$, Appendix 8).

The shoots obtained were vigorous within the first week but soon became stunted, despite weekly media replacement. Eventually, the shoots started showing signs of hyperhydricity and died by the sixth week of culture (Image 3.10).

Table 3.8: Shoot regeneration under different combinations of BAP and IAA(n=80)

Treatment	Regeneration (%)
No PGR	10
1 µm BAP + 0.25 µm IAA	15
2 µm BAP + 0.25 µm IAA	0
3 µm BAP + 0.25 µm IAA	0
Mean	6.25
C.V %	6.68*
LSD (5%)	21.33
P value (5%)	>0.05

*%C.V. values calculated after data transformation using Log_{10}

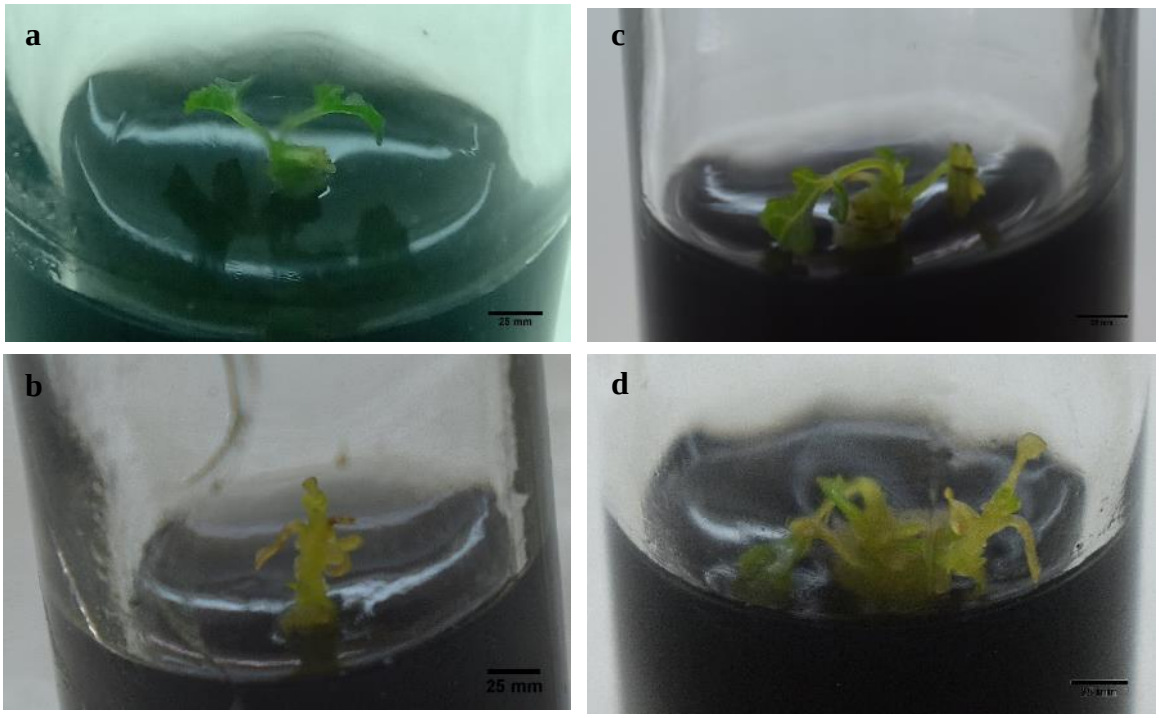


Image 3.10: Developing shoot (a) after 2 weeks of culture and (b) after 4 weeks in MS media with no PGRs. Shoot (c) after 2 weeks and (d) after 5 weeks of culture in MS media with 1 µm BAP + 0.25 µm IAA. Note the hyperhydricity in (b) and (d).

3.4 Discussion

3.4.1 Germination and viability of *B. sacra* seeds are affected by lack of embryos

Many *B. sacra* trees from Exdad and Af Yare Dawl-dawl had no fruits at time of seed collection, while others had very few, which was attributed to season of seed collection. Although there is no information times of flowering, fruit set and fruit maturity in the study area, studies on *B. sacra* from Oman indicate that flowering occurs over winter with fruits ripening from March to May (Lippi et al., 2011). Sample collection for this study was carried towards the end of summer in September and the trees could have already shed the fruits resulting in the low numbers of fruits hence seed collected. Secondly, low fruit set could have been attributed to the re-allocation of photosynthates since all sampled trees had previously been severely tapped and photosynthates could have been directed towards the healing of wounds at the expense of fruit formation. Similar results were reported by Rijkers et al. (2006) who concluded that tapping for frankincense resulted in limited production of fruits in *B. papyrifera* in Ethiopia.

Results from this study revealed that most fruits collected did not have seed, with a characteristic shriveled appearance in contrast to fruit capsules with a seed that appeared plump indicative of poor seed set and loading. Seed loading is affected by allocation of resources, excessive tapping of these trees may have limited resources needed for development of fertilized ovules into seeds. The negative effect on seed loading due to limitation of resources has been reported in other species such as *Cassia fasciculata* (Martin and Lee, 1993). Another reason for low seed set is reproduction biology, which is unknown since there is no documentation on it being self or cross pollinated. It could therefore be self-incompatible or cross pollinated, which requires pollinators but could have not been present. Self-incompatibility has been reported in other Boswellia species. Studies on

species like *B. serrata* and *B. ovalifoliolata* revealed no fruit and therefore seed formation in controlled self-pollination but higher fruit set in controlled cross-pollinated flowers due to the inability to form embryos (Sunnichan et al., 2005; Raju et al., 2012; Tandon, et al., 2010).

Seeds collected from Exdad and Af Yare Dawl-dawl had a thousand seed weights of 2.4 and 1.9 g, respectively, but this was low compared to previously reported *B. papyrifera* with 15.8 g (Eshete et. al., 2012). Many fruits were empty after dissection and the seeds had no embryos or endosperm, which could explain the low 1000 seed weight. A similar report by Swartout and Solowey (2018) noted empty shells on *B. sacra* seeds. High seed weight indicates that seeds have food reserves resulting in higher germination rates and seedling vigour upon establishment (Seiwa and Kikuzawa, 1996). This is particularly important to species such as *B. sacra* that grow in the wild in areas with limited resources for seedling establishment.

The results from sink tests revealed 26.5% of the seeds sinking after five minutes indicating that only this percentage had a high mass from embryos and endosperm. The rest of the seeds that did not sink did not have embryos. Similar observations were made by Swartout and Solowey (2018). Seeds that sank were viable as determined by viability tests leading to tetrazolium and germination tests with 3.3% and 4.4% viability, respectively, although the values were low. Considering seeds collected from continuously tapped trees from the two sources in this study, these results are in line with less than 10% germination rate described by Swartout and Solowey (2018), which were collected seeds from well-maintained orchards. Al- Harrasi et al. (2019) described *B. sacra* to have the lowest germination rate in genus *Boswellia*. Nonetheless, there are no previous reports on seed viability screening of *B. sacra* using tetrazolium but this method has proven reliable to determine germination potential regardless of presence or absence of dormancy.

Results from seed viability screening, therefore, suggest that low fruit set and poor seed loading were caused by lack of embryos for *B. sacra* seeds hence the need to determine seed reproductive biology for future research to enhance germplasm enhancement efforts. This in turn leads to challenges in offspring establishment whether naturally or in controlled conditions. A five-minute sink test could be used in the field during seed collection as a preliminary discrimination standard. However, due to the overall low seed viability, efforts for reforestation and domestication using the currently used methods are not applicable.

3.4.2 Regeneration of *B. sacra* via organogenesis

Due to poor seedling establishment, leaf and axillary bud explants were used for regeneration via direct organogenesis to avoid destroying the mother plants. Leaf explants cultured in MS media with different cytokinins revealed no regeneration despite differences in explant survival. When explants are placed in nutrient media, four phases of growth are expected including lag phase where induction of cell division takes place, exponential growth, reduced growth period and finally stationary growth period (George et al., 2008). In this study, explants did not get to the exponential growth stage since there was no growth observed. For direct organogenesis, after week 2, media started browning and by week 5, all explants had died and media turned brown suggestive of polyphenol oxidation. Plants produce polyphenols as a response to wounding (Ahmad et al., 2013; North et al., 2012). Phenolic compounds released inhibit cellular growth and activate polyphenol oxidase in explants. Activities of polyphenol oxidase have been known to cause explant and media browning and limits explant morphogenic responses (Ahmad et al., 2013). When polyphenol oxidation occurs, quinines are produced (Preece and Compton, 1991) and they are responsible for media and explant browning. Quinines spread to other parts of the explant and cause inactivity of

enzymes thereby halting cell division and growth (Loomis and Battaile, 1966). Polyphenols are a major setback to micro-propagation of many woody plants (Ahmad et al., 2013).

The study repositioned to find ways to minimize the effect of polyphenols on the cultures. One of the commonly used substances to reduce effects of polyphenols is polyvinylpyrrolidone (PVP). After PVP incorporation, leaf explant survival increased from 4 to 6 weeks. Despite minimizing production of polyphenols, no organogenesis was observed, which is contrary to reports on *B. ovalifoliolata* and *B. serrata*, where PVP addition successfully reduced media browning during micropropagation leading to shoot production (Chandrasekhar et al., 2005; Purohit et al., 1995). Failure of regeneration persisted even with use of axillary bud explants for *B. sacra*. This failure of plant tissues to respond to tissue culture referred to as recalcitrance is attributed to donor plant physiology, in vitro manipulations and stress physiology (Benson, 2000). In vitro manipulations include growth media components while in vitro plant stress may be attributed to light regimes and temperature.

3.4.3 Regeneration via somatic embryogenesis

Callus formed in all leaf explants in all treatments except MS with no PGRs within two weeks of culture in total darkness, attributed to low endogenous PGR levels as well as absence of polyphenol activity. Explant morphogenic responses are triggered by an interaction between endogenous and exogenous PGRs (Benson, 2000). Calli formation on leaf explants cultured in 5 µm TDZ was 100% but a higher concentration led to decreased callus formation. TDZ has been described as more effective than other cytokinins such as BAP and kinetin in promoting morphogenic responses in woody plants (George et al., 2008; Murthy et al., 1998). However, higher concentrations of TDZ have been reported to reduce growth (Murthy et al., 1998). MemTR and BAP alone had low callus induction frequencies compared to TDZ while combined TDZ with MemTR decreased callus

formation, therefore indicating that TDZ is more potent while combining it with other cytokinins reduces its potency in callus induction on *B. sacra* leaf explants. Most calli was formed on the cut end of the petiole compared to midribs indicating wounding triggers and increases rate of callus formation (Ikeuchi et al., 2017). There was no regeneration using somatic embryogenesis when leaf explants were cultured in BAP in combination with IAA despite its success in species like *Digitalis trojana* (Verma et al., 2012).

Incubating leaf explants in darkness helped overcome recalcitrance for somatic embryogenesis. Light regimes and light quality are major determinants in success of micro-propagation of many plant species (Stefano and Rosario, 2003). Light is reported to intensify polyphenol oxidation leading to explant browning, reduced growth and necrosis (Birmeta and Welander, 2004; Naidoo, 2016). Naidoo (2016) overcame this problem in micropropagation of *Scadoxus puniceus* by incubating cultures in continuous darkness. Recalcitrance was overcome in the same manner in micro-propagation of *Ensete ventricosum* (Birmeta and Welander, 2004).

Formation of somatic embryos was achieved by culturing calli on MS consisting NAA and BAP while calli cultured on PGR-free MS media did not form somatic embryos, which was different for species like *Eleutherococcus koreanum* that require no exogenous PGRs to induce embryogenesis (Park et al., 2005). Most species, however, require the addition of PGRs notably cytokinins and auxins (Jiménez, 2005). Though some species can undergo somatic embryogenesis with auxins or cytokinins alone, others require a combination of the two (Raemakers et al., 1995; Gaj, 2004).

Maturation of somatic embryos into plantlets was achieved in basal MS media and MS media consisting BAP and IAA, similar to *Pices asperata* and *Phoenix dactylifera* species with shoot regeneration on PGR-free media (Mazri et al., 2017; Xia et al., 2017). Efficient regeneration in

species such as *Coffee liberica w*as achieved using cytokinins on MS supplemented with 0.5 mg/L BAP (Ardiyani et al., 2020). Although high auxin levels inhibit conversion of somatic embryos (Jiménez, 2005; Gaj, 2004) shoot regeneration from somatic embryos in *Phellodendron amurens* was achieved in MS containing 2 µm BAP and 1 µm NAA (Azad et al., 2009). Manokari et al., (2021) reported 97.4% somatic embryo germination rate using MS medium with 2 mg/L BAP and 0.25 mg/L IAA in micropropagation of *Spathoglottis plicata.*

Shoots obtained were vigorous within the first week of culture but succumbed to hyperhydricity. Hyperhydricity, previously known as vitrification, leads to abnormal plant growth and is a major problem in plant regeneration from somatic embryos (Debergh et al., 1992; Gaspar et al., 1987). It is characterized by glassy, translucency and turgid appearance of in-vitro regenerated shoots (Kevers et al., 2004) same as observed in *B. sacra*. Hyperhydricity is attributed to excess intake of water and reduced transpiration, sometimes leading to excess production of H_2O_2 (Dewir et al., 2006)

Despite being complicated, somatic embryogenesis is considered much more efficient compared to organogenesis as only a small amount of plant material is required to initiate embryonic cultures which can also be used as a source of embryogenic protoplasts for genetic engineering (Bach and Pawłowska, 2003; Lu and Thorpe, 1987).

3.5 Conclusion and recommendations

The current status of *B. sacra* forests in Somaliland calls for immediate interventions for conservation and sustainable utilization of the species. Improper tapping for frankincense leaves the existing trees vulnerable to diseases and insect pest attacks. Illegal harvesting, where unauthorized harvesters scrap the entire bark for sale in the black market leading to increased mortality of these trees as witnessed in Af Yare Dawl-dawl. If no actions are taken, in the near future this important resource could become extinct, leaving thousands of families with no source of income. Some of these actions include re-introduction of government policies governing frankincense value chains, introduction of sustainable frankincense harvesting methods and reforestation efforts.

From this study tetrazolium test revealed 3.3% seed viability while germination was 4.15% indicative of low seed viability. This was ascribed to over-tapping and low number of natural pollinators. Therefore, to increase yield of viable seeds, there is a need to introduce sustainable frankincense harvesting methods that will ensure some of the trees are left untapped for use as a source of seed. However, more work needs to be done on the flowering and pollination patterns of the species to develop breeding programs for genetic improvement and sustainable conservation. This study also revealed that a 5-minute sink test can be used in the field as a quick preliminary to discriminate non-viable seeds.

Direct organogenesis was not effective due to recalcitrance but other explants could be used as well as younger explants to address this since only leaf and axillary bud explants were used. More explants such as cotyledonary shoots, apical buds and stem could be investigated. Addition of PVP in growth media at the rate of 1g/L can help reduce media browning in the culture of *B. sacra*.

This study has demonstrated an efficient protocol for callus induction, subsequent somatic embryogenesis and shoot regeneration. However, more research is needed to overcome hyperhydricity leading to normal shoot and root development. Also, more research is needed to determine the development of synthetic seeds from somatic embryos. These could be instrumental in providing mass propagation material for reforestation and germplasm conservation.

CHAPTER FOUR

GENETIC DIVERSITY OF *BOSWELLIA SACRA* ACCESSIONS FROM SOMALILAND

Abstract

Boswellia sacra is distributed in Oman and southern Somaliland but facing extinction partly due to overexploitation of existing stands and poor establishment of new stands. In addition, there is no information available on the genetic diversity of the existing stands, which is a major hindrance for conservation and germplasm improvement efforts. In this study, genetic diversity was investigated through morphological and molecular markers. Six morphological traits: stem type, tree height, height to branching, bottom swelling, growth surface and number of stems were recorded for 32 genotypes from Af Yare dawl-dawl and 25 from Exdad. The morphological data showed no significant ($p>0.05$) differences for all traits but correlation was present between some traits ($p<0.05$). The dendrogram from hierarchical cluster analysis of principal components for the morphological data revealed two main clusters each with two sub-clusters but failed to cluster the genotypes according to geographical region indicative of low variability. DNA extraction was done using a modified CTAB protocol for woody plants resulting to quantities ranging from 105-3046 ng/µL. DNA from Exdad site was degraded and downstream processes were not successful. Thirteen ISSR primers were screened for polymorphism but 8 produced polymorphic bands though they were not distinct enough to be used for diversity studies, which led to the use of SRAP markers. Twelve SRAP marker combinations were screened for polymorphism out of which 8 produced clear polymorphic bands and were suitable for genetic diversity studies for *B. sacra.* Very low heterozygosity was found between Af Yare Dawl-dawl genotypes ($H_e=0.053$) indicative of low variability within the population. Hierarchical cluster analysis revealed two major clusters each with 2 sub-clusters suggesting that genotypes from the sub-clusters could be used as distinct

parental groups in hybridization programs aimed at germplasm enhancement. Nonetheless, genetic enrichment programs like introduction could be done by getting plant material from other regions. Since there are many regions within the Sanaag region, Somaliland, where *B. sacra* is endemic, more research focussed on determination of the genetic structure and diversity status of these populations should be undertaken.

4.1 Introduction

Worldwide, woody plant species are facing threats such as climate change, habitat fragmentation, forest fires and unsustainable utilization by humans (Hall et al., 1996; Aerts et al., 2016). These threats have led to population decrease and as a result loss of genetic diversity (Ouinsavi et al., 2009) and this includes *B. sacra.* *Boswellia sacra* populations in Somaliland have been declining at an alarming rate due to unsustainable exploitation and its poor natural regeneration capacity. (Bongers et al., 2019; Groenendijk et al., 2012). The seeds have a very low germination rate, <10%, under controlled conditions (Swartout and Solowey, 2018). Poor tapping techniques coupled with frequent tapping have led to the failure of existing trees to regenerate. Improper tapping also leads to attacks by pests and diseases leading to eventual death of existing stands (Groenendijk et al., 2012). Young trees are also prone to grazing by wild and domestic animals reducing their chances of survival into mature trees. *Boswellia sacra* has been listed on IUCN's red list as near threatened (IUCN, 2020). Due to the disturbing current status of *B. sacra*, there is a need for conservation and domestication measures to be put in place. There is also need for the development of sustainable exploitation measures, or else the species might become extinct sooner than later (Bongers et al., 2019), thereby putting the livelihoods of thousands of Somali families that rely on frankincense value chains at stake.

For effective domestication and conservation purposes, it is crucial to determine genetic diversity for populations (Van Dyke, 2008). Genetic diversity is vital in enhancing germplasm improvement programs. Genetic diversity can be determined through morphological, biochemical and molecular markers. Major morphological differences between *B. sacra* trees have been described where the single and multiple stems were differentiated originating from the base and characteristic swelling at the base for trees growing on flat rock, faces of rock and cliffs (Svoboda et al., 2001; Thulin and Warfa 1987). *Boswellia sacra* is endemic in the Sanaag region eastwards towards the border with Puntland. This area is associated with frequent community clashes and for this reason, not much research has been done (DeCarlo et al., 2020). The morphological descriptions available have not been quantified. No information is available on the genetic diversity of *B. sacra* populations in Somaliland. In Oman, the genetic diversity between *B. sacra* populations in the Dhofar region using ISSR and ITS markers has been determined, where total genetic diversity (H_t) and population diversity (H_s) averaged at 0.22 and 0.136 respectively, which were fairly low. However, sequence related amplified polymorphism markers have not been previously used. This study thus sought to determine the genetic diversity within and between two populations of *B. sacra* from Af Yare Dawl-dawl and Exdad regions in El Afweyn, Somaliland through morphological, ISSRs and SRAP marker analysis in efforts to aid future germplasm enhancement, conservation and domestication.

4.2 Materials and Methods

4.2.1 Morphological characterization of *B. sacra* from Exdad and Af Yare Dawl-dawl sites

4.2.1.1 Study areas, sampling and data collection

Sampling was done for the B. sacra populations from Exdad and Af Yare Dawl-dawl, El Afweyn based on availability of leaves and seed from the respective trees but the trees must be at least 10 m apart. For both Exdad and Af Yare sites, collection was done in three quadrats as a representative for the sites and to avoid bias. In Exdad, 25 trees were sampled while in Af Yare Dawl-dawl, 32 trees were sampled. The two regions are characterized by a hot and dry climate. Trees grow wildly in rugged rocky valleys.

The type of tree base, type of stem, height to branching, number of stems and heights of the trees were recorded. Two types of stem bases were evident, swollen and normal bases and this was recorded for each tree. Tree height and height of stem(s) to first branching were recorded for each tree sampled. Two distinct growth surfaces were observed, with some of the trees growing on the edge of rocks and cliffs while the others grew on flat rock surfaces. Two types of stems were evident namely multiple and single stems at the base (Image 4.1). For trees that had multiple stems at the base, the number of stems was recorded. GPS coordinates of each tree were recorded.

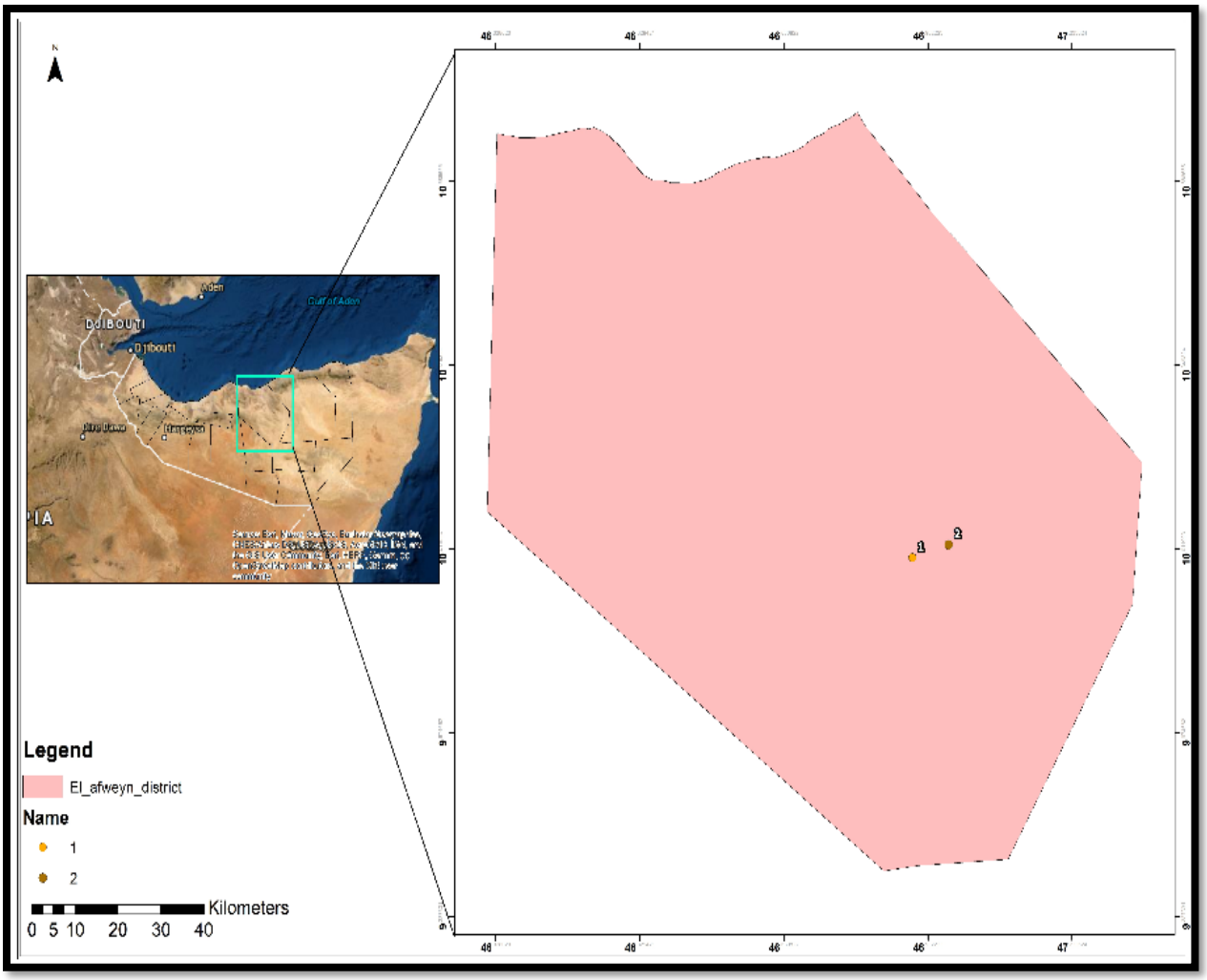


Figure 4.1: *B. sacra* sample collection sites in Cel Afweyn district 1). Exdad 2). Af Yare Dawl-dawl.

Image 4. 1: Morphological and growth surface for the *B. sacra* trees. a, c and d have multiple stems originating from base in contrast to b. a and d are growing on faces of a cliff and rock respectively while c is growing on a flat rock surface. Also note the bottom swelling in a and c.

4.2.2 Molecular characterization of *B. sacra* genotypes from Exdad and Af Yare Dawl-dawl

4.2.2.1 Sample collection

Young leaves were collected from 25 trees in Exdad and 32 trees from Af Yare Dawl-dawl regions in El Afweyn, Somaliland. Random sampling was used to select the trees and collect plant material from them since the trees were scattered on these two sites and the areas are prone to bandit attacks hence limiting proper sampling. Leaves were dried on silica gel and taken to Kenya Forestry Research laboratories, Muguga in Kenya for DNA extraction and molecular characterization.

4.2.2.2 DNA extraction and quantification

Leaves were removed from silica gel and kept in an oven at 40ºC for 24 hours. CTAB protocol (Doyle, 1991); was used for DNA extraction as follows: 0.2 g leaf tissue was ground using a mechanical grinder then transferred to 2 ml microfuge tubes with 500 µl isolation buffer (10% PEG, 0.35M sorbitol, 0.1 M Tris-HCL and 0.5% mercaptoethanol), mixed by vortexing, followed by centrifugation at 10000 rpm at 4ºC for 3 minutes. The supernatant was discarded, followed by addition of 800 µl IB and mixing by vortexing centrifugation at 10000rpm at 4 ºC for 3 minutes. This step was repeated 3 times. After removing supernatant IB, 800 µl CTAB solution (1% CTAB, 0.05 M Tris-HCL, 0.7 M NaCl, 0.5% mercaptoethanol and RNase) was added followed by incubation at 65 ºC for 45 min and 37 ºC for 45 min. Chloroform: isoamyl alcohol (CIA) at 24:1 ratio, 800 µl was added and mixed by inversion. Centrifugation was done at 14000 rpm for 10 mins RT and the upper aqueous phase transferred to a new 2 ml microfuge tubes. In the new microfuge tube, 800 µl of CIA was added and mixed by inversion for 10 mins followed by centrifugation at 14000 rpm at room temperature for ten mins. The upper aqueous phase was transferred into a new 1.5 ml microfuge tube. 8 µl 3 M NaOAc and isopropanol were added followed by centrifugation at 15000 rpm at 4 ºC for five minutes. All supernatants were discarded followed by washing of the DNA pellet with 800 µl ethanol and centrifugation at 15000 rpm at 4

ºC for 5 min. All the supernatant was discarded and the DNA pellet dried. The DNA pellet was dissolved in 100 µl DNase- free water. The extracted DNA was quantified using a nano-spectrophotometer and 1% w/V agarose gel, stained using cyber green. The gel was run in 0.5x TBE and 100 v for 45 mins.

4.2.2.3 ISSR primer optimization

Eight random DNA samples were selected used in optimization where 13 primers were screened (Table 4.1). The 12.5 µl reaction volume was used consisting 1 µl DNA, 0.3µl dNTPs, 0.08 µl *taq* polymerase, 0.13 µl BSA, 0.5 µl PVP, 2.71 µl PCR water and 0.4 µl primer. The PCR program used is initial denaturing at 95 ºC for 3 min. This was followed by 35 cycles of denaturation at 95 ºC for 30 sec, annealing at 47.5 ºC for 30 sec, extension at 72 ºC for 1 min. the final extension at 72 ºC for 10 min. PCR was done in a 96 well thermocycler (Applied Biosystems). PCR products were run in a 2% w/V agarose gel in 0.5x TBE buffer for 45 min and 100 v. Gel was stained using cybersafe dye. Visualization was done on UV transilluminator.

Table 4. 1: University of British Columbia (UBC) ISSR primers screened and their sequence

Primer	Primer sequence 5' to 3'
UBC 802	ATATATATATATATATG
UBC 806	TATATATATATATATAG
UBC 808	AGAGAGAGAGAGAGAGC
UBC 811	GAGAGAGAGAGAGAGAC
UBC 813	CTCTCTCTCTCTCTCTT
UBC 817	CACACACACACACACAA
UBC 818	CACACACACACACACAG
UBC 820	GTGTGTGTGTGTGTGTC
UBC 822	TCTCTCTCTCTCTCTCA
UBC 824	TCTCTCTCTCTCTCTCG
UBC 825	ACACACACACACACACT
UBC 829	TGTGTGTGTGTGTGTGC
UBC 849	GTGTGTGTGTGTGTGTYA

4.2.2.3 SRAP marker optimization

Two random DNA samples were selected and used to run PCR with 12 SRAP marker pairs (Table 4.2). The PCR reaction mixture in 15 µL total volume consisted 5 µL of PCR water, 2.5 µL PCR buffer, 2.5 µL MgCl 2 (25 mM), 1 unit taq polymerase, 0.5 µL dNTPs (10 µm), 1 µL forward primer, 1 µL reverse primer and 2 µL DNA (50 ng).

The PCR reactions were carried out using a thermocycler (Applied Biosystems ^{TM}) with an initial denaturation at 94 ºC for 5 min followed by 5 cycles of denaturation at 94 ºC for 1 min, annealing at 35 ºC for 1 min and extension at 72 ºC for 1 min. This was followed by 35 cycles of denaturation at 94 ºC for 1 min, annealing at 50 ºC for 1 min and extension at 72 ºC for 1 min. The final extension was carried out at 72 ºC for 10 min. The PCR products were then analysed on 2.5 % agarose gel stained with ethidium bromide in 1X TAE buffer for 3.5 h at 110 V. Fragment patterns were photographed under UV light for further analysis.

Table 4. 2: Primer sequences for SRAP primers used for *B. sacra* genotypes

Primer pair	Forward primer (5' to 3')	Reverse primer (5' to 3')
ME 1/ EM 7	TGAGTCCAACCGGATA	GACTGCGTACGAATTAGC
ME 1/ EM 11	TGAGTCCAACCGGATA	GACTGCGTACGAATTCCA
ME 2/EM 5	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTAAC
EM 2/EM 9	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTAAT
ME 5/EM 7	TGAGTCCAAACCGGAAG	GACTGCGTACGAATTAGC
ME 5/EM 9	TGAGTCCAAACCGGAAG	GACTGCGTACGAATTAAT
ME 8/EM 10	TGAGTCCAAACCGGCTG	GACTGCGTACGAATTCAG
ME 8/EM 12	TGAGTCCAAACCGGCTG	GACTGCGTACGAATTCTA
ME 11/EM 9	TGAGTCCAAACCGGTCC	GACTGCGTACGAATTAAT
ME 11/EM 11	TGAGTCCAAACCGGTCC	GACTGCGTACGAATTCCA
ME 12/ EM 12	TGAGTCCAAACCGGAGA	GACTGCGTACGAATTCTC
ME 12/EM 10	TGAGTCCAAACCGGAGA	GACTGCGTACGAATTCAG

4.2.3 Data analysis

4.2.3.1 Data collection and analysis for morphological data

Data collection was based on the following traits: type of tree base, type of stem, height to branching, tree height, number of stems and growth surface. Population means were calculated and standard deviations determined using GenStat© statistical software ver. 15 (Payne, 2009). Means were used as a descriptive tool without any statistical inferences. The correlation matrix between type of tree base (swollen or normal), type of stem (multiple or single), tree height, the number of stems, height to branching, and growth surface (edge of a cliff or flat rock) was determined. The morphological parameters were used to form a similarity matrix by the Euclidean method (Lele, 1993). This similarity matrix was used for hierarchical cluster analysis to generate a dendrogram using the nearest neighbour method in GenStat software.

4.2.3.2 Molecular data analysis

Amplified bands were scored as present (1) and regarded as dominant genetic markers while the latter scored as absent (0). The obtained SRAP profiles were converted to a binary matrix. For the interpopulation genetic analyses, band frequencies, estimated allele frequencies and estimated heterozygosity were calculated. The binary matrix was also used to generate a genetic distance trimatrix using GenALEx software (Peakall and Smouse, 2006). The genetic distance trimatrix was used for principal coordinate analysis.

Owing to degradation of DNA samples from Exdad, the samples from Af Yare Dawl-dawl were subdivided according to their stem morphology. The two sub-populations, trees with single stems and trees with multiple stems were used to generate a genetic distance matrix which was then subjected to an analysis of molecular variance (AMOVA) using GenALEx based on 999

permutations. AMOVA was used to determine molecular variance within and among the sub-populations.

4.2.3.3 Combined morphological and molecular data analysis

Trees with both morphological and molecular data were used to create a third data set, which were 31 trees from Af Yare Dawl-dawl. The six morphological measurements from the 31 individuals were subjected PCoA extraction using Infostat software (Di Rienzo et al., 2009) to generate six principal components. Nei's genetic matrix was calculated from the binary matrix to generate genetic distance matrix which was subjected to PCoA to generate 27 principal components. The principal components from molecular data and six from morphological data were combined into a single data set with 33 principal components. This data set was used for hierarchical cluster analysis to generate a dendrogram in GenStat software.

4.3 Results

4.3.1 Morphological characterization

4.3.1.1 Stem type

Two types of stems were observed, single stems and multiple stems originating from the base. Of the trees sampled in Af Yare Dawl-dawl, 60% had multiple stems while Exdad had 44% (Fig. 4.1). Overall, 55% of all the trees sampled had single stems 42% had multiple stems (Fig. 4.1).

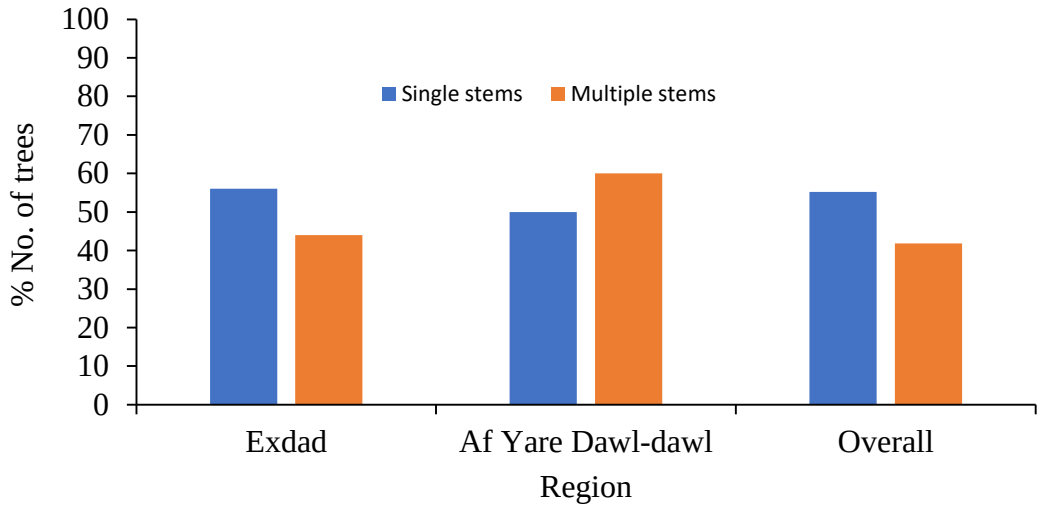


Figure 4.1: Distribution of single and multiple stems in *B. sacra* trees in Exdad and Af Yare Dawl-dawl.

4.3.1.2 Number of stems

The number of stems on trees with multiple stems ranged from 1 to 8. Trees sampled in Af Yare Dawl-dawl the mean number of 3.84 stems while Exdad, had a mean of 3.32 stems but they were not significantly different ($p > 0.05$, Appendix 11). Overall mean number of stems was 3.61 (Fig 4.2).

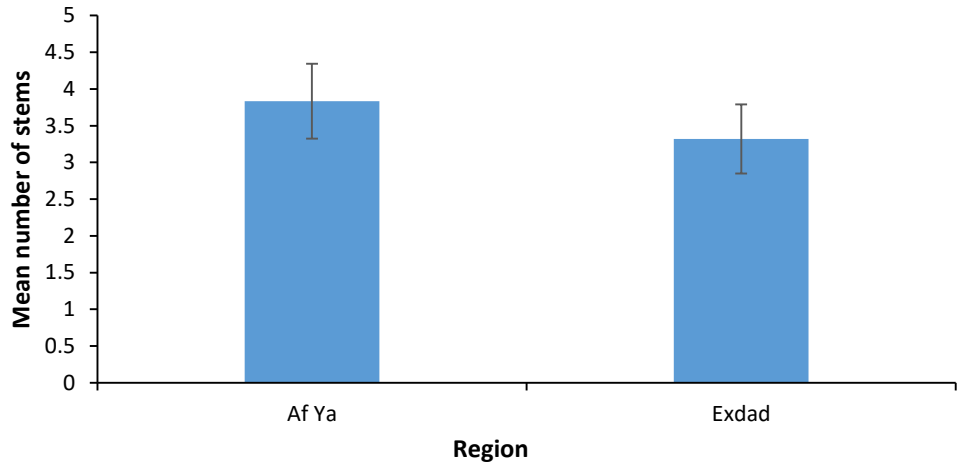


Figure 4.2: Mean number of stems on *B. sacra* trees sampled in Exdad and Af Yare Dawl-dawl.

4.3.1.3 Tree height

The mean height observed in Af Yare Dawl-dawl was 6.1 m while 5.9 m for Exdad but they were not significantly ($p>0.05$, Appendix 12) different.

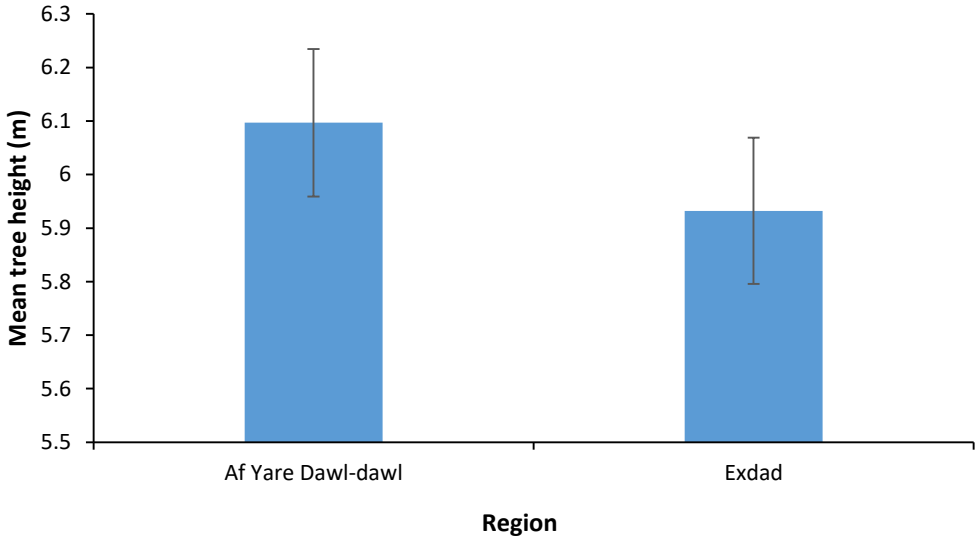


Figure 4.3: Performance in height for the sampled trees from Exdad and Af Yare Dawl-dawl.

4.3.1.4 Height to branching

The mean height to branching was 1.55 m on trees sampled in Af Yare Dawl-dawl while 1.79 m in Exdad but they were not significant (Fig. 4.4, Appendix 13) different.

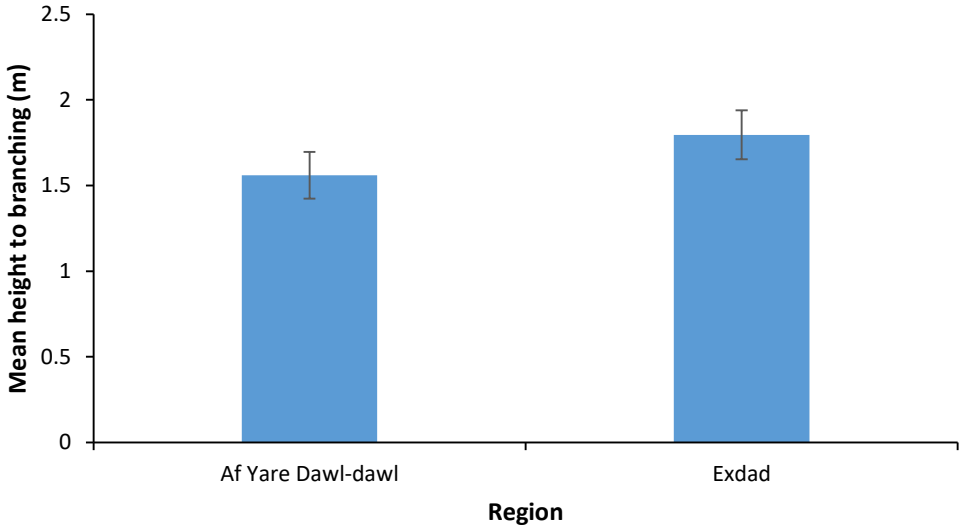


Figure 4.4: Mean height to branching on *B. sacra* trees in Exdad and Af Yare Dawl-dawl.

4.3.1.5 Swelling at the base of the stem

Swelling at the base of the stem was evident for both sites but in Exdad, most trees, 67.3% had a swollen base while Af Yare Dawl-dawl had 52% (Fig. 4.5). The mean number of trees in both regions with bottom swelling was 57.6%.

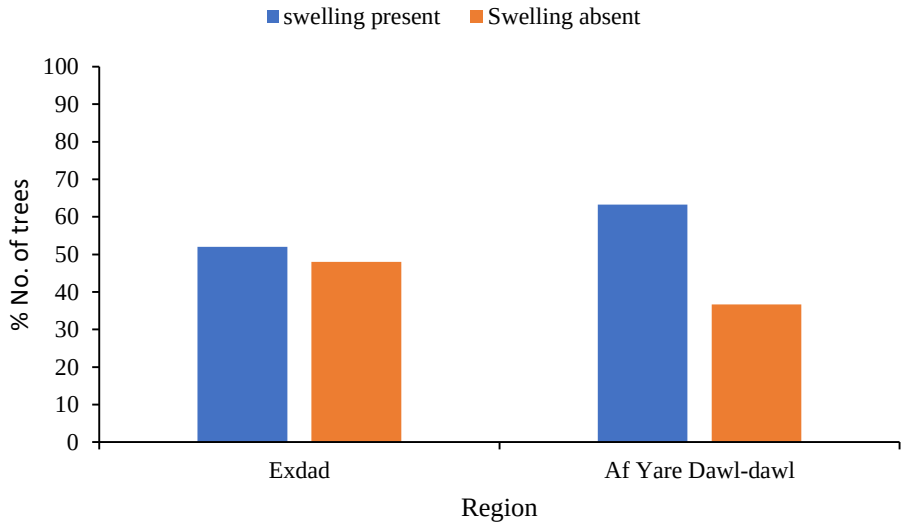


Figure 4.5: Percentage number of trees with and without base swelling in Exdad and Af Yare Dawl-dawl.

4.3.1.6 Growth surfaces of *B. sacra* trees in Exdad and Af Yare Dawl-dawl

In Exdad, 52% of trees sampled grew on cliffs while 48% on flat rock surfaces. In Af Yare Dawl-dawl, 63% of *B. sacra* trees grew on cliffs while 37% on flat rocks (Fig. 4.6).

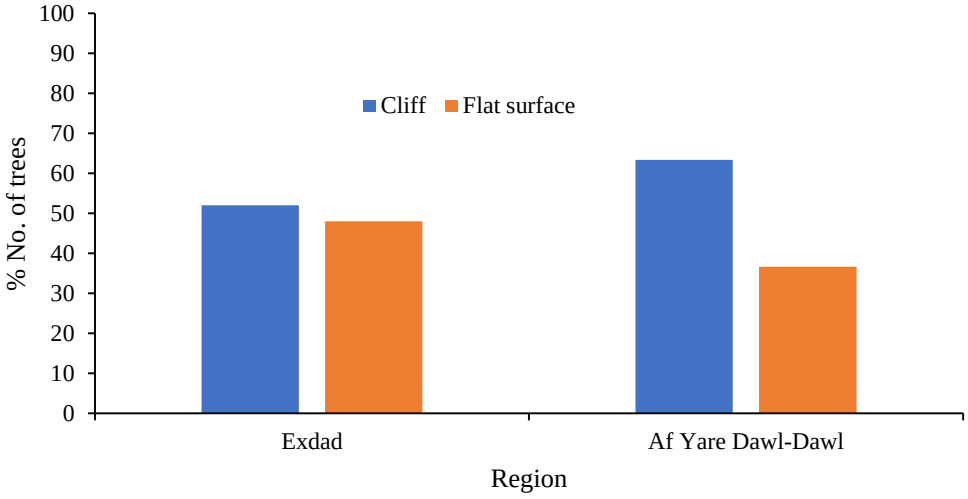


Figure 4.6: Growth surface characteristic of *B. sacra* trees in Exdad and Af Yare Dawl-dawl

4.3.1.7 Correlation between traits of *B. sacra* trees in Af Yare Dawl-dawl site

Strong correlation between stem type and height to branching was observed (r=0.75, p<0.05). There was also positive correlation between stem type and tree height, (r=0.56, p<0.05). Strong positive correlation was also observed between height to branching and tree height (r=0.75, p<0.05). Negative correlation was observed between number of stems and stem type (r=-0.87, p<0.05) as well as height to branching (r=-0.72, p<0.05)

Table 4.3: Correlation matrix between stem type, number of stems, tree height, bottom swelling and growth of *B. sacra* trees in Af Yare Dawl-dawl**.**

Correlation Matrix							
		Stem type	No of stems	Tree Height	Height to branching	Trunk swelling	Growth surface
Correlation	Stem type	1	-0.877	0.562	0.753	-0.198	0.367
	No of stems	-0.877	1	-0.424	-0.727	0.284	-0.356
	Tree Height	0.562	-0.424	1	0.767	-0.09	0.329
	Height to branching	0.753	-0.727	0.767	1	-0.1	0.307
	Trunk swelling	-0.198	0.284	-0.09	-0.1	1	0.282
	Growth surface	0.367	-0.356	0.329	0.307	0.282	1
Sig. (0.05)	Stem type		0	0.001	0	0.148	0.023
	No of stems	0		0.01	0	0.064	0.027
	Tree Height	0.001	0.01		0	0.319	0.038
	Height to branching	0	0	0		0.299	0.05
	swelling at the bottom	0.148	0.064	0.319	0.299		0.065
	Growth surface	0.023	0.027	0.038	0.05	0.065	

4.3.1.8 Correlation between traits of *B. sacra* trees in Exdad

In Exdad, the stem type was correlated positively to height to branching (r=0.75, p<0.05) and tree height (r=0.56, p<0.05). There was a strong correlation between tree height and height to branching (r=0.763, p<0.05). Negative correlation existed between number of stems and stem type (r= -0.82, p<0.05). Strong positive correlation was observed between the growth surface and trunk swelling (r=0.599, p<0.05). No notable correlation was observed between the other traits. (Table 4.4).

Table 4. 4: Correlation matrix between stem type, number of stems, tree height, bottom swelling and growth of *B. sacra* trees in Exdad

Correlation Matrix						
		Stem type	No. of stems	Tree height	Height to branching	Trunk swelling
Correlation	Stem type	1	-0.82	0.486	0.517	0.033
	No. of stems	-0.82	1	-0.446	-0.525	0.317
	Tree height	0.486	-0.446	1	0.763	0.169
	Height to branching	0.517	-0.525	0.763	1	0.005
	Trunk swelling	0.033	0.317	0.169	0.005	1
	Growth surface	-0.036	0.163	0.19	0.111	0.599
Sig. (0.05)	Stem type		0	0.007	0.004	0.438
	No. of stems	0		0.013	0.004	0.061
	Tree height	0.007	0.013		0	0.209
	Height to branching	0.004	0.004	0		0.49
	Trunk swelling	0.438	0.061	0.209	0.49	
	Growth surface	0.431	0.217	0.182	0.299	0.001

4.3.1.9 Correlation between traits of *B. sacra* trees in Exdad and Af Yare Dawl-dawl.

When all traits from both Exdad and Af Yare Dawl-dawl were pooled together, a strong positive correlation was observed between the stem type and tree height ($r=0.552$, $p<0.05$) as well as the height to branching ($r=0.672$, $p<0.05$). A strong negative correlation was present between the number of stems and the height to branching ($r=-0.65$, $p<0.05$) as well as stem type ($r=-0.878$ $p<0.05$). Strong positive correlations existed between tree height and stem type ($r= 0.672$, $p<0.05$) as well as the height to branching ($r=0.731$, $p<0.05$). No traits were correlated to trunk swelling as well as the growth surface.

Table 4. 5: Correlation matrix between stem type, number of stems, tree height, bottom swelling and growth of *B. sacra* trees in Exdad and Af Yare Dawl-dawl.

Correlation Matrix							
		Stem type	No. of Stems	Tree Height	Height to branching	Trunk swelling	Growth surface
Correlation	Stem type	1	-0.878	0.552	0.672	-0.121	0.212
	No. of Stems	-0.878	1	-0.414	-0.65	0.28	-0.178
	Tree Height	0.552	-0.414	1	0.731	0.01	0.212
	Height to branching	0.672	-0.65	0.731	1	-0.033	0.263
	Trunk swelling	-0.121	0.28	0.01	-0.033	1	0.434
	Growth surface	0.212	-0.178	0.212	0.263	0.434	1
Sig. (0.05)	Stem type		0	0	0	0.19	0.06
	No. of Stems	0		0.001	0	0.019	0.097
	Tree Height	0	0.001		0	0.47	0.06
	Height to branching	0	0	0		0.406	0.026
	Trunk swelling	0.19	0.019	0.47	0.406		0
	Growth surface	0.06	0.097	0.06	0.026	0	

4.3.1.10 Hierarchical cluster analysis for morphological data

Hierarchical cluster analysis of the principal components obtained from morphology data of Af Yare Dawl-dawl populations revealed two major clusters each with 2 sub-clusters. Most genotypes were concentrated in sub-cluster one. Similar clustering pattern was observed in hierarchical cluster analysis of morphological traits observed on *B. sacra* populations from Exdad (Fig 4.8).

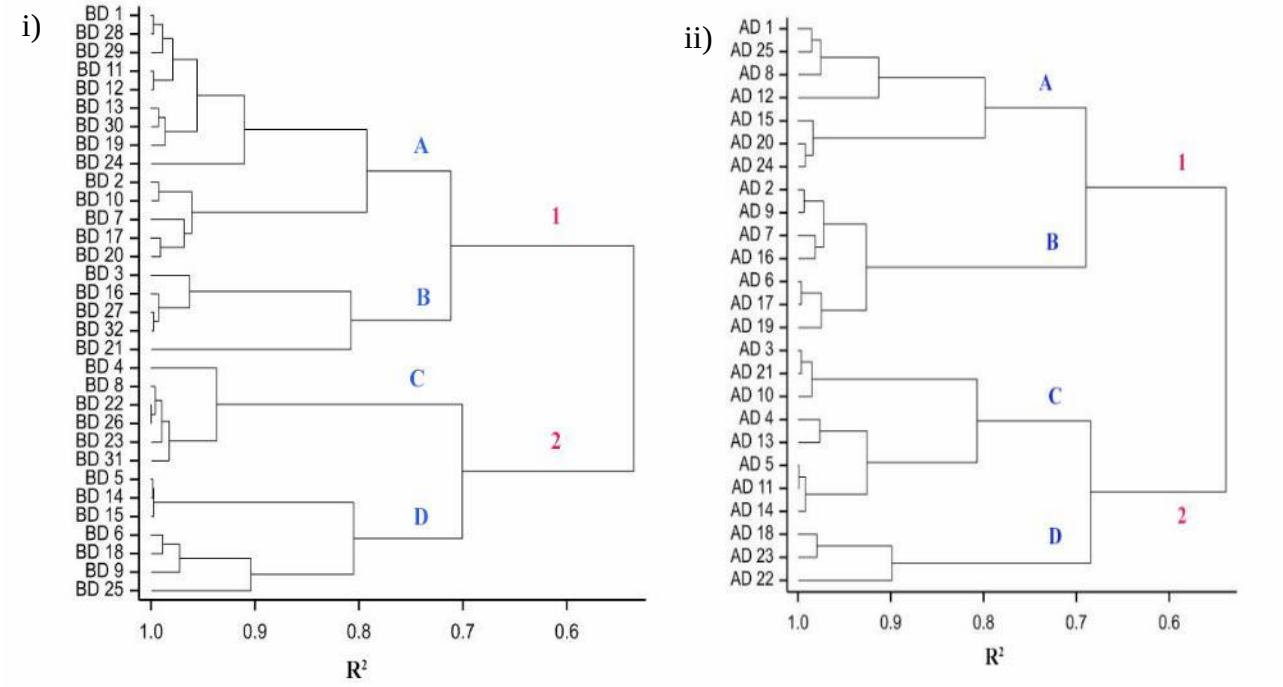


Figure 4.7: Dendrograms showing hierarchical cluster analysis of accessions from i) Af Yare Dawl-dawl and ii) Exdad. Both dendrograms show two main clusters (1 and 2) each with two sub-clusters (A, B, C and D).

When morphological data from Exdad and Af Yare Dawl-dawl populations was pooled and subjected to hierarchical cluster analysis, a dendrogram with two main clusters each with two distinct subclusters was obtained (Fig. 4.8). However, the clusters did not correspond to genotype origin. Genotypes from both regions were distributed in all clusters (Fig. 4.8).

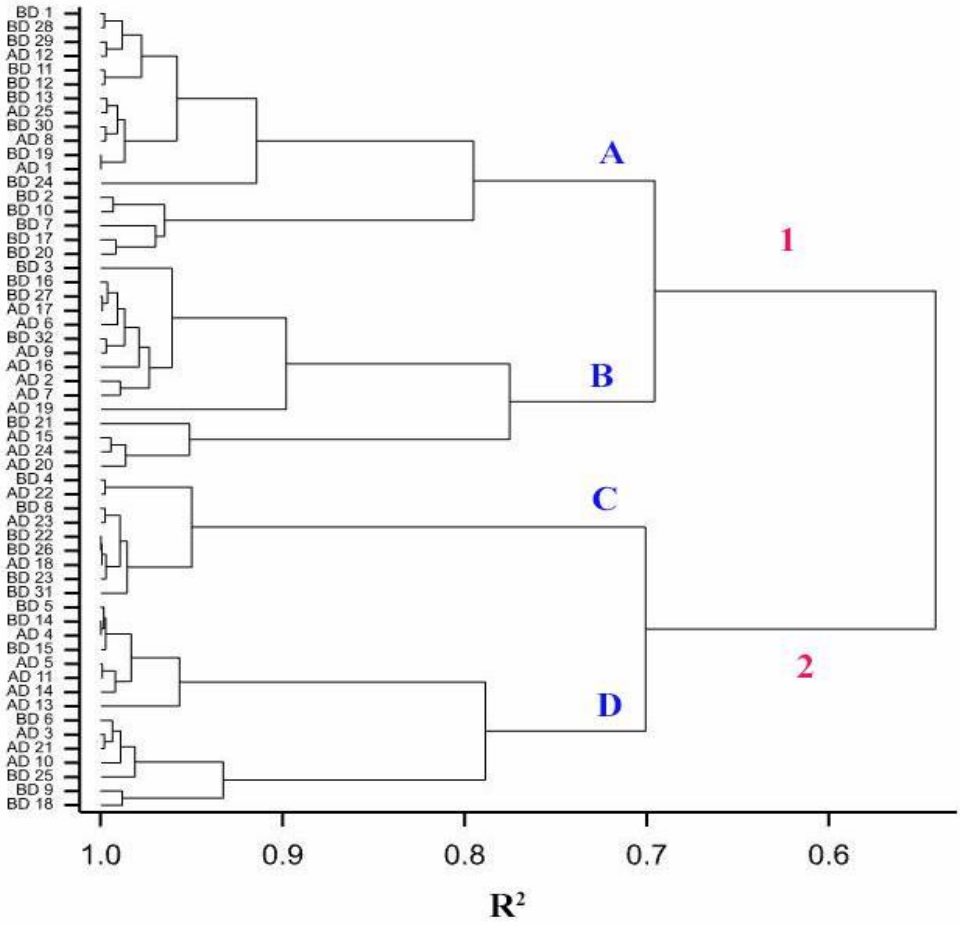


Figure 4.8: Dendrogram showing hierarchical cluster analysis of similarity matrix obtained from six morphological traits in *B. sacra* ecotypes from Af Yare Dawl-dawl (BD) and Exdad (AD) showing two major clusters (1 and 2) each with two (A, B, C, D) subclusters. All clusters consisted of genotypes from both Af Yare Dawl-dawl and Exdad.

4.3.2 Molecular characterization

4.3.2.1 DNA quantification

a) Agarose gel quantification

When the extracted DNA samples were run on 1% agarose gel with 100 bp ladder as the standard, almost all the samples did not pass the 1500 bp marker (image 4.2). This size is estimated to be 45 ng/µl. Thus, the concentration of extracted DNA for most samples was no less than 45 ng/l. The band intensity varied across all the samples. Most of the DNA extracted from samples collected in Exdad appeared degraded as evident in-band shearing patterns on the gels.

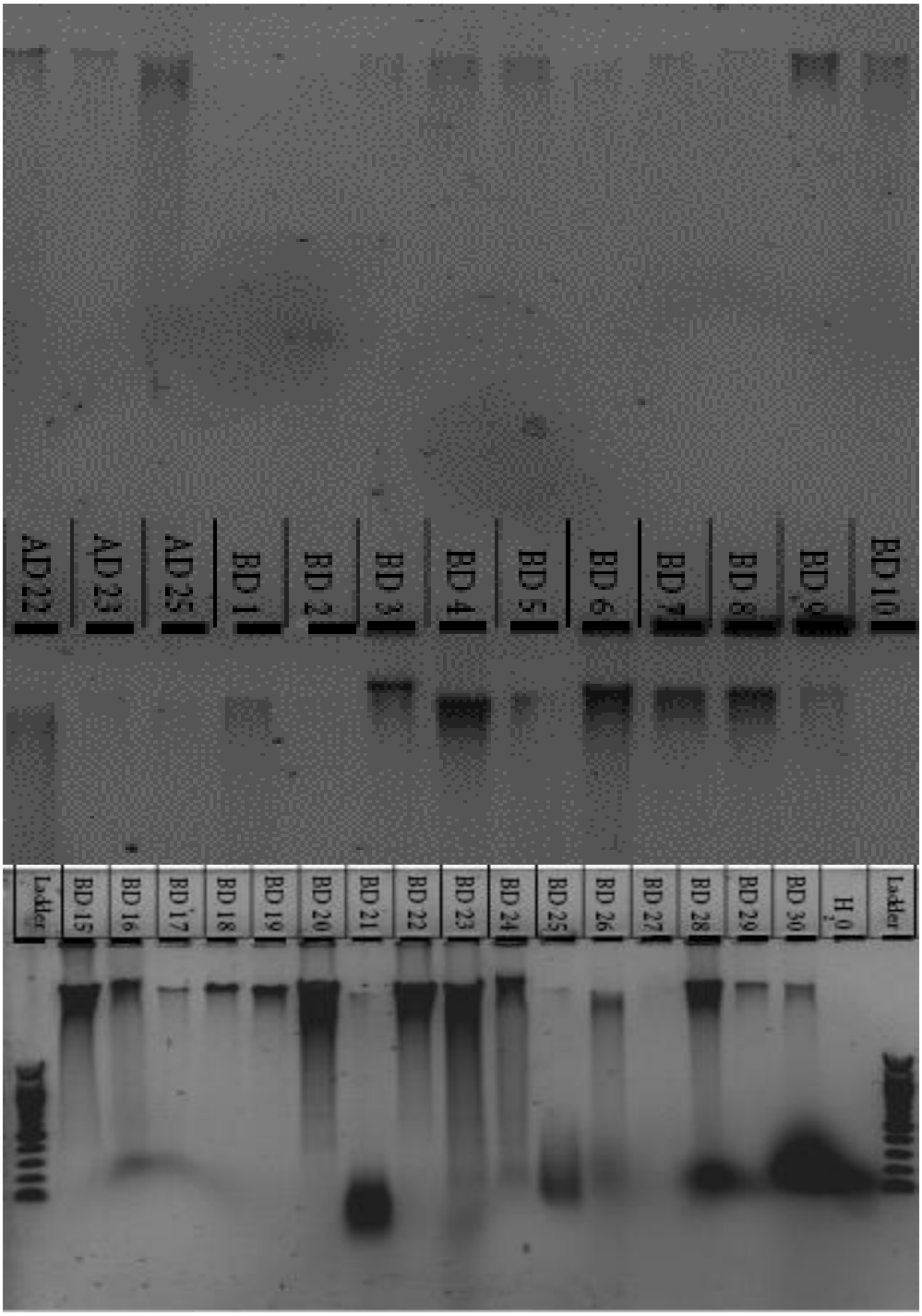


Image 4.2: Gel images showing the quantity of DNA from 55 samples from Af Yare Dawl-dawl (BD) and Exdad (AD). Note the smearing, an indication of DNA shearing due to degradation.

b) Nanodrop DNA quantification

Spectrophotometry revealed a high concentration of polyphenols with OD260/230 ranging from 0.4 to 1.11 while high protein levels with OD260/280 of 0.9 to 1.89 present in DNA (Table 4.5). OD260/230 is expected to range between 2 to 2.2 while OD260/280 from 1.8 to 2 for good quality DNA. DNA concentration ranged from 105 to 3046 ng/µl. However, the DNA was good enough for PCR for ISSR and SRAP marker analysis.

Table 4.6: Quantity and quality of DNA extracted from leaf samples collected in Af Yare Dawl-dawl (BD) and Exdad (AD).

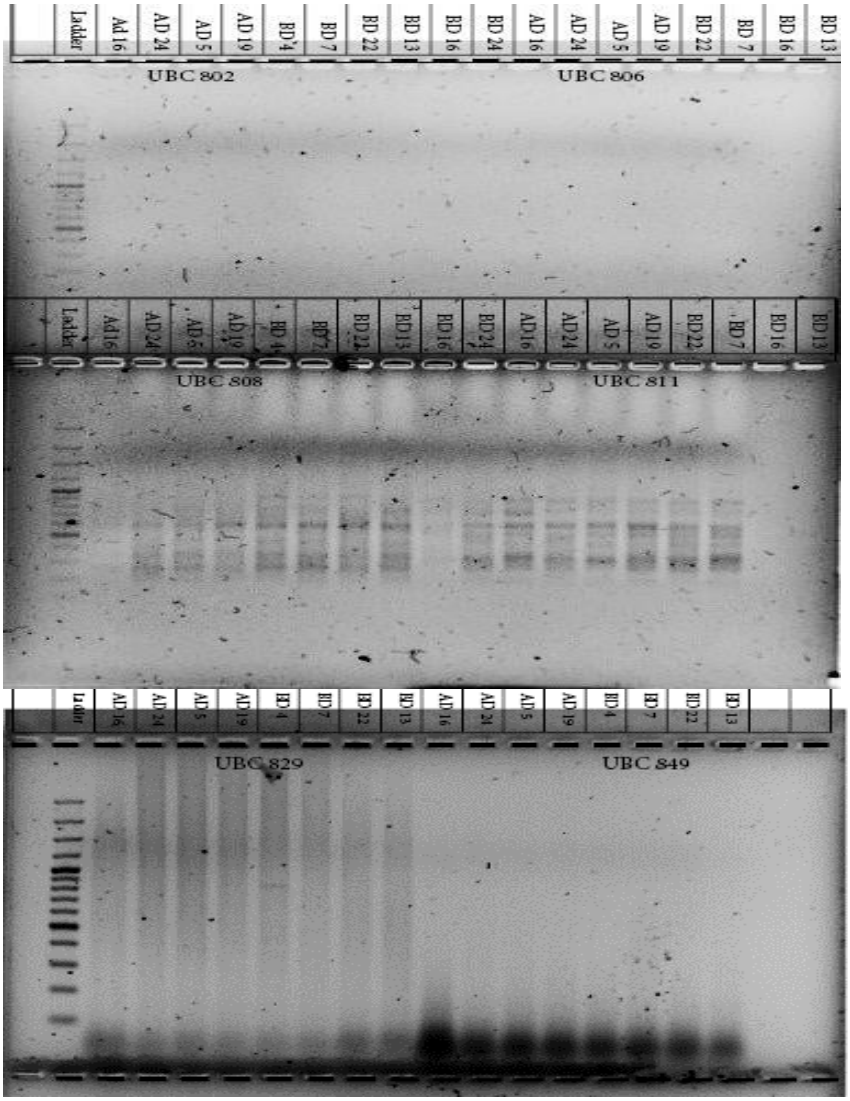
Sample ID	Nucleic Acid(ng/µl)	260/280	260/230	Sample ID	Nucleic Acid(ng/µl)	260/280	260/230
BD 1	612.5	1.8	1.12	AD 1	353.6	1.83	0.49
BD 2	202	1.11	0.54	AD 2	176.9	1.17	0.44
BD 3	201.7	1.87	1	AD 3	418.8	1.78	1.11
BD 4	1448	0.89	0.53	AD 4	447.1	1.48	0.64
BD 5	439.7	1.12	0.57	AD 5	378.8	1.66	0.84
BD 6	697.5	0.9	0.45	AD 6	550	1.69	1.01
BD 7	393.9	1.5	0.7	AD 7	225.1	1.23	0.52
BD 8	279.2	1.46	0.61	AD 8	181.9	1.69	0.83
BD 9	361.9	1.56	0.7	AD 9	754.4	1.63	0.9
BD 10	625.9	1.6	0.83	AD 10	387.7	1.51	0.77
BD 12	105	1.29	0.44	AD 11	549.9	1.18	0.48
BD 13	238.1	1.3	0.48	AD 12	464.8	1.57	0.82
BD 14	347.2	1.15	0.46	AD 13	361.7	0.97	0.46
BD 15	306.8	1.46	0.61	AD 14	298.2	1.37	0.53
BD 16	569.9	1.29	0.49	AD 15	363	1.42	0.67
BD 17	338.7	1.69	0.92	AD 16	395.4	1.48	0.7
BD 18	487.5	1.25	0.49	AD 17	466	1.76	1.11
BD 19	449.7	1.29	0.46	AD 18	741.2	0.95	0.41
BD 20	370.7	1.26	0.55	AD 19	458.7	1.09	0.59
BD 21	356.1	1.21	0.57	AD 20	154.6	1.33	0.43
BD 22	613.1	1.46	0.62	AD 21	993.5	1.08	0.44
BD 23	504	1.39	0.59	AD 22	347.9	1.63	0.75
BD 24	267.9	1.29	0.56	AD 23	119.5	1.34	0.56
BD 25	419.8	1.46	0.65	AD 24	171.1	1.4	0.4
BD 26	3046.9	1.89	1.65	AD 25	1722.5	0.84	0.6
BD 27	367.5	1.62	0.85				
BD 28	313.4	1.6	0.78				
BD 29	226.5	1.38	0.54				
BD 30	220.5	1.61	0.88				
BD 31	255	1.36	0.92				
BD 32	301.9	1.55	0.55				

4.3.2.1 ISSR primer optimization

Thirteen UBC ISSR primers were screened and only 8 produced polymorphic bands (Image 4.3). These bands, however, were not clear enough and thus could not be used effectively for diversity studies (Table 4.6).

Table 4.7: ISSR primer optimization results

Primer	Primer sequence 5' to 3'	Remarks
802	ATATATATATATATATG	No bands
806	TATATATATATATATAG	No bands
808	AGAGAGAGAGAGAGAGC	Polymorphic bands
811	GAGAGAGAGAGAGAGAC	Polymorphic bands
813	CTCTCTCTCTCTCTCTT	Polymorphic bands
817	CACACACACACACACAA	Polymorphic bands
818	CACACACACACACACAG	Polymorphic bands
820	GTGTGTGTGTGTGTGTC	Smears, no distinct bands
822	TCTCTCTCTCTCTCTCA	Clear polymorphic bands
824	TCTCTCTCTCTCTCTCG	Polymorphic bands
825	ACACACACACACACACT	Polymorphic bands
829	TGTGTGTGTGTGTGTGC	Smears, no bands
849	GTGTGTGTGTGTGTGTYA	Smears, no bands


Ladder
Ad16
AD24
AD5
AD19
BD4
BD7
BD22
BD13
BD16
BD24
AD16
AD24
AD5
AD19
BD22
BD7
BD16
BD13
UBC 802
UBC 806
Ladder
Ad16
AD24
AD5
AD19
BD4
BD7
BD22
BD13
BD16
BD24
AD16
AD24
AD5
AD19
BD22
BD7
BD16
BD13
UBC 808
UBC 811
Ladder
AD16
AD24
AD5
AD19
BD4
BD7
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BD13
AD16
AD24
AD5
AD19
BD4
BD7
BD22
BD13
UBC 829
UBC 849

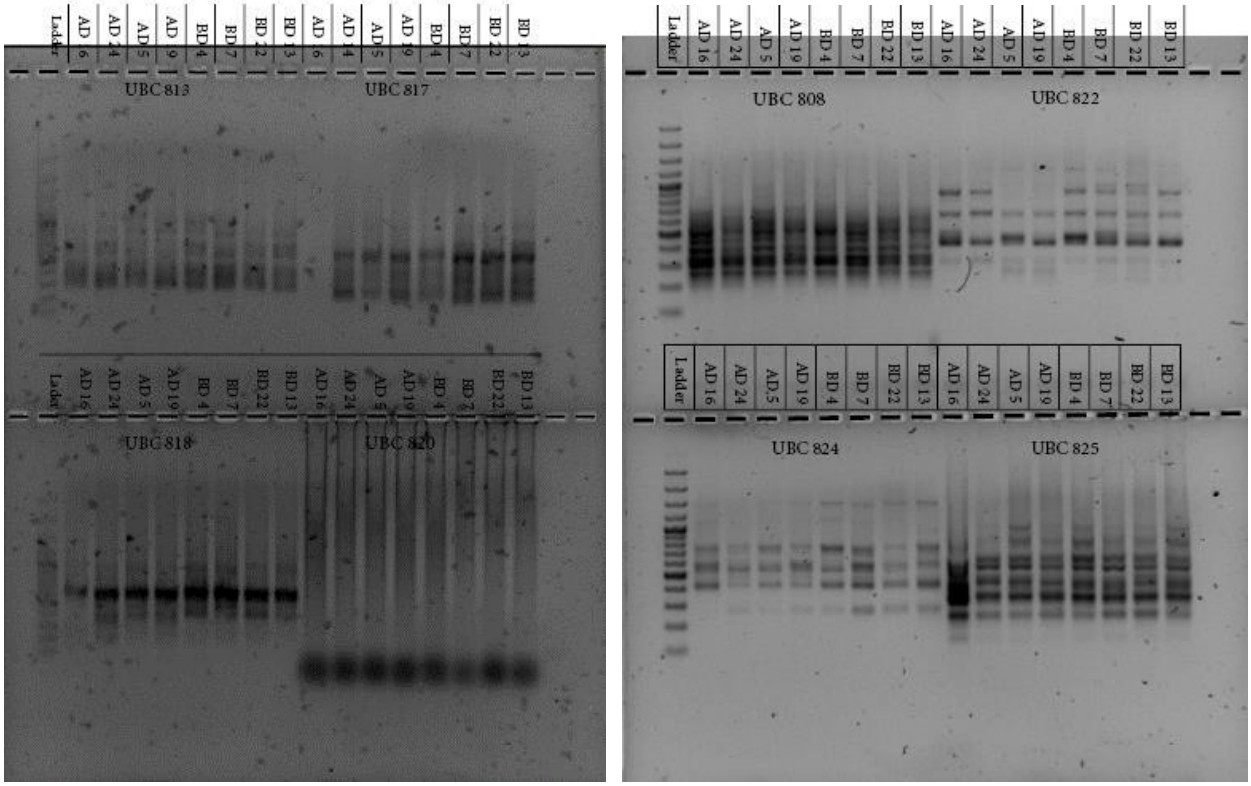

Image 4.3: Gel images showing polymorphism and amplification of UBC ISSR primers 808, 822, 824, 825, 802, 806, 808, 811, 813, 817,818, 820, 829 and 849. Although there were several primer amplifications, note the inconsistencies in band intensity.

Due to inconsistencies in ISSR PCR results of the DNA samples, SRAP markers were used for genotyping.

4.3.2.2 SRAP marker optimization

From 12 primer pairs screened, 8 combinations produced clear polymorphic bands suitable for diversity analysis (Table 4.8, Image 4.4).

Table 4. 8: SRAP primer pairs selected for assessment of genetic diversity for *B. sacra* genotypes

Primer pair	Forward primer (5' to 3')	Reverse primer (5' to 3')
ME 1/EM 7	TGAGTCCAACCGGATA	GACTGCGTACGAATTAGC
ME 1/EM 11	TGAGTCCAACCGGATA	GACTGCGTACGAATTCCA
ME 2/EM 9	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTAAT
ME 8/EM 12	TGAGTCCAAACCGGCTG	GACTGCGTACGAATTAAT
ME 8/EM 10	TGAGTCCAAACCGGCTG	GACTGCGTACGAATTCAG
ME 11/EM 11	TGAGTCCAAACCGGTCC	GACTGCGTACGAATTCCA
ME 12/EM 10	TGAGTCCAAACCGGAGA	GACTGCGTACGAATTCAG
ME 12/EM 12	TGAGTCCAAACCGGAGA	GACTGCGTACGAATTCTC

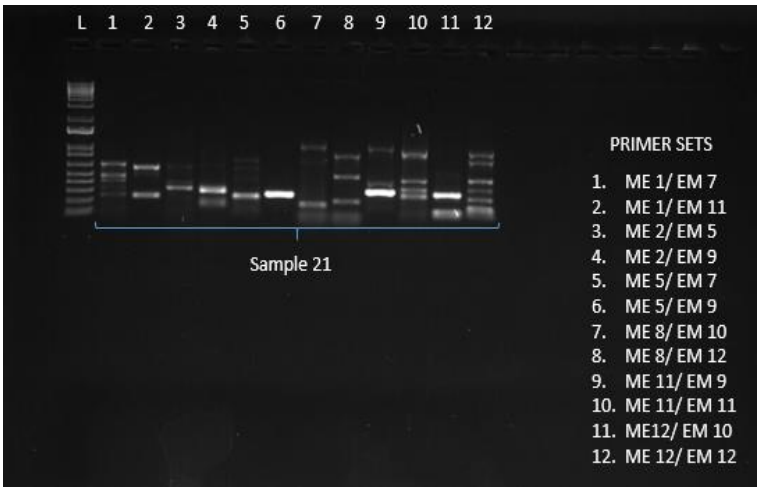

Image 4.4: Gel image of 12 SRAP marker pairs over two samples with primers 1, 2, 4, 7, 8, 10, 11 and 12 showing polymorphism.

4.3.2.3 SRAP marker analysis

A total of 152 clear DNA fragments were amplified from 31 samples from Af Yare Dawl-dawl. No amplification was observed on samples from Exdad. The percentage polymorphic loci were 31.58%. The number of different alleles (Na) was 0.632, number of effective alleles (Ne) 1.078

while the expected heterozygosity (He) and Shannon's information index (I) were 0.053 and 0.09 respectively (Table 4.8).

Table 4. 9: Mean and SE over loci for *B. sacra* genotypes from Af Yare Dawl-dawl.

	N*	Na*	Ne*	I*	He*
Mean	31.000	0.632	1.078	0.090	0.053
SE	0.000	0.076	0.015	0.014	0.009

*Na = No. of different alleles, Ne = No. of effective alleles, I = Shannon's information index, He = Expected heterozygosity.

The genetic data matrix obtained from the binary SRAP matrix was used for PCoA. The percentage variation explained by the first three axes after principal coordinate analysis was 35.14%, 11.42% and 9.08% respectively grouping the genotypes into three major clusters (Fig. 4.9).

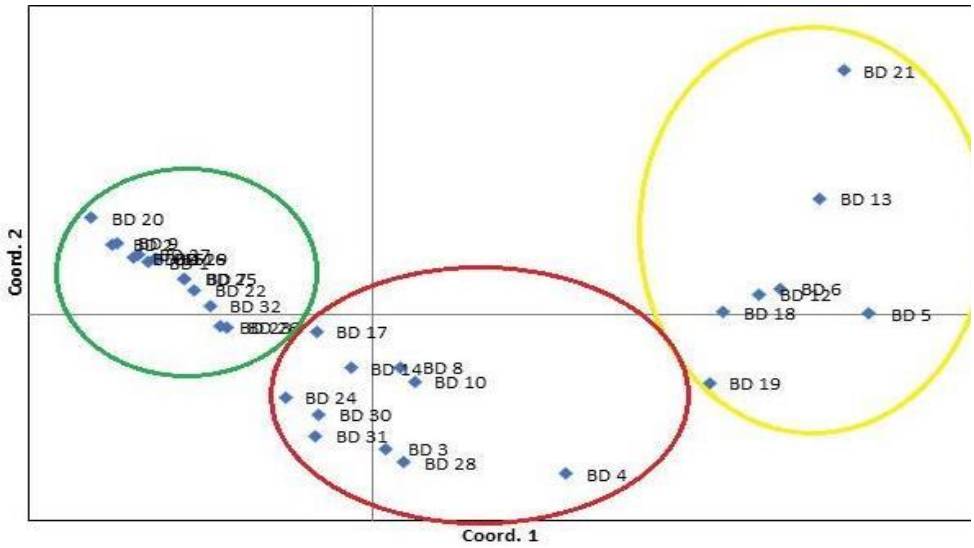

Figure 4.9: PCoA analysis from genetic data matrix of 31 *B. sacra* genotypes from Af Yare Dawl-dawl with three main clusters.

Unweighted neighbour joining dendrogram revealed three main clusters (Fig 4.10). One of the clusters had only one genotype while the second and third clusters consisted of 15 genotypes each. The second and third clusters consisted several subclusters (Fig. 4.10)

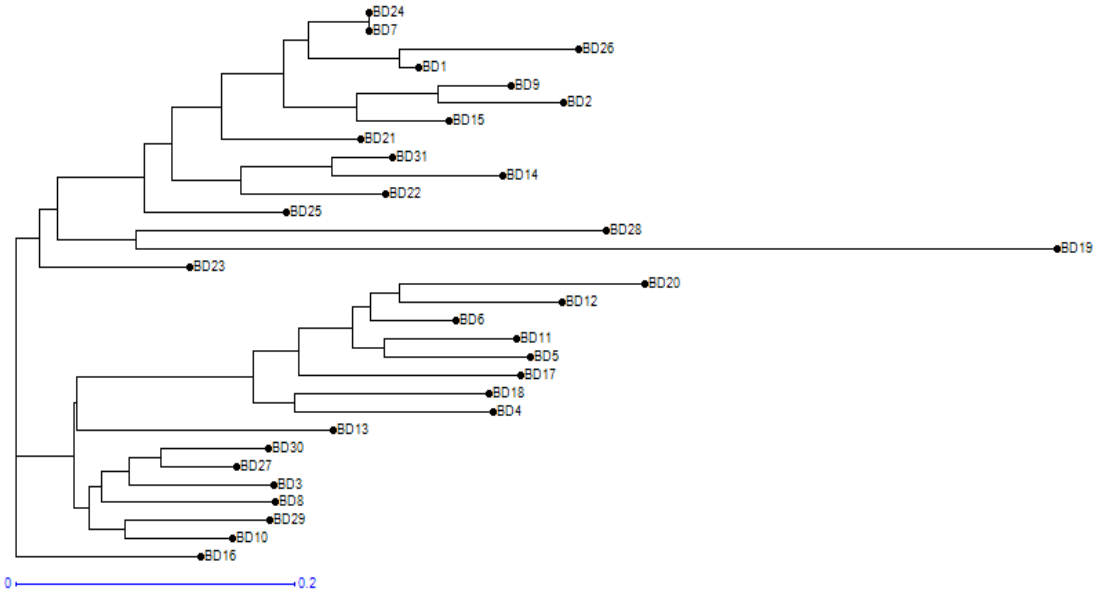

Figure 4.10: Unweighted neighbour-joining dendrogram for 31 B. sacra accessions from Af Yare Dawl-dawl.

a) SRAP marker analysis for Af Yare Dawl-dawl subpopulations

The 31 *B. sacra* genotypes from Af yare Dawl-dawl were grouped into 2 sub-populations consisting trees with multiple and single stems. Genetic distance between the two sub-populations (Nei's distance) was 0.04. Nei's genetic identity was 0.996. The observed number of alleles (Na) was 0.454 while effective number of alleles (Ne) was 1.087, expected heterozygosity (He) was 0.056 While Nei's gene diversity and Shannon's information index (I) was 0.09 for the genotypes with multiple stems. For trees with single stems the observed number of alleles (Na), effective number of alleles (Ne), expected heterozygosity (He), and Shannon's information index were 0.539, 1.069, 0.056 and 0.079 respectively (Table 4.9).

Table 4.10: Mean and SE over loci for multiple-stemmed and single-stemmed genotypes from Af Yare Dawl-dawl

Population		N*	Na*	Ne*	I*	He*
Multiple stems	Mean	12.000	0.454	1.087	0.090	0.056
	SE	0.000	0.067	0.017	0.015	0.010
Single stem	Mean	19.000	0.539	1.069	0.079	0.046
	SE	0.000	0.072	0.015	0.013	0.009
Total	Mean	15.500	0.497	1.078	0.084	0.051
	SE	0.201	0.049	0.011	0.010	0.007

*Na = No. of different alleles, Ne = No. of effective alleles, I = Shannon's information index, He = Expected heterozygosity.

Analysis of molecular variance between the two sub-groups showed that there was no variation among the two sub-populations and 100% variation within the sub-populations (Table 4.10).

Table 4.11: Analysis of variance for single-stemmed and multi-stemmed *B. sacra* genotypes from Af Yare Dawl-dawl.

Source	df	SS	MS	Est. Var.	%
Among Pops	1	4.051	4.051	0.000	0%
Within Pops	29	159.303	5.493	5.493	100%
Total	30	163.355		5.493	100%

Principal coordinate analysis based on genetic similarity coefficient matrix of the 2 sub-populations =11.42% and 9.08% of the total genetic variation leading to three main clusters each comprising genotypes with single and multiple stems (Fig. 4.9).

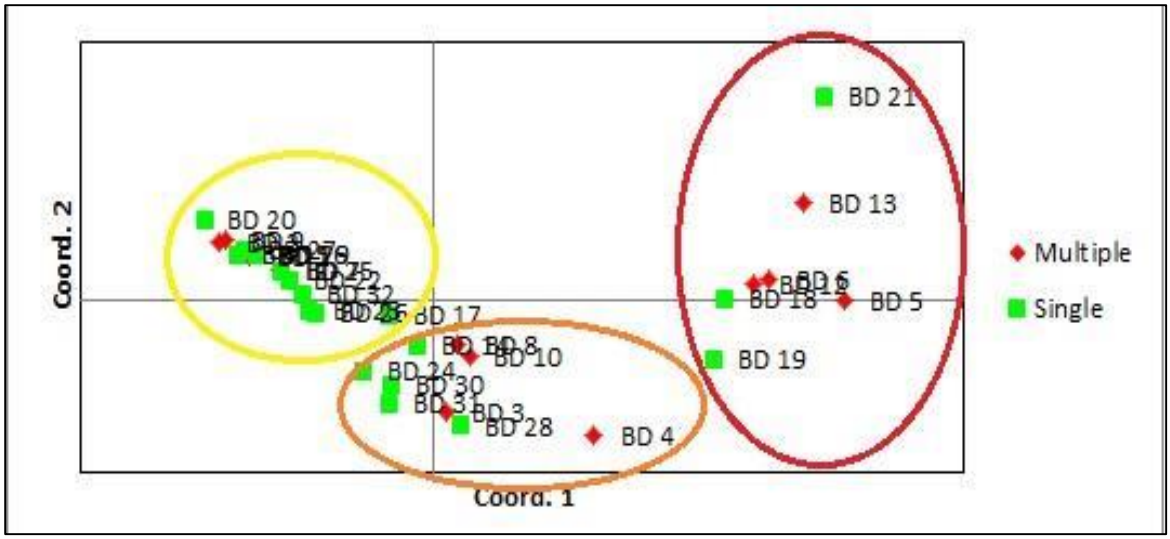

Figure 4.11: Principal coordinates analysis from genetic distance matrix of B. sacra genotypes with multiple and single stems from Af Yare Dawl-dawl.

4.3.3 Combined molecular and morphological characterization

Six principal components extracted from morphological data merged with 27 components extracted from the genetic distance matrix data formed a principal component matrix with 33 components. These components were subjected to hierarchical cluster analysis to generate a dendogram. Two main clusters each with two sub-clusters were generated. It was observed that 80.65% of the genotypes were grouped into one cluster “1”.

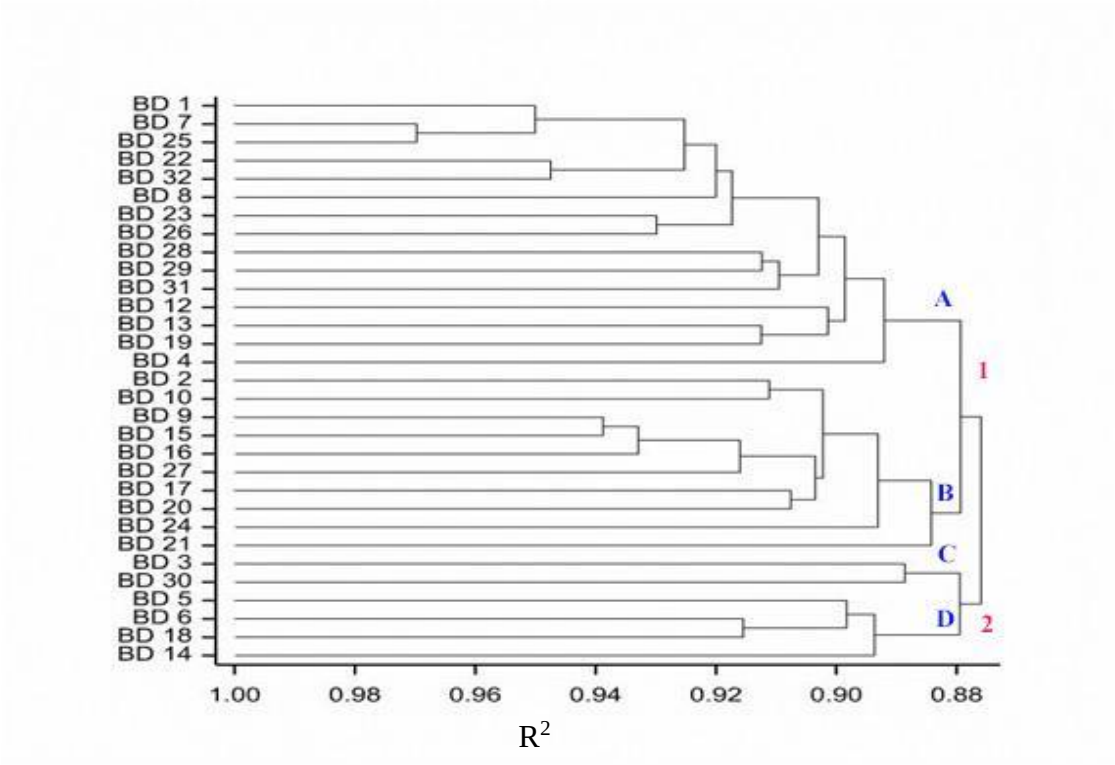

Figure 4.12: Dendrogram showing hierarchical cluster analysis of a combined data set consisting morphology and molecular attributes of B. sacra genotypes from Af Yare Dawl-dawl. Two main clusters (1 and 2) each having two subclusters (A, B, C and D) are shown. Most genotypes fell on cluster 1.

4.4 Discussion

4.4.1 Morphological diversity

Morphological parameters used in this study failed to discriminate distinctively *B. sacra* genotypes from Exdad and Af Yare Dawl-dawl since no differences were observed. However, correlation between traits revealed that the presence of one trait affected the expression of other traits. Tree height was dependent on stem type whereby those with multiple stems were shorter compared to trees with single stems suggesting that these two parameters could be used for tree selection to enhance breeding programs. Many stems are advantageous especially in cases where stems die due to biotic or abiotic stress, the surviving stems will ensure that the tree continues growing (Götmark et al., 2016). In sustainable frankincense harvesting, some stems could be left untapped for a season allowing for regeneration and seed production (DeCarlo et al., 2018; Lemenih and Kassa 2011).

The number of stems was strongly correlated to tree height, r=0.5, suggesting that selection of both height and number of stems concurrently could be done in breeding programs. Height to branching was also strongly correlated to tree height, r=0.7 indicating that these two parameters could be selected together in a breeding program (de Souza et al., 1998; Toker & Ilhan Cagirgan, 2004). However, the negative correlated traits observed could complicate selection programs because they imply an unfavourable response in one trait while selecting for the other (Bernardo, 2002; Neyhart et al., 2019). Such antagonistic traits have been observed in other breeding programs, posing setbacks to breeders, for instance, seed oil and seed protein content in soybean (Bandillo et al., 2015) and grain yield and grain protein content in wheat (Simmonds, 1995)

The weak correlation between swollen tree trunks and growth surface suggests that there was no relationship between trunks that grew on cliffs and those that grew on flat rock. Thulin and Warfa

(1987) described a *B. sacra* tree growing on a vertical limestone rock face with a swollen bottom. Similar trunk swelling habits are reported on *B. occulata* growing on rock edges (Thulin et al., 2019) and *B. frerreana* (Thulin and Warfa 1987), which could be not only be attributed to anchorage of trees on steep rock surfaces but as well as storage features for water, owing to the arid conditions where this species is distributed (Bobich & North, 2009).

Hierarchical cluster analysis did not classify genotypes according to their geographical location therefore indicating that there were few similarities in their genetic composition. Variation within the populations was observed but it was not high, suggestive of phenotypic plasticity since clustering did not distinguish between them. Phenotypic plasticity is the existence of more than one form of morphology in a single genotype in response to environmental conditions, which could have played part in expression of *B. sacra* genotypes (West-Eberhard, 1989; Whitman and Agrawal, 2009). Another reason could be attributed to the few traits used for clustering, which could have affected the groupings since only 6 traits were used to determine these groupings as a result low discriminative power (Babic et al., 2016; Chesnokov et al., 2020). This is evident as genotypes from both regions did not group according to their morphological traits. Future studies should therefore involve more traits for better clustering results.

4.4.2 DNA quality was affected by polyphenolic compounds and moisture build-up in silica gel

The quality of DNA of *B. sacra* leaves was affected by polyphenol levels from spectrophotometry readings, which were below optimum values of 260/230=1.8-2 while those of this study ranged from 260/230= 0.4-1.11. Polyphenols are known to affect DNA quality in plants during extraction (Varma et al., 2007). During lysing stage of DNA extraction, polyphenols are released from cell

vacuoles and are readily oxidized by cellular oxidases. Oxidized polyphenols may interreact with nucleic acids affecting their integrity (Couch and Fritz 1990; Varma et al., 2007). Although a modified CTAB protocol was used for *B. sacra* for the extraction process, it was not effective. This could also have been affected by the age of the leaf samples since younger leaves are reported to contain low levels of polyphenols to inhibit extraction. Despite existence of many plant DNA extraction protocols, it is concluded that none can be used effectively across all species (Varma et al., 2007) due to variation in factors that affect DNA quality including plant age.

Gel electrophoresis revealed that DNA from Exdad samples was degraded evident on persistent smearing, in addition to lack of amplification when subjected to PCR using SRAP markers. The degradation could be attributed to moisture build up in the silica gel during transport since the container with Exdad samples had initially been opened at the airport for security checks when going for sampling. Most DNA extraction methods require fresh leaves to be dried immediately using liquid nitrogen or freezing at -80 ºC, though rapidly drying leaves in silica gel can also be done in cases where the equipment is not available or where sample collection is in far and remote areas (Aljanabi and Martinez, 1997).

4.4.3 SRAP markers generated polymorphism for Exdad DNA samples

Inter-simple sequence repeats markers did not amplify the DNA from either Exdad or Af Yare, which could have been affected by high polyphenol content in the samples (De Boer et al., 1995; Varma, 2007) despite inclusion of PVP in the master mix (Zhang et al., 2007). Since none of the ISSRs used by Coppi et al., (2010) were screened, it cannot be concluded that ISSRs are ineffective and more ISSRs could be included in future studies. On the other hand, SRAP markers amplified these samples producing clear reproducible polymorphic bands for *B. sacra* suggesting that they

were effective for genotyping the species. A total of 152 scorable bands were amplified for the 31 samples from Af Yare Dawl-dawl which is within the range of other tropical woody species like *Tectona grandis* (Thakor et al., 2019) and eucalyptus (Zhang et al., 2010).

4.4.4 Low heterozygosity of the *B. sacra* genotypes indicated low genetic diversity

Total heterozygosity (H_e =0.053) within genotypes from Af Yare Dawl-dawl was low compared to He=0.22 from Oman as described in previous study (Coppi et al. 2010). Nonetheless, it is reported that long-lived species which have out-crossing for reproduction, with seed dispersed by wind or water have H_e values above 0.136 (Nybom, 2004) but what was observed in *B. sacra* in Af Yare Dawl-dawl was lower (H_e =0.053). In Af Yare Dawl, *B. sacra* trees are found at the bottom and slopes of steep valleys with an estimated population size of less than 80 trees, which could have contributed to the low genetic diversity. The level of heterozygosity in small populations decreases due to increased incidences of inbreeding among genotypes of common ancestry and this could have been the case for Af Yare Dawl-dawl (Young et al., 1996). In species that exhibit self-incompatibility mechanisms, of which *B. sacra* is thought to be (Coppi et al., 2010) inbreeding may lead to increased instances of sterility thereby reducing chances of establishment of new stands (Glémin et al., 2001). Increased inbreeding for small populations could have influenced the population fitness due to increased homozygosity leading to an increased frequency of deleterious alleles (Coppi et al., 2010; Ellstrand and Elam, 1993). Thus, the current stands may be unable to adapt in a bid to overcome biotic and abiotic stresses. Decreased variability also increases the chances of extinction in contrast to species with high variability (Huenneke, 1991). Therefore, a quick response is needed for the conservation of *B. sacra* populations in Af Yare Dawl-dawl.

4.4.5 Sub-clustering in Af Yare Dawl-dawl population indicated variation for use in hybridization programs

The analysis of the overall pattern of genetic diversity among germplasm accessions facilitates the selection of parents with diverse genetic background. The genetic diversity of the base population determines the success of the breeding program (Subramanian & Subbaraman, 2010). Cluster analysis provides an efficient mechanism for highlighting the nature of relationship between any genotypes within the population (Osawaru et al., 2015). In this study, hierarchical cluster analysis of genetic distance matrix obtained from SRAP marker analysis revealed three main clusters. Two of these three clusters had subclusters within which the genotypes were distributed at varying coefficients. Therefore, these genotypes have different parentage origins, an indicator of a diverse genetic base. This shows that despite the low heterozygosity observed, the genotypes from the different clusters that show greater variation can be used as progenitors in hybridisation and domestication programs in Af Yare Dawl-dawl.

4.5 Conclusion and recommendations

Genetic diversity studies on *B. sacra* populations from Af Yare dawl-dawl and Exdad, El Afweyn using morphological traits revealed small genetic differences between the two regions. However, SRAP marker analysis of genotypes from Af Yare Dawl-dawl revealed very low heterozygosity, an indication of very low variability. These stands are vulnerable to environmental change and a sudden change in climatic conditions could lead to their extinction. Radical measures should be taken immediately for conservation. Bringing in stands from other populations may enrich the genetic basis of this population. This will reduce risk of bottlenecks and genetic drift, eventually reducing the risk of extinction as a result of environmental changes.

The Sanaag region in Somaliland has many areas where *B. sacra* grows. However, due to security situation in this region, particularly in the South, not much research and documentation has been done concerning the exact population status and utilisation trends. More research should be done to ensure the genetic diversity of *B. sacra* populations from all Somaliland is established. This will give a clearer picture on the diversity status which could lead to better decision making on conservation and establishment of breeding programs. Meanwhile, it is crucial to enforce sustainable exploitation measures.

CHAPTER FIVE

5.0 General discussion, conclusion and recommendations

Boswellia sacra is an important non-timber forest product (NTFP) resource that provides a livelihood to a large number of communities and families in Somaliland (Farahani et al., 2008; Farah, 2008). However, due to overexploitation and poor natural regeneration, the species faces extinction if appropriate conservation and sustainable exploitation measures are not put in place (DeCarlo et al., 2020; Bongers et al., 2019; DeCarlo et al., 2018). Some conservation measures include development of an efficient micro-propagation protocol and investigation of the genetic diversity between and within population where the species is endemic.

This is the first reported successful attempt for micropropagation of *B. sacra*. Its micropropagation, like many medicinal plants, is mainly hindered by heavy presence of polyphenols whose oxidation upon explant wounding leads to explant and media browning (Ahmad et al., 2013; North et al., 2012). Thus, the explant becomes recalcitrant. Some of the methods used to reduce the negative effects of polyphenol oxidation during micropropagation include the addition of compounds in culture media that inhibit polyphenol oxidation such as PVP and change of culture conditions such as duration of illumination (Ahmad et al., 2013). Inclusion of PVP in culture media reduced media browning and at the same time increased the duration of explant survival in culture media, despite the persistence of recalcitrance and explant browning. However, culturing explants in media consisting PVP and culturing them in total darkness in contrast to 16/8 h light and darkness cycles prevented both explant and media browning.

Direct and indirect micro-propagation were reported in this study. Direct micro-propagation is development of adventitious shoots on the explant upon culture in appropriate media and has been

reported in other *Boswellia* species such as *B. ovalifoliolata* (Chandrasekhar et al., 2005) and *B. serrata* (Purohit et al., 1995). In attempts for direct micropropagation of *B. sacra,* leaf and axillary bud explants were cultured in MS media supplemented with the PGRs mTR, BAP and TDZ at different levels but it was not successful due to media and explant browning resulting from polyphenol oxidation. Since only leaf and axillary bud explants were used, it cannot be concluded that direct organogenesis is not possible in *B. sacra* and for this, more explants including cotyledonary meristems, apical meristems and axillary buds with stem segments should be tried on different media with different PGRs.

Indirect micropropagation involves in-vitro plant regeneration through somatic embryogenesis. If embryogenesis is preceded by a callus phase, then the process is known as indirect embryogenesis and if not, direct embryogenesis (Gaj, 2004; Thorpe, 1983). In this study successful indirect somatic embryogenesis was achieved. *B. sacra* leaf explants formed calli under MS containing cytokinins BAP, MemTR and TDZ which after culturing in MS consisting BAP and NAA formed somatic embryos which were then matured in PGR-free MS as well as BAP and IAA. The shoots obtained were hyperhydritic and soon became necrotic. Hyperhydricity, which has also been reported in other species is a major setback in indirect micropropagation. Several ways that have been used to reduce hyperhydricity include ventilation of culture vessels (Lai et al., 2005), addition of silicon (Sivanesan and Park, 2014) and silver nitrate (Vinoth and Ravindhran, 2015) among other compounds to regulate osmotic pressure. Imposing these methods during micropropagation of *B. sacra* can help obtain healthy shoots which can then be successfully acclimatized and should be investigated for *B. sacra* indirect micropropagation suitability.

Differences in morphology between *B. sacra* trees have been described that include a characteristic bottom swelling present in some genotypes and absent in some. *Boswellia sacra* trees have also

been described as having either single or multiple stems. Some trees grow on flat rocks and others on faces of rock cliffs (Thulin & Warfa, 1987; Thulin et al., 2019). Hierarchical analysis of these traits, together with tree height and height to branching failed to cluster the genotypes with their corresponding geographical area. This is an indication that no distinct genetic diversity exists between stands sampled in Exdad and Af Yare Dawl-dawl that can be revealed by the morphological traits studied.

Genetic diversity of Exdad genotypes as revealed by SRAP marker analysis was very low indicated by the low level of heterozygosity (H_e=0.053). This may be attributed to the fragmented nature of this ecosystem which may have led to inbreeding of closely related genotypes. The harsh climatic conditions and lack of adequate biodiversity may lead to low numbers of pollinators further limiting cross-pollination. Genetic diversity should be investigated deeply by using a large number of samples and different molecular markers as well as all populations in Somaliland to get a clearer overview of the exact diversity status as was done in Oman (Coppi et al., 2010). The low genetic diversity among *B. sacra* stands in Exdad is a cause of worry, as since a form of self-sterility exists in *Boswellia* species, inbreeding may lead to reduced production of viable seeds reducing the number of possible offspring in the subsequent generations. This together with over-exploitation being observed may lead to extinction of the existing stands soon.

Conclusion

This study has laid a pathway for possible in-vitro mass propagation of *B. sacra* through indirect micropropagation. Inclusion of PVP in growth media and incubating initiation cultures in total darkness have demonstrated effectiveness in reducing polyphenol oxidation in *B. sacra* cultures. Direct organogenesis of *B. sacra* using both leaf and axillary bud explants was not possible, despite axillary buds showing promising results during initial stages. Leaf explants can be used

successfully in media consisting 5 µm TDZ for callus induction. The calli can be cultured in MS supplemented with 2 µm BAP + 1 µm NAA for somatic embryogenesis and conversion done in 0.25 µm IAA + 1 µm BAP. This study has demonstrated that growth regulators impact both direct and indirect regeneration of *B. sacra.*

Morphological traits analysed in this study failed to group the populations from Exdad and Af Yare Dawl-dawl according to their geographical origin. Despite this, cluster analysis of these traits revealed that individual genotypes were grouped in various clusters indicating differences in parentage. Similar results were obtained through analysis of SRAP marker data. Despite the low diversity revealed at the population level, the individual genotypes show varying differences in coefficient of parentage an indicator of sufficient genetic diversity base for establishment of hybridisation programs as revealed by Hierarchical cluster analysis

Recommendation

Direct micropropagation of *B. sacra* was not successful. However, only leaf and axillary buds were experimented as explants and following this, we recommend the use of other explants such as cotyledonary nodes which have been used in other *Boswellia* species such as *B. serrata* (Purohit et al., 1995) and *B. ovalifoliolata* (Chandrasekhar et al., 2005; Mohammad, 2014). Further, different growth media such as woody plant media (WPM) with different PGRS should be tested. Despite success in callus induction and somatic embryo induction, poor shoot regeneration was observed in this study. The shoots obtained were not vigorous and succumbed to hyperhydricity. In this regard, we recommend further studies be conducted to improve shoot regeneration and eliminate hyperhydricity.

Since it was only possible to sample populations from two regions in Somaliland, it is not possible to capture the diversity level at the country level. We recommend sampling of all the frankincense production regions within Somaliland for genetic diversity establishment.

In the conservation of *B. sacra,* breeding programs that will involve in-depth determination of genetic diversity in all populations followed by mass propagation of stands that show greater variation and their translocation to populations that show low genetic diversity should be initiated. Thereafter, natural pollination should be promoted to ensure gene transfer and restoration of genetic diversity in subsequent generations. We also recommend if this is done, seeds of the species can be bulked and new stands established preferably in protected areas with community involvement. Communities should also be trained on sustainable frankincense harvesting methods to conserve the existing stands.

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Appendices

Appendix 1: ANOVA table for explant survival rate on BAP and mTR.

	Sum of Squares	df	Mean Square	F	Sig.
% Survival week 1	320	4	80	3	0.053
	400	15	26.667		
	720	19			
% Survival week 2	3080	4	770	1.242	0.335
	9300	15	620		
	12380	19			
% Survival week 3	3680	4	920	1.453	0.266
	9500	15	633.333		
	13180	19			
% Survival week 4	1080	4	270	0.81	0.538
	5000	15	333.333		
	6080	19			

Appendix 2: ANOVA table for the effect of TDZ on explant survival

	Sum of Squares	df	Mean Square	F	Sig.
% Survival week 1	600	2	300	1	0.405
	2700	9	300		
	3300	11			
% Survival week 2	1816.667	2	908.333	0.828	0.468
	9875	9	1097.222		
	11691.67	11			
% Survival week 3	466.667	2	233.333	0.42	0.669
	5000	9	555.556		
	5466.667	11			
% Survival week 4	1266.667	2	633.333	3.167	0.091
	1800	9	200		
	3066.667	11			

Appendix 3: ANOVA table for the effect of PVP on media non-browning

	Sum of Squares	Df	Mean Square	F	Sig.
% Non-browning week 1	300	3	100	1	0.426
	1200	12	100		
	1500	15			
% Non-browning week 2	9700	3	3233.333	8.435	0.003
	4600	12	383.333		
	14300	15			
% Non-browning week 3	12600	3	4200	10.957	0.001
	4600	12	383.333		
	17200	15			
% Non-browning week 4	6900	3	2300	5.52	0.013
	5000	12	416.667		
	11900	15			
% Non-browning week 5	4600	3	1533.333	4.842	0.02
	3800	12	316.667		
	8400	15			
% Non-browning week 6	1400	3	466.667	2.154	0.147
	2600	12	216.667		
	4000	15			

Appendix 4: ANOVA table for the effect of TDZ on callus induction

	Sum of Squares	df	Mean Square	F	Sig.
Total week 2	2268.75	3	756.25	19.105	0
	475	12	39.583		
	2743.75	15			
Petiole week 2	2268.75	3	756.25	19.105	0
	475	12	39.583		
	2743.75	15			
Midrib week 2	368.75	3	122.917	6.556	0.007
	225	12	18.75		
	593.75	15			
Total week 3	22425	3	7475	163.091	0
	550	12	45.833		
	22975	15			
Petiole week 3	20118.75	3	6706.25	82.538	0
	975	12	81.25		
	21093.75	15			
Midrib week 3	13550	3	4516.667	51.619	0
	1050	12	87.5		
	14600	15			

Total week 4	26768.75	3	8922.917	99.605	0
	1075	12	89.583		
	27843.75	15			
Petiole week 4	25318.75	3	8439.583	86.191	0
	1175	12	97.917		
	26493.75	15			
Midrib week 4	16250	3	5416.667	56.522	0
	1150	12	95.833		
	17400	15			
Total week 5	26768.75	3	8922.917	99.605	0
	1075	12	89.583		
	27843.75	15			
Petiole week 5	25318.75	3	8439.583	86.191	0
	1175	12	97.917		
	26493.75	15			
Midrib week 5	16250	3	5416.667	56.522	0
	1150	12	95.833		
	17400	15			

Appendix 5: ANOVA table for effect MemTR on callus induction

	Sum of Squares	df	Mean Square	F	Sig.
Total week 2	116.667	2	58.333	0.913	0.435
	575	9	63.889		
	691.667	11			
Petiole week 2	116.667	2	58.333	0.913	0.435
	575	9	63.889		
	691.667	11			
Midrib week 2	150	2	75	1.929	0.201
	350	9	38.889		
	500	11			
Total week 3	816.667	2	408.333	2.882	0.108
	1275	9	141.667		
	2091.667	11			
Petiole week 3	1350	2	675	4.765	0.039
	1275	9	141.667		
	2625	11			
Midrib week 3	66.667	2	33.333	0.245	0.788
	1225	9	136.111		
	1291.667	11			
Total week 4	1066.667	2	533.333	2.667	0.123
	1800	9	200		

	2866.667	11			
Petiole week 4	1516.667	2	758.333	3.845	0.062
	1775	9	197.222		
	3291.667	11			
Midrib week 4	716.667	2	358.333	2.58	0.13
	1250	9	138.889		
	1966.667	11			
Total week 5	1066.667	2	533.333	2.667	0.123
	1800	9	200		
	2866.667	11			
Petiole week 5	1850	2	925	4.269	0.05
	1950	9	216.667		
	3800	11			
Midrib week 5	716.667	2	358.333	2.58	0.13
	1250	9	138.889		
	1966.667	11			

Appendix 6: ANOVA table for the effect of MemTR and TDZ on callus induction

	Sum of Squares	df	Mean Square	F	Sig.
Total week 2	116.667	2	58.333	0.913	0.435
	575	9	63.889		
	691.667	11			
Petiole week 2	116.667	2	58.333	0.913	0.435
	575	9	63.889		
	691.667	11			
Midrib week 2	150	2	75	1.929	0.201
	350	9	38.889		
	500	11			
Total week 3	816.667	2	408.333	2.882	0.108
	1275	9	141.667		
	2091.667	11			
Petiole week 3	1350	2	675	4.765	0.039
	1275	9	141.667		
	2625	11			
Midrib week 3	66.667	2	33.333	0.245	0.788
	1225	9	136.111		
	1291.667	11			
Total week 4	1066.667	2	533.333	2.667	0.123
	1800	9	200		
	2866.667	11			
Petiole week 4	1516.667	2	758.333	3.845	0.062
	1775	9	197.222		

	3291.667	11			
Midrib week 4	716.667	2	358.333	2.58	0.13
	1250	9	138.889		
	1966.667	11			
Total week 5	1066.667	2	533.333	2.667	0.123
	1800	9	200		
	2866.667	11			
Petiole week 5	1850	2	925	4.269	0.05
	1950	9	216.667		
	3800	11			
midrib week 5	716.667	2	358.333	2.58	0.13
	1250	9	138.889		
	1966.667	11			

Appendix 7: ANOVA table for the effect of BAP on callus induction

	Sum of Squares	df	Mean Square	F	Sig.
Total week 2	200	2	100	1.44	0.287
	625	9	69.444		
	825	11			
Petiole week 2	16.667	2	8.333	0.167	0.849
	450	9	50		
	466.667	11			
Midrib week 2	50	2	25	1.286	0.323
	175	9	19.444		
	225	11			
Total week 3	266.667	2	133.333	0.667	0.537
	1800	9	200		
	2066.667	11			
Petiole week 3	266.667	2	133.333	0.667	0.537
	1800	9	200		
	2066.667	11			
Midrib week 3	266.667	2	133.333	3	0.1
	400	9	44.444		
	666.667	11			
Total week 4	266.667	2	133.333	0.706	0.519
	1700	9	188.889		

	1966.667	11			
Petiole week 4	350	2	175	0.84	0.463
	1875	9	208.333		
	2225	11			
Midrib week 4	466.667	2	233.333	2.625	0.126
	800	9	88.889		
	1266.667	11			
Total week 5	266.667	2	133.333	0.706	0.519
	1700	9	188.889		
	1966.667	11			
Petiole week 5	350	2	175	0.84	0.463
	1875	9	208.333		
	2225	11			
Midrib week 5	466.667	2	233.333	2.625	0.126
	800	9	88.889		
	1266.667	11			

Appendix 8: ANOVA table for effect of BAP and IAA on callus induction

	Sum of Squares	df	Mean Square	F	Sig.
Total week 2	816.667	2	408.333	5.654	0.026
	650	9	72.222		
	1466.667	11			
Petiole week 2	866.667	2	433.333	9.176	0.007
	425	9	47.222		
	1291.667	11			
Midrib week 2	116.667	2	58.333	3.5	0.075
	150	9	16.667		
	266.667	11			
Total week 3	1350	2	675	3.627	0.07
	1675	9	186.111		
	3025	11			
Petiole week3	866.667	2	433.333	2.294	0.157
	1700	9	188.889		
	2566.667	11			
Midrib week 3	150	2	75	0.529	0.606
	1275	9	141.667		
	1425	11			
Total week 4	2866.667	2	1433.333	6.143	0.021
	2100	9	233.333		
	4966.667	11			
Petiole week 4	2450	2	1225	4.282	0.049

	2575	9	286.111		
	5025	11			
Midrib week 4	416.667	2	208.333	1.471	0.28
	1275	9	141.667		
	1691.667	11			
Total week 5	2866.667	2	1433.333	6.143	0.021
	2100	9	233.333		
	4966.667	11			
Petiole week 5	2450	2	1225	4.282	0.049
	2575	9	286.111		
	5025	11			
Midrib week 5	416.667	2	208.333	1.471	0.28
	1275	9	141.667		
	1691.667	11			

Appendix 9: ANOVA table for the effect of BAP and NAA on somatic embryogenesis from callus.

Embryo development week 1					
Source of variation	d.f.	Sums of Squares.	Mean Square.	v.r.	F pr.
Treatment	6	110.44	18.407	12.3	<.001
Residual	21	31.417	1.496		
Total	27	141.857			
Embryo development week 2					
Source of variation	d.f.	Sums of Squares.	Mean Square.	v.r.	F pr.
Treatment	6	168.824	28.137	22.7	<.001
Residual	21	26.033	1.24		
Total	27	194.857			
Embryo development week 3					
Source of variation	d.f.	Sums of Squares.	Mean Square.	v.r.	F pr.
Treatment	6	170.69	28.448	19.02	<.001
Residual	21	31.417	1.496		
Total	27	202.107			
Embryo development week 4					
Source of variation	d.f.	Sums of Squares.	Mean Square.	v.r.	F pr.
Treatment	6	183.262	30.544	23.4	<.001
Residual	21	27.417	1.306		
Total	27	210.679			

Appendix 10: ANOVA table for the effect of BAP and IAA on shoot regeneration from somatic embryos.

Source of variation	d.f.	Sums of Squares.	Mean Square.	v.r.	F pr.
Treatment	3	1.6875	0.5625	1.17	0.36
Residual	12	5.75	0.4792		
Total	15	7.4375			

Appendix 11: ANOVA table for the number of stems observed on *B. sacra* genotypes from Af Yare Dawl-dawl and Exdad.

Source of variation	d.f.	Sums of Squares.	Mean Square.	v.r.	F pr.
No. of stems	1	3.85	3.85	0.59	0.447
Residual	55	361.659	6.576		
Total	56	365.509			

Appendix 12: ANOVA table for tree heights of *B. sacra* genotypes from Exdad and Af Yare Dawl-dawl

Source of variation	d.f.	Sums of Squares.	Mean Square.	v.r.	F pr.
Tree height	1	0.411	0.411	0.8	0.374
Residual	55	28.0841	0.5106		
Total	56	28.4951			

Appendix 13: ANOVA table for height to branching for *B. sacra* populations from Exdad and Af Yare Dawl-dawl

Source of variation	d.f.	Sums of Squares.	Mean Square.	v.r.	F pr.
Height to branching	1	0.8067	0.8067	1.57	0.216
Residual	55	28.3484	0.5154		
Total	56	29.1551			

Appendix 14: ANOVA table for the number of seeds obtained from capsules collected in Exdad and Af Yare Dawl-dawl.

Source of variation	Sums of Squares	d.f.	Mean Square.	v.r.	F pr.
Region	104.895	1	104.895	0.263	0.613
Residual	8789.105	22	399.505		
Total	8894	23			

Appendix 15: ANOVA table for the 1000 seed weight for seeds collected from Exdad and Af Yare Dawl-dawl.

Source	Sums of Squares.	d.f.	Mean Square.	v.r.	F pr.
1000 seed weight	1.559	1	1.559	0.983	0.332
Residual	34.895	22	1.586		
Total	36.453	23			