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OPTIMIZING *IN-VITRO* CONSERVATION AND RAPID MULTIPLICATION REGIMES FOR EXOTIC AND LOCAL TARO GENOTYPES

BY

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(B.SC. AGRICULTURE)

A THESIS IN THE DEPARTMENT OF CROP AND SOIL SCIENCES EDUCATION, FACULTY OF AGRICULTURE EDUCATION

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DECLARATION

I hereby declare that except for references to other peoples work which have been duly cited, this work is the results of my own original research and this thesis has neither in whole nor in part been prescribed for another degree elsewhere.

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DEDICATION

TO THE LORD JESUS CHRIST FOR HIS FAITHFULNESS. To my mother; Ama Serwaah and my siblings; Benjamin Kufuor, Bofah Vandyke, Atobrah Richard, James Appiah Kontoh and Mr George Amoateng (King George, CFC).

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ABSTRACT

Taro (*Colocasia esculenta* (L) Schott) is an important staple food crop grown throughout many part of the world for it fleshy corms and nutritious leaves. Suckers are the main planting materials used in West Africa; therefore, it is traditionally conserved in field collections. This is not an effective conservation strategy for medium to long term preservation of taro genetic resources due to extreme climatic conditions, pests and diseases. Tissue culture is one of the *in-vitro* techniques which have in recent years become major agricultural importance in the area of plant propagation, disease elimination and production of secondary metabolites *in-vitro*. However, with tissue or micropropagation conservation, challenges include: High growth rate of both the local and exotic genotypes of taro which causes sub-culturing in every eight weeks and also high shoot proliferation and all axillary buds produce shoots, leading to a dense population competing for limited nutrients in the glass vessel. Taro is a crop that is amenable to tissue culture.

A study was conducted to optimize rapid multiplication and establish slow growth systems for *Colocasia* genotypes *in-vitro*. Thus, taro genotypes used for the experiment were obtained from the Taro Breeding Programme at Crop Research Institute which includes SA0 002, CE/MAL/32, SA0 006 and CE/MAL/ 14, BL/SM/80, KA 019 as corms. The study was carried out at the Tissue culture labouratory-Crops Research Institute (CRI), Fumesua.

The experiment was categorized into 5 stages thus: *In-vitro* Rapid Multiplication, *In-vitro* Medium-Term Conservation; *In-vitro* Recovery, Fourier Transform Infra-red analysis for concentration of Mannitol absorption content in *Colocasia* genotype and Biochemical analyses. The genotypes were evaluated in a complete random design. Means were separated using LSD at 5%. Rapid multiplication experiment cultures were maintained at 25 ± 1^{0}C at 16hr photoperiod.

Two modes of explant excision (Transverse Section (TS) and Longitudinal Section (LS)) were used. For the rapid multiplication set up results, the highest number of shoots (26.00) and leaves (40.00) were produced on the multiplication medium of transverse section (TS) shoot excision for BL/SM/80 genotype, and lowest number of shoots (24.00) and leaves (39.67) occurred on medium culture of longitudinal section (LS) shoot excision. However, for KA 019 genotype, the minimum number of shoots (30.60) and maximum leaves (38.13) were observed in the culture of LS excision, while the maximum number of shoots (32.00) and minimum leaves (37.40) were observed on culture of TS of shoot.

The *in-vitro* cultures were evaluated for their growth, re-growth, mannitol absorption rate and enzyme activity (Catalase and peroxidase activities) as a response to the conservation conditions of the *in-vitro* collection. The four genotypes studied showed a good *in-vitro* response, when the mannitol was used as a growth retardant. The medium term conservation cultures were incubated at 20 ± 1^{0}C and 12 hours photoperiod (3000lux) conditions. Explants were subjected to different concentrations of mannitol (0, 20, 25 and 30g/l) and White medium (which is a supposed low nutrient as compare to MS basal media). No growth regulators were added to the various media. Survival rate was high and explants remained almost green and healthy on all mannitol treated media when conserved for 6 months on conservation medium. However, with White medium, the growth decreased as the conservation period increased. High number of shoots (6.33) and leaves (22.67) occurred on medium containing 20 and 25g/l mannitol in genotype SAO 006, whereas 30g/l mannitol was the best to restrict growth for the entire 6 months period in terms of shoot height (22.50cm) among all the genotypes conserved. Results regarding recovery and regrowth showed that regeneration percentage, number of shoots per ex-plant and their length (cm) decreased with increasing concentration of mannitol from control to 30g/l. Determination

of mannitol absorption rate by the plantlet, enzyme activities were performed by FTIR spectroscopy and UV/VIS spectrophotometer respectively. The analysis of FTIR and UV spectroscopy both confirm the rate of absorption of mannitol and enzyme activities after 6 months period of conservation.

This showed that Transverse (TS) section of explants has more axillary and accessory buds occurring at the base than Longitudinal (LS) section, therefore rapid multiplication can be achieved, whereas mannitol supplemented culture media could reduce the growth of *Colocasia* plantlets especially in stem height. Results showed that conserved explants can be regenerated after transferring and culturing on recovery medium and incubating under regular usage conditions.

The osmolite mannitol, at all concentrations tested, did not affect the viability of the whole explants, probably influenced ROS scavenger system, affecting the antioxidant enzymes activity. The time of FTIR analysis is considerably reduced compared to the classical methods; this demonstrates that FTIR analysis of mannitol offers a quick and efficient means of sugar analysis.

ABBREVIATIONS

MS	Murashige and Skoog medium
UV	Ultra Violet spectrophotometer
FTIR	Fourier Transform Infrared spectroscopy
ROS	Reactive Oxygen Species
PGR	Plant Growth Hormones
CSIR-CRI	Council for Scientific and Industrial Research- Crops Research Institute
USDA	United State Department of Agriculture
FAOSTAT	Food and Agriculture Organization Statistics
SSR	Simple Sequence Repeat
FAO	Food and Agriculture Organization
UNCED	United Nation Conference on Environment and Development
AFLP	Amplified Fragment Length Polymorphism

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Taro as a monocotyledonous root crop

Taro is commonly referred to as cocoyam, dasheen, eddoe, *kooko*, or *broobe*. Its distribution and cultivation in the wet tropics is wide. Yields are about 4 t/ha in Ghana, but with good management, yield of 25 - 37 t/ha have been recorded in the Pacific (Sar *et al*., 1998). Farmers cultivate about 200 hectares per year. It is normally found growing along river banks or low-lying areas on small scale. In Ghana, a few farmers grow it on a large scale for the local and export markets, and rank it as their main source of income. The common use is to boil, roast and fry its edible tubers, and to a lesser extent, its foliage as a leafy vegetable. Taro starch grain are small and very digestible and, therefore, used for foods (FAO, 1989). Flour made from taro is used as substitute for pastries and beverages.

Using isozyme analysis, Lebot and Aradhya (1991), reported the existence of two gene pools for cultivated taro; one in Asia and the other in Pacific. Studies with SSR markers (Simple Sequence Repeats) (Noyer *et al*., 2003) and AFLP markers (Amplified Fragment Length Polymorphism) (Kreike *et al*., 2004) have confirmed the existence of these two distinct gene pools. This indicates that taro was domesticated in Asia as well as in the Pacific; therefore, it can be considered as a native plant of the Pacific. This ancient crop, first domesticated in Southeast Asia, has continued to spread throughout the world and is now an important staple food crop grown throughout many Pacific Island countries, parts of Africa, Asia and the Caribbean for its fleshy corms and nutritious leaves. The plant can fit well into tree crop and agro-forestry systems and some types are particularly well adapted to unfavourable land and soil conditions such as poor drainage. As such taro is grown under intensive cultivation as a starch crop (Jianchu *et al*.,

2001). Worldwide, taro ranks fourteenth among staple vegetable crops with about 12 million tonnes produced globally from about 2 million hectares with an average yield of 6.5 t/ha. (FAOSTAT, 2010 estimates).

1.2 Taro Genetic Resources

Traditional germplasm conservation under natural condition methods suffers from many drawbacks. Plants are exposed to destructive biotic and abiotic stresses. Moreover, the maintenance of clonal taro is labour intensive and expensive (Shibli *et al*., 2006). Slow growth culture, for medium-term preservation, and cryopreservation techniques, for long-term preservation, are two kinds of *In-vitro* germplasm conservation methods which could offer practical means of germplasm storage of plant species (Shatnawi *et al*., 2007). Slow growth techniques are recommended for the storage of shoot cultures in many laboratories, as well as in many regional and international germplasm conservation centers (Shibli *et al*., 2006).

Due to the above-mentioned difficulties, tissue culture is the only alternative method for rapid propagation of *C. esculenta.* Plant tissue culture has some advantages over other propagation systems, as it provides rapid clonal propagation of uniform plants throughout the year (Durkovic and Misalova, 2008). Hence *In-vitro* propagation appears as an alternative technique for rapid multiplication of *Colocasia* within a short span of time. Micropropagation of *C. esculenta* can be carried out through direct organogenesis and shoot multiplication provides a rapid reliable system for a production of large number of genetically uniform disease free plantlets, irrespective of the season. Although earlier attempts for micropropagation have been carried out, considerable effort is still required to make it more practical (Kornilova and Kalashnikova, 1997; Ahmed *et al*., 2007; Ibrahim *et al*., 2008).

1.3 Role of Plant Tissue Culture

The term plant tissue culture denotes genetically all cell, tissue and organ cultures, and employs the basic dogma totipotency. The technique essentially involves separation of a cell/tissue/organ from the donor plant under aseptic conditions and growing it on a synthetic medium in a suitable container under controlled environment. Among other applications, tissue culture has been successfully dovetailed to conservation and exchange of germplasm in horticulture.

In the past decades, *In-vitro* techniques have been extensively developed for over 1000 different species. Tissue culture systems allow for axenic propagation of plant material with high multiplication rates. Virus-free plants can be obtained through meristem culture in combination with thermotherapy. The miniaturization of explants reduces space requirements and consequently labour costs for maintenance of germplasm collections. It is now mandatory to exchange taro germplasm across countries using embryo cultures because of the obvious advantages. However, it may be noted that mere transfer to *In-vitro* culture does not automatically confer the disease-free status. Specific pathogenicity tests need to be carried for certification of the *In-vitro* plant material as being disease-free.

At present, biotechnological methods have been used to conserve endangered, rare, crop ornamental, medicinal and forest species, allowing the conservation of pathogen-free material, elite plants and genetic diversity for short, medium and long-term. *In-vitro* conservation is especially important for vegetatively propagated and for non-orthodox seed plant species (Engelmann, 2012). Furthermore, *In-vitro* techniques offer a safe means to internationally exchange plant material, enable the establishment of extensive collections using minimum space,

allow supply of valuable materials for wild population recovery and facilitate molecular investigations and ecological studies (Tandon *et al.*, 2005).

1.4 *In-vitro* Technologies for Conserving Plant Biodiversity

The material to be collected depends on each species. Due to cell totipotency, in theory, almost any part of the plant is sufficient to regenerate a whole organism under the appropriate growth conditions. For vegetatively propagated species, it is necessary to collect stakes, pieces of bud wood, tubers or corms, (Engelmann, 2011). The different factors that must be considered during the *In-vitro* collection of plant tissue are: the appropriate tissue for *In-vitro* collection, the size of the tissue, soil residues, presence of diseased tissue, sterilization of plant tissue, removal of the disinfectant, nutrient medium and the conditions of storage, including light, temperature and humidity (Withers, 2002). Since *In-vitro* collections are based on tissue culture techniques, its limitations are based on the recalcitrance of some species to regenerate or even to grow *In-vitro* (Pence, 2002).

In-vitro techniques used to achieve medium-term conservation allow the storage of biological materials from several months to 2–3 years without sub-culturing, depending on the technique used and on the plant material.

Growth reduction is generally attained by modifying the culture medium and/or the environmental conditions. Modifications of the culture medium can include dilution of mineral elements, manipulation of sugar concentration, changes in the type and/or concentration of growth regulators and addition of osmotically active compounds (Engelmann, 2011). As regards the culture environment, it can be changed by reducing the temperature, combined or not, with a decrease in light intensity or by keeping cultures in complete darkness. The most frequently used

combination of physical and chemical factors involves decrease of temperature, reduction of mineral elements and carbon source concentration in the medium and the use of low light intensity (Holobuic *et al*., 2007). The temperatures reported for medium-term conservation are usually from 4°C to room temperature (Tassy *et al*., 2006). However, tropical plant species are often cold-sensitive and have to be stored in the range of 15–20°C or even higher, depending on their sensitivity (Paunescu, 2009). Therefore, the procedure to enable extending subculture periods will mainly focus on modifying the chemical composition of culture medium. Other additional parameters may also influence the efficiency of slow growth storage, such as the type of explants, their physiological state, as well as the type, volume and the kind of closure of culture vessels (Engelmann, 1991).

Standard *In-vitro* culture conditions can also be used for medium-term storage when dealing with species that have a naturaly slow growing habit. Alternatively, the explants may be covered with paraffin, mineral oil or with liquid medium to reduce the growth rate. Modifications in gaseous environment, desiccation and/or encapsulation are other possible options (Engelmann, 2012). Short- and medium-term conservation is routinely used in many laboratories in order to increase the intervals between subculturing required under the multiplication procedure. At the end of a storage period, cultures are transferred onto fresh medium and usually placed for a short period in optimal conditions to stimulate regrowth before entering the next storage cycle (Engelmann, 2012).

The advantage of slow growth techniques is that they use the same basic facilities used for plant micropropagation and that the storage regimes are based on modifying the conditions previously established for rapid multiplication. However, they do not alleviate the main problem associated

with the high costs of labour and space requirements of any micropropagation system, in addition to the potential risks of somaclonal variation for some species (Blakesly *et al*., 1996).

1.5 Methods of *In-vitro* Conservation

Prolonged maintenance of *in-vitro* materials also provides an effective system for establishing both active and base germplasm collections particularly for the conservation and maintenance of those plant species that cannot be stored as true seed and are amenable to micropropagation technology (Fay 1992). The properties required of a successful storage system are the ability to:

1. Reduce the growth rate of *in-vitro* plants, resulting in extension of intervening sub-culture frequencies.

2. Retention of *in-vitro* plant viability during storage at maximum simultaneously maintaining genetic germplasm collection.

Several *in-vitro* techniques have been developed for storage of vegetatively propagated and recalcitrant seed producing species. In general, they fall under two categories: (i) slow growth procedures, where germplasm accessions are kept as sterile plant tissues or plantlets on nutrient gels; and (ii) cryopreservation where plant material is stored in liquid nitrogen. Slow growth procedures provide short and medium term storage options, while cryopreservation enables long-term storage of the plant material. This review briefly presents the *In-vitro* techniques, which can be efficiently used to improve the conservation of plant biodiversity such as Taro.

Problem Statement and justification

Tissue or micropropagation conservation, challenges includes;

i. High growth rate in the lines of both the local and exotic accession of taro which causes sub-culturing to be done every eight weeks.
ii. High shoot proliferation and all axillary buds produce shoots leading to a dense population competing for limited nutrients in the glass vessel.

There is therefore the need for slow growth method to conserve from short to medium term period to save time and reduce cost of maintaining cultures *In-vitro*.

Minimal growth storage is the most direct way of restricting growth and development of *In-vitro* materials, and is usually applied to differentiated plantlets and developing meristem cultures.

A recent status report on the use of *In-vitro* techniques for the conservation and use of plant genetic resources (Ashun, 1996) concluded that slow growth techniques are now successfully and routinely applied to a range of species and across a range of genotypes within species.

OBJECTIVES

Main objective

To optimize rapid multiplication and establish slow growth systems for taro genotypes *In-vitro*

Specific objectives

i. Evaluate effect of explant excision through LS and TS on *In-vitro* growth rates of culture during rapid multiplication

ii. Assess the growth rate of taro *In-vitro* for germplasm conservation by addition of osmotic represent such as Mannitol to growth medium.

iii. Determine the rate of recovery and re-growth after the conservation period

iv. Determine Mannitol absorption of the taro plantlets after the conservation period through FTIR analysis method.

v. Determine the effect of mannitol on anti-oxidative enzymes *In-vitro* medium-term cultures of *Colocasia* genotypes (i.e., UV spectrometry analysis)

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Origin and distribution

Taro (*Colocasia esculenta* (L.) Schott) is an ancient and important vegetatively propagated root crop species, belonging to the monocotyledonous family Araceae, grown for its edible corms and leaves (Plucknett, 1976; Coates *et al*., 1988) and dated for more than 2000 years in cultivation. This dating is based on the earliest written records for taro (Matthews 2006) and is open-ended because the plant is difficult to detect archaeologically. Taro is thought to have originated in North Eastern India and Asia (Kuruvilla and Singh, 1981; Hanson and Imamuddin, 1983; Ivancic, 1992) and gradually spread worldwide by settlers. As such, it is now cultivated in more than 65 countries worldwide (USDA, 2001).

2.2 Classification and Morphology

Taro (*Colocasia esculenta*) is a herbaceous plant, which grows to an average height of 1-2 m. The plant consists of a central corm lying just below the soil surface, with leaves growing from the apical bud at the top of the corm and roots growing from the lower portion. Cormels, daughter corms and runners grow laterally. The leaf is peltate; the root system is fibrous and lies mainly in the top one metre depth of soil. The corm is a nutrient storage organ and shares the following characteristics with food storage organs in carrot, sweetpotato: abundance of periderm, food storage in large, thin-walled parenchymatous cells, poorly developed vascular bundles that are few in number, presence of latex cells, mucilage cells and ergastic substances, such as druses and raphides (Miyasaka, 1979).

Cultivated taro is classified as *Colocasia esculenta*, but the species is considered to be polymorphic (Purseglove, 1972). There are eight recognized variants within *Colocasia esculenta,* of which two are commonly cultivated (O'Sullivan *et al*., 1996): i) *Colocasia esculenta* (L.) Schott var. *esculenta* which possesses a large cylindrical central corm and only few cormels, agronomically it is referred to as the, dasheen type of taro and, ii) *Colocasia esculenta* (L.) Schott var. *antiquorum* which has a small globular central corm with several relatively large cormels arising from the corm, agronomically this variety is referred to as the, eddoe type of taro (Purseglove, 1972; Lebot and Aradhya, 1991). Most of the taro grown in Asia Pacific region is of the dasheen type. In places where taro is grown primarily for leaves, *C. esculenta* var. antiquorum is preferred (O'Sullivan *et al*., 1996).

At Kilu Cave in the Solomon Islands, near Papua New Guinea, starch granules on stone tools have been tentatively identified as coming from *C. esculenta*. The tools were found in a stratigraphic unit dated to between ca. 28 700 years BP and 20 100 years BP (Loy *et al*., 1992). The tools were apparently used to scrape and/or cut fresh (i.e. raw) taro, and it was assumed that taro formed part of the prehistoric diet. This evidence may relate to wild or cultivated plants, and the possibility of medicinal or other non-food uses cannot be excluded. At the Kuk archaeological site, in a swamp in the Western Highlands Province of Papua New Guinea, earthworks, geomorphological evidence, and plant remains indicate a sequence of agricultural practices from around 9000 years ago. Taro is one of the main candidates for early use and cultivation at this site (Bayliss-Smith, 1996; Denham *et al*., 2003).

Presumably, the earliest cultivation of taro was preceded by the gathering and use of wild-type (natural) forms from wild habitats. These could have been used in multiple ways as food (for the starch and green leaves), as medicine and as a cooked fodder (perhaps for pigs). While the

history appears to be complex, present-day methods and tolerances permit the safe consumption of all cultivated forms of taro, with the exception of wild-types (natural forms) that are cultivated as ornamental plants. Chromosome numbers reported for taro from various regions include 2n = 22, 26, 28, 38 and 42 (Onwueme, 1978). The most commonly reported chromosome numbers are: diploids 2n = 28 and triploids 3n = 42 (Kuruvilla and Singh, 1981; Wang, 1983; Lebot and Aradhya, 1991; Lee, 1999). Furthermore, plants with 3n = 42 are referred to as 'alowane' (male, large plant) and those of 2n = 28 are referred to as alokine (female, short plant) by Solomon Island farmers (Jackson *et al*., 1977; Wang, 1983).

2.3 Propagation

Taro is mainly vegetatively propagated (Shaw, 1975; Strauss *et al*., 1979), but may also reproduce sexually (Ivancic, 1992). Due to consumer's preferences, propagation is mainly vegetative/clonal; there is almost no genetic variation within the cultivars.

Sexual hybridization of taro is well documented and techniques for pollinating and growing seedlings have been established (Wilson, 1990; Tyagi *et al*., 2004). Sexual hybridization is one way to generate new cultivars with improved qualities (Straus *et al*., 1979). Extensive breeding programmes (sexual propagation) have been carried out in Samoa, Papua New Guinea and Hawaii to produce cultivars with resistance to Taro Leaf Blight (TLB) and with high yields (Lebot and Aradhya, 1991; TaroGen, 1999; Singh *et al*., 2001). From this breeding programme, promising taro cultivars resistant to TLB have been produced in Hawaii (Trujillo *et al*., 2002), PNG and Samoa (SPC, 2002b).

Even though sexual hybridization of taro is promising, it is a labour intensive and lengthy process in terms of field preparation, planting of parents, induction of flowering, pollination, development and maturation of fruit heads and seed harvesting. In addition, the germination and planting of seedlings and screening processes take several years. “It often takes 10 years or more from the time you make a pollination, until the new, improved cultivar finally reaches a large number of farmers” (Wilson, 1990). Further, viable seed production depends on the availability and compatibility of resistant germplasms as well as the vagaries of weather and pests and diseases.

2.4 Diseases and pests of taro

In many countries taro is being replaced by sweet potatoes and cassava largely due to pests and disease problems, which are becoming a limiting factor for taro production (Ivancic, 1992). Viruses are one of the most important pathogens with some infections resulting in severe yield reductions and plant death. The main effect of virus infection is a reduction in corm size and quality, with yield losses of up to 20% being reported. There are currently five viruses reported to infect taro with varying distribution throughout the Pacific Islands.

Dasheen Mosaic Virus (DsMV) is a potyvirus with flexuous, rod shaped virions, which infects both the edible and ornamental aroids spread by aphids. It is characterized by chlorotic and feathery mosaic patterns on the leaf, distortion of leaves and stunted plant growth. There is some evidence that it decreases the yield.

Amongst the pests, taro beetle belonging to the genus Papuana is of great concern. The adult beetles which are black, shiny and 15-20mm in length fly from the breeding sites to the taro field and tunnel into the soil just at the base of the taro corm. They further proceed to feed on the

growing corm leaving large holes that reduce the eventual market quality. Further, the wounds they create while feeding promote the attack of rot-causing organisms. The feeding activity can cause wilting and even death of the affected plant.

Other insect pests include taro leafhopper (*Tarophagus proserpina*), which transmits viruses and may also cause wilting and death of the plant in heavy infestations and the sweet potato hawk-moth whose larvae defoliate the plant and the armyworm or cluster caterpillar, which also do extensive damage to the leaves. These diseases and pests are becoming a threat to taro industry in Ghana. Thus exploring potential of elite varieties and generating substantial numbers of disease-free planting material and/or breeding resistant taro varieties are necessary.

2.5 Production practices in West Africa

The top world producers of taro (*Colocasia esculenta*) are Nigeria, China, Cameroon, and Ghana. The world's total production of taro in 2011 was 9,532,427 tons while more than 74 percent of the total production comes from Africa FAOSTAT (2012). Nigeria being the leading world's producer of taro produced more than 27 percent of the total world's production in 2011. The highest intensity of production, utilization and dependence on taro is in the Pacific islands. The highest areas of production, however, are in West Africa.

Land resources are a major factor in the production of taro. Year-round taro production is possible in the tropics, provided that water is continuously available (Dudal, 1980). In most of West Africa, taro production is under rain-fed conditions. Large areas in West Africa are not suitable for upland taro production due to high risk of crop failure. Production is thus limited to rainy season in areas along streams and marshy areas, especially in valley bottoms, where water may be available for most part of the year. Taro has been grown in West Africa for several

generations in southern and eastern Nigeria, southern Ghana and Benin along forest streams and in swamps (Irvine, 1974). In heavy rainfall areas of western Ghana and eastern Cote d'Ivoire, marshy areas are common, usually adjacent to coconut plantations. In south-western Ghana, for instance, the lethal yellow disease has destroyed most of the coconut trees and a booming taro industry has sprung up over the past six years. This area is wet throughout the year and hence yields are high. The farmers use very little of the crop but the bulk is sold for cash.

Suckers are the main planting materials used in West Africa. Most of the cultivars produce numerous suckers. Tops of corms are also used as planting material to a limited extent. Manual weed control with cutlass or machete is the common practice. Some farmers when overwhelmed by weeds cut down weeds and taro plants. Subsequently, the taro plants outgrow the weed re-growth and smother weeds. In Nigeria, 25 % of labour needs are used for weeding (Knipscheer and Wilson, 1980). There is very little herbicide usage, though it has been found that herbicides cost only 45-55% of cost of hand weeding (Abasi and Onwueme, 1984). Use of herbicide is slowly increasing in the south-western part of Ghana. After harvest of plant crop, weed control in the ratoon is rather difficult manually, due to the high plant population from the suckers. Optimum plant populations have been reported for Nigeria (16,667 plants ha.-1) (Igbokwe and Ogbonnaya, 1980) and Ghana (20,000 plants ha.-1) (Safo-Kantanka, 1986).

Low organic matter and deficiency in major soil nutrients, without fertilizer application or manuring limit growth and yield of taro in West Africa (Igbokwe and Ogbonnaya 1980). This is aggravated by continuous production in the same location. Various components of the crop are used with preference depending on the location and food habits. In Ghana and Nigeria, corms are preferred, while in Liberia, corms and leaves are used for food. Post-harvest losses are high, especially cases of corm rot. Markets are not well organized, with farmers receiving poor prices

from middle men and women. Processed products have not been developed, but the potential for products such as taro chips look bright.

Taro has evolved with the cultures of the people of the Asia and Pacific region; therefore, it has acquired considerable socio-cultural importance. It is considered a prestige crop and the crop of choice for royalty, gift-giving, traditional feasting and the fulfillment of social obligations. It features prominently in the folklore and oral traditions of many cultures in Oceania and South-east Asia. Samoa and Tonga each have prominent depictions of taro on their currencies (Onwueme, 1999). Moreover, in Hawaii, images of taro and taro farmers can be found throughout the islands, in murals, posters, original arts and other visuals, where its symbolic importance reflects its continuing role as a common food and common element in the agricultural landscape (Matthews, 1998). The socio-cultural attachment to taro has meant that taro itself has become a symbol of cultural identification, such that the people of Pacific Island origin continue to consume taro wherever they may live in the world. This is one of the means of maintaining links with their culture; consequently, this cultural attachment to taro has spawned a lucrative taro export market to ethnic Pacific Islanders living in Australia, New Zealand and western North America (Onwueme, 1999). Taro is also used as a traditional medicine with root extract used to treat rheumatism and acne, while leaf extract is used for blood clotting at wound sites, neutralizing snake poison and as a purgative medicine (Thinh, 1997).

2.6 Economical importance and uses of taro

In Pacific Island countries such as Fiji and parts of Africa, taro is a staple food crop (Lebot and Aradhya, 1991; Opara, 2001). In Tonga, for example, tubers represent almost half the nations‟ calory intake of which about 40% is contributed by taro. Similarly, in Solomon Islands, about

10% of people‟s dietary calories come from taro and 30% from other tubers. Moreover, in Samoa, prior to the devastating spread of taro leaf blight (TLB), virtually all the populations‟ dietary intake from tubers (one-fifth of the overall diet) came from taro (CTA, 2003). Taro is one of the few major staple foods where both the leaf and underground parts are important in the human diet (Lee, 1999). As such, it has attained considerable economic importance as a fresh crop in many large islands in the region such as Samoa, Fiji and others (Hanson and Imamuddin, 1983). It is now becoming one of the major export commodities providing substantial foreign exchange to some of the Pacific Island countries.

Large quantities of taro are produced in Asia/Pacific region, with the corm being boiled, baked or fried and consumed with fish and coconut preparations. A favorite and peculiarly Pacific way to prepare taro is to roast it on hot stones in dug-out earth ovens.

This is quite common when taro is used in feasts and ceremonies. Young taro leaves are used as a main vegetable throughout Melanesia and Polynesia where they are usually boiled or covered with coconut cream, wrapped in banana or breadfruit leaves and cooked on hot stones. The processed and storable form of taro is the taro chip and Poi.

Taro chips are prepared by peeling the corm, washing, slicing into thin pieces and blanching; the pieces are then fried in oil, allowed to cool, then drained and packed. ‘Poi’ is a sour paste made from boiled taro and its production and utilization is quite limited – mainly in the Hawaiian Islands. Griffin (1982) has emphasized other important economic uses of taro. For example, the development of taro silage and its use as animal feed especially for swine, the potential of taro alcohol as a fuel for remote islands and the potential of taro starch as a raw material in cosmetic and plastic manufacture. Furthermore, taro flour and other products are used extensively for

infant formulae in the United States and have formed an important constituent of proprietary canned baby foods (Lee, 1999).

2.7 Nutritive value of taro

Tables 2.1, 2.2 and 2.3 show the nutrient composition of taro, chemical characteristics of taro leaves, mineral contents of *Colocasia esculenta* and chemical composition of differently processed taro corms. *Colocasia esculenta* is one of the six most important root and tubers crops worldwide (Jennings, 1987). Leaves of taro are eaten as vegetable by human, having B- carotene, iron, protein, vitamins and folic acid which protects against anemia (FAO, 1990; Sukkamoto, 2003; Afris, 2004). Nutritional composition of roots and tubers vary from place to place depending on climatic conditions, variety of crop cultivated, as well as soil conditions (FAO, 1990). The result of the study carried out by Abdulrashid *et al*. (2009) revealed that taro meal contained 7.87% crude protein, 31% dry matter, 4.75% crude fiber, and 3214.91 Kcal/kg metabolisable energy on dry matter basis. The result of proximate analysis carried out on *Colocasia esculenta* by Ogunlakin *et al*. (2012) showed that *Colocasia esculenta* contained 89.53-90.57% dry matter, 4.93-5.17% crude protein, 0.50-0.57% ether extract, 2.70-2.97% crude fiber, 78.7-79.0% carbohydrate and 2.47-2.87% ash content on dry matter basis. *Colocasia esculenta* in its raw form is toxic. The toxin is however destroyed by processing techniques such as cooking, soaking, ensiling and drying (Olajide *et al*, 2011; Onu and Madubuike, 2006; Ogunlakin *et al*., 2012). Cooking has however been shown to reduce the protein content of taro (Olajide *et al*., 2011; Onu and Madubuike, 2006). Ogunlakin *et al*. (2012) noted that the physicochemical properties of sun-dried sample of *Colocasia esculenta* were more acceptable than oven-dried and cabinet-dried samples, having greater values in most physico-chemical properties considered. It was said that sundried sample retained the best starch structure.

Table 2.1. Nutrient composition of taro corm

Parameters	Raw oven dried	Raw sundried	Raw cabinet dried	SWCC	CWCC	FWCC
Dry matter (%)	90.57	88.42-89.53	89.87	88.06	88.64	87.90
Crude protein (%)	5.17	4.93-7.07	5.07	6.56	6.13	7.44
Crude fiber	2.97	2.70-3.90	2.83	3.75	3.55	3.45
Ether extract (%)	0.57	2.47-2.93	0.50	2.86	2.76	2.63
Ash (%)	2.87	0.50-1.10	2.77	0.95	0.75	0.88
Carbohydrate (%)	79.00	73.43-78.93	78.70	73.90	75.46	73.50
ME (Kcal/kg)	NA	2958.34	NA	2943.70	2966.82	2956.52

SWCC= soaked taro; CWCC= cooked taro; FWCC= fermented taro Source: Olajide *et al*. (2011).

Table 2.2 Chemical characteristics of taro leaves

Parameters	Ensiled taro leaves	Dried taro leaves
Dry matter (%)	18.30-22.60	92.20
Crude protein (%)	25.90-26.30	26.70
Crude fiber (%)	17.10	15.20
Organic matter	8.30-8.53	8.70
Calcium oxalate	0.11	1.10

Source: Chhay *et al*. (2007); Pheng *et al*. (2008)

Table 2.3. Mineral contents (mg/100g DM) of *Colocasia esculenta* corm

Mineral content	*Colocasia esculenta*
Potassium	715.39
Phosphorus	134.30
Copper	0.19
Iron	3.48
Zinc	4.32
Maganese	3.68
Calcium	68.67
Magnesium	83.76
Sodium	13.18

2.8 Anti-nutritional factors in taro

Colocasia esculenta is a cheaper carbohydrate source than grains (Onu and Madubuike, 2006; Adejumo, and Baruidele Oladeji, 2012). It has low production cost, high caloric yield per hectare and are not easily susceptible to pests and disease attack. Its major limitations in its use in animal nutrition are storage and the presence of anti-nutritional factors such as tannins, saponins, phytates, oxalates it contains.

Some anti-nutritional factors serve as defense mechanisms against pests and diseases. For example, oxalates have been observed to play defensive role in the plant as well as a storage reserve for Calcium (Smith, 1982). A toxicant is a substance which under practical circumstances can impair some aspect of animal metabolism and produce adverse biological or economic effects in animal product (Udeybir *et al*., 2008). Anti-nutritional factors are, however,

described as substances in the diets which by themselves or their metabolic products arising in the system interfere with the feed utilization, thereby reducing production or affect the health of the animals. Toxicants can be classified based on their chemical properties of effect of their utilization on nutrients as alkaloids, glycosides (such as saponins, cyanogens), phenols (gossypol, tannins), mycotoxins, metal binding (oxalates) and proteins (protease inhibitors and haemoglutinins).

2.9 *In-vitro* Propagation

Tissue culture technology has proved to be a versatile tool for propagating elite clones. Plant propagation through culture technology has emerged from a century old concept, i.e. the totipotency of cells established by the German scientist Haberlandt (1902). Experiments of Laibach (1929), White (1934), van Overbeek *et al*. (1941), Loo (1945) and Murashige and Skoog (1962) led the way to lay a strong foundation, which brought the technology and its versatile application to the forefront especially in the field of agriculture and horticulture. The successful application of plant tissue culture technology for plant improvement is based upon the mass regeneration of plants from cultured cells or tissues.

Micropropagation is the true to type propagation of selected genotype using *In-vitro* culture technique. The use of apical meristem culture for simultaneous virus elimination and shoot proliferation in sugarcane was assesed by van Ramgareeb *et al*. (2010). Shoot induction and proliferation via direct organogenesis were achieved on Murashige and Skoog nutrient medium supplemented with 0.1 mg/l Benzyladenine and 0.015 mg/l Kinetin. Approximately 1,300 shoots were propagated from a single 2 mm meristem in 11 weeks.

2.10 Mode of Explant Excision

Among cut modes, transversal cut, the most popular cut mode (Moore *et al*., 1992; Pena *et al*., 2004) is simple to manipulate but produces the fewest adventitious buds. Longitudinal cut, a newly developed but infrequently used cut mode produced the most adventitious buds (Kayim *et al*., 2004). Some studies have shown that explant characteristics such as type, source, genotype, and history affect the success and commercial viability of tissue culture systems (Bhau and Waklu, 2001; Chan and Chang, 2002; Hoy *et al*., 2003). The effect of explant type on successful tissue culture of various plants has been reported (Gubis *et al*., 2003; Blinstrubiene *et al*., 2004; Tsay *et al*., 2006; Gitonga *et al*., 2010).

2.11 *In-vitro* conservation of plant genetic resource

The germplasm of all organisms manifests differing degrees of variability in the characteristics or traits that they possess as a result of heritable changes called mutations. It is this variability that is designated as biodiversity. Biodiversity is a fundamental property of life and without it no evolutionary changes are possible (Solbrig, 1991; Okigbo, 1994).

Biodiversity conservation is of prime importance and the aim is to ensure the availability of useful germplasm at any given time. The most widely used method for conserving plant genetic resources is seed storage. However, many categories of crops face problems with regard to seed storage. At present, the most common method to preserve the genetic resources of these problem crop species is as whole plants in the field, which are called field genebanks. There are, however, serious problems with field genebanks especially when there is disease and pest outbreak (Withers and Engels, 1990). There are different ways by which the growth rate of *In-vitro* cultures can be reduced, so that the life of the cultured tissue is extended. The techniques, which

have been used to maintain collections in slow growth include the use of reduced temperature, reduced light conditions, modifications in media, particularly the addition of osmotic inhibitors or growth retardants, dehydration of tissue, or modification of gaseous environment. Combinations of these techniques are used for many species, such as cassava, potato, Musa, coffee, etc. (Ashmore, 1997).

One of the factors, which causes tissue death, or drying of *In-vitro* cultures is moisture loss, due to evaporation both from the tissue and from the culture medium. This can be prevented or minimized by the use of suitable culture vessel closures. Instead of closures, which allow more evaporation, caps or films which allows gaseous exchange but prevent moisture loss should be used. By closing the culture tube with screw caps, the storage period of Mentha spp., blackberry, Rubus, Vaccinium, Pistachio and *Coffea arabica* cultures could be extended for 12 months under normal culture conditions (Kartha *et al*., 1981; Barghchi, 1986; Gunning and Lagerstedt, 1986). White spruce embryogenic tissues withstood at 1 year storage period in hermetically sealed serum capped flasks (Joy *et al*., 1991). The use of heat-sealable polypropylene bags instead of glass test tubes or plastic boxes was beneficial for the storage of several strawberry varieties (Reed, 1991; 1992). Sharma (2001) reported storage of *In-vitro* cultures of a medicinal plant, *Gentiana kuroo* up to 7 months at 25^{O}C by replacing the cotton plugs with polypropylene caps as culture tube closures.

Shoots and plantlets of many plant species have been stored successfully at low temperatures. Optimal conditions usually vary from one genus to another. Temperatures in the range of 0-5^{0}C have been found to be suitable for conservation of *In-vitro* cultures of cold tolerant species. Temperate woody species like apple, pear and Pinus, etc. could be stored at 0-5^{0}C (Lundergan and Janik, 1979; Chun and Hall, 1986; Aitken-Christie and Singh, 1987). Apple and Prunus

shoots survived 52 weeks at 2°C (Druart, 1985). Strawberry (Fragaria x ananassa) plantlets have been stored at 4°C in the dark and kept viable for 6 years with regular addition of a few drops of liquid medium (Mullin and Schegel, 1976). Tropical species are often cold sensitive and have to be stored at higher temperatures, which depend on the cold sensitivity of the species. For many tropical and sub-tropical species in which low temperature storage reduces the viability of shoot cultures, a temperature of 14-20°C is suitable for inducing minimal growth (Henshaw *et al*., 1980). Kiwi fruit shoots could be conserved at 8^0C (Monette, 1986) and taro tolerates 3 years of storage at 9^OC (Staritsky *et al*., 1986). Banerjee and De Langhe (1985) maintained proliferating cultures of seven cultivars of Musa for more than 1 year at 15^0C and low light level (1000 lux). Jarret *et al*. (1986) stored 38 Musa accessions for up to 18 months at 18^0C under low light intensity in liquid media on filter paper bridges. Cultures of cassava deteriorated if stored at temperatures lower than 20^0C (Roca *et al*., 1984). Shoot cultures of *Actinidia chinensis*, Vitis species, sugarbeet and potato are reported to be stored at 12^0C (Hussey and Hepher, 1978; Westcott, 1981; Henshaw, 1982; Monette, 1986; Galzy and Compan, 1988). Oil palm, another tropical species, is more cold sensitive and their somatic embryos do not withstand even a short exposure to temperatures lower than 18^OC (Corbineau *et al*., 1990).

An alternative but less common approach is the modification of culture media by reducing the nutrient concentration of the basal medium or by adding an osmoticum to the culture medium or by removal of growth regulators from the culture medium or by adding growth retardants to the medium. Embryogenic cultures of carrot could be conserved on a medium without sucrose for 2 years, and proliferated if a sucrose solution was supplied (Jones, 1974). Zamora *et al*. (1986; 1987) reported storage of Musa cultures up to 4 months on media containing sorbitol or mannitol.

Replacement of sucrose by ribose allowed the conservation of banana plantlets for 24 months (Ko *et al*., 1991). Addition of osmotic growth inhibitors such as mannitol or hormonal growth inhibitors (abscisic acid) is also employed successfully to reduce growth (Westcott, 1981 Staritsky *et al*., 1986; Ng and Ng, 1991; Viterbo and Rabinowitch, 1994; Vysotskaya, 1994). Induction of slow growth by altering the constituents of the culture media was reported in many crop species like Cinchona, cassava, coffee, strawberry, tomato, potato, Vitis, etc. (Henshaw *et al*., 1980; Kartha *et al*., 1981; Gunning and Lagerstedt, 1986; Hunter *et al*., 1986; Schnapp and Preece, 1986; Moriguchi and Yamaki, 1989). Kartha *et al*. (1981) could conserve coffee plantlets on a medium devoid of sugar and with only half of the mineral elements of the standard medium. The type of explant used can influence the duration of storage achieved. In chrysanthemum nodal segments showed higher survival rates than apical buds (Roxas *et al*., 1995). The presence of a root system generally increases the storage capacities, as observed by Kartha *et al*. (1981).

Low temperature regimes are used for the storage of sugarcane shoots at 18^0C on reduced mineral salts, potato microtubers at 10°C (Kwiatkowski *et al*., 1988) strawberry and Rubus shoots at 4^0C in the presence of plant growth substances (Reed, 1992), and banana shoots at 16°C in the presence of plant growth substances (Van den houwe *et al*., 1995). The subculture interval ranged from every 6 months for *Coffea spp*. to every 21 months for potato microtubers.

In ginger and turmeric, the cultures could be stored up to 12 months with 70-80% survival in half strength MS medium supplemented with 15 gl^{-1} each of sucrose and mannitol in sealed culture tubes. Storage of these cultures beyond 12 months was impossible as the aerial shoot exhibited yellowing and symptoms of deficiency by then.

In *Kaempferia galanga* and *Kaempferia rotunda*, the cultures could be stored up to 12 months with 80-90% survival in half strength MS medium supplemented with 15g/l each of sucrose and mannitol in .sealed culture tubes. In *K. galanga*, the cultures could be stored up to a maximum period of 18-19 months with 60% survival, whereas in *K. rotunda* further storage was not possible as the culture growth was comparatively high and the cultures exhibited drying.

In all these species minimal growth could be induced and cultures were maintained up to 12 months without subculture in half strength MS medium supplemented with $10gl^{-1}$ each of sucrose and mannitol also. But in this medium cultures were not very healthy and showed symptoms of deficiency; hence the medium with $15gl^{-1}$ each of sucrose and mannitol was used for *In-vitro* conservation of these species.

Few attempts have been made earlier to use tissue culture for conservation of ginger germplasm. The subculture interval could be extended up to 7-12 months by using mannitol as osmoticum and polypropylene caps to minimize evaporation loss (Balachandran *et al*., 1990). Dekkers *et al*. (1991) reported that ginger shoot cultures could be maintained over 1 year at ambient temperatures of 24-29°C in a medium containing 25 gl^{-1} mannitol with an overlay of mineral oil. Babu (1997) reported medium term storage of *In-vitro* cultures of ginger in mannitol and sucrose containing medium in culture tubes closed with aluminium foil and he reported the deterioration of ginger cultures under low temperatures of 5°C and 10°C.

2.12 Antioxidant defense system in plants

The antioxidative defense system of plants comprise of antioxidants and antioxidative enzymes. In plants, ROS like superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and alkoxyl radical (RO^-) are continuously produced predominantly in chloroplast, mitochondria

and peroxisomes. Normally, production and removal of ROS are balanced. However, the production and scavenging of ROS may be disturbed by number of biotic and abiotic factors or stresses (Apel and Heribert, 2004). These ROS initiate lipid peroxidation and degrade proteins, lipids and nucleic acids which may lead to cell death (McCord, 2000). ROS are removed by enzymatic and non-enzymatic antioxidative defence systems. Non-enzymatic antioxidative system includes the ascorbate, reduced glutathione (GSH), tocopherol, flavonoids, alkaloids and carotenoids. Ascorbate and α-tocopherol are extremely effective antioxidants as they are relatively poor electron donors and effectively scavenge OH^-, O_2^- and singlet oxygen.

Enzymatic antioxidative system consists of various antioxidative enzymes like of superoxide dismutase, peroxidase, Catalase, monodehyroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR). SOD acts as first line of defence against ROS, dismutating O_2^- to H_2O_2. Subsequently Catalase, Peroxidase detoxify H_2O_2 to H_2O. H_2O_2 is also converted into water by ascorbate glutathione cycle. Ascorbate peroxidases (APX) detoxify the H_2O_2 to H_2O by using ascorbate as reducing agent, which is oxidized into mono-dehydroascorbate. Later, it can be regenerated into ascorbate with the help of MDHAR using nicotinanide adenine dinucleotide phosphate (NADPH) as the reducing power. Mono-dehydroascorbate can be spontaneously dismutated into dehydroascorbate. Then ascorbate regeneration is mediated by DHAR driven by oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG). Finally, glutathione reductase (GR) regenerates GSH from GSSG using NADPH as the reducing agent. The extent of oxidative stress in a cell is determined by the amount of O_2^-, H_2O_2 and OH^-. Therefore, the balance of antioxidative enzymes activities is vital for suppressing the toxic levels of ROS. All component of enzymatic and non-enzymatic defence system act in a co-ordinated manner and constitute the pathway called as “Asada-Halliwell pathway” (Noctor and Foyer,

1998; Apel and Heribert, 2004). Photosynthetic pigments (Chlorophylls a,b, and carotenoids) are important factors of photosynthesis and are very important for plant growth and development and protect the plant from various stresses (Tanaka *et al.*, 2006). Carotenoids protect the chlorophyll by absorbing excess of excitation energy and quenching singlet oxygen (Vernoux *et al.,* 2000a, b). Therefore, decrease in carotenoid levels increase the possibility of OH formation. It is already known that mannitol can induce moderate osmotic stress, reducing the osmotic potential in culture medium, besides being a compatible solute, which does not disturb proteins structure, protecting SH-enzymes from oxidative inactivation (Shen *et al*., 1997a). Mannitol can be absorbed from culture media at the cell level. The intracellular accumulation contributed to the osmotic adjustment potential and improved tolerance to desiccation.

In counteracting reactive oxygen species (ROS) resulted from oxidative stress generated by desiccation and for the restoration of the cells homeostasis, the plants have antioxidant equipments, which inactivated ROS by transformation in stable compounds, to avoid the cellular damages. The antioxidant enzyme as catalases and peroxidases, can act together with other endogenous reducing agents, as ROS scavengers in the plant cell.

2.13 Application of FTIR in the study of biological tissues

The dried state of desiccation-tolerant tissues limits the number of techniques that can be applied to study conformation and stability of intracellular biomolecules. One of the few suitable techniques for dried tissue analysis is FTIR spectroscopy. The advantage of FTIR is that it can be used, irrespective of the hydration state of the tissue. On account of characteristic molecular vibrations that absorb in the infrared region, information can be derived on the molecular conformation and the inter-molecular interactions of biomolecules in their native environment.

With an FTIR microscope, a specific sample area as small as 100 μm2 can be selected for FTIR analysis. For a macromolecule, there are many vibrational transitions absorbing in the IR region, which can be assigned to particular bonds or groupings. This forms the basis of characteristic group frequencies. The main experimental parameter is the position of the maximum of the absorption band, often presented as wavenumber (cm^{-1}). The band position of a molecular group depends on the intrinsic molecular vibration and on the micro-environment of the oscillating atoms. Information can be obtained about the molecular structure and interaction with other molecules. Characteristic group frequencies also form the basis for the analysis of biological tissues. It should be realized that the observed group frequencies in IR spectra of biological tissues may contain contributions from various types of biomolecules. Nevertheless, some of the *in situ* IR bands are dominated by one type of biomolecule. The characteristic CH_2 stretching vibrations of lipids have been used to detect lipid phase transitions in isolated biological membranes and in whole cells (Cameron *et al*., 1983; Crowe *et al*., 1984, 1989). Lipids contain a relatively high proportion of CH_2 groups compared to other biomolecules, which show up as two characteristic absorption bands at around 2924 and 2854 cm^{-1}, denoting the asymmetric and symmetric stretching mode of the lipid CH_2 groups, respectively. Proteins in biological tissues can be detected on account of two characteristic absorption bands at around 1650 cm^{-1} (Amide-I band) and 1550 cm^{-1} (amide II band), arising from the peptide backbone. The Amide-I band, which is most often used for protein analysis, is usually a complex band, because the different types of protein secondary structure have different IR transitions in the Amide-I region. The Amide-I band has been extensively studied to determine the relative proportion of the different types of protein secondary structure (Bandekar, 1992; Goormaghtigh *et al*., 1994; Surewicz *et al*., 1993). The observed protein bands in biological tissues are the average of all the proteins in

the cell, but are often dominated by one type of protein. For example, in *Lathyrus sativus* seeds, globulins and albumins comprise 60% and 30% of the total protein fraction, respectively (Rosa *et al*., 2000). The OH stretching band between 3600 and 3000 cm^{-1} is dominated by water in hydrated biological tissues, but in dried, desiccation-tolerant tissues, this band arises from carbohydrates and proteins (Wolkers *et al*., 1998).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1. Plant material (Source of the explants)

All taro genotypes used in the experiments were obtained from the Taro Breeding Programme at CSIR-CRI, Kumasi as corms. The taro genotypes used for the experiment are presented in table below.

PASSPORT DATA OF TARO GERMPLASM					
Collecting number	**Country**	**Locality**	**Source**	**Type of Material**	**Date of Collection**
KA 2011/019	Ghana	Ankokrom	Garden	Cormel	15-09-11
SAO 2011/002	Ghana	Okyerekrom	Farm	Cormel	23-09-11
SAO 2011/006	Ghana	Okyerekrom	Farm	Cormel	23-09-11
BLSM/80	Western Samoa	Alafua	Laboratory	Tissue culture	14-08-11
CEMAL /14	Malaysia	Kluang	Laboratory	Tissue culture	14-08-11
CEMAL/32	Malaysia	Kluang	Laboratory	Tissue culture	14-08-11

During initiation *In-vitro* mother plants were obtained from stock plant which had been grown in the field (Plate 3.1a).

3.2. Preparation of Plant Material and Surface Sterilization

The excised corms (Plate 3.1b) were thoroughly washed under running tap water for 10-15 minutes and then soaked in 70% ethanol for 5 minutes and rinsed with distilled water, followed by disinfecting with 20% and 10% NaOCl which had 2 drops of tween20 added for 20 and 10 minutes respectively (Plate 3.1c). The excised corms were washed with distilled water and trimmed at each level of disinfection, finally rinsed with sterile distilled water and trimmed to its shoot meristem (Plate 3.1d). This was done to get rid of the micro-organisms that might be

present. Corm cuttings of length approximately 0.5cm were used for initiation before sub-culturing to multiplication and conservation media.

Plate 3.1 Taro stock plant (A), excised corm (B), sterilized trimmed corm (C) and (D) shoot meristem length (0.8-1cm) for initiation culture *In-vitro*

3.3 Culture media preparation

When preparing medium, the needed volume of the stock solutions were mixed and made up to three-quarters of the required volume by the addition of distilled water. Stock cultures were maintained on Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium supplemented with 30 g/l sucrose and stirred by the use of magnetic stirrer. Growth regulators and other chemicals were added to the medium as required. The medium was adjusted to the correct volume with distilled water and the pH was adjusted to 5.7± 0.1 using 0.1M and 1M solutions of NaOH and HCl. 0.8% purified agar (phytoblend) was then added to the medium and allowed to dissolve by micro waving before dispensing into culture vessels. The medium was autoclaved at a pressure of 105.4kg/cm^2 and temperature of 121^{0}C for 15-20 minutes.

3.4 Sterilization Procedures for the apparatus and laminar air flow chamber

The laminar air flow chamber was first switch on and cleaned with technical alcohol (70% ethanol) and then left for about 10-15 minutes before use. During this period, the following were arranged in the chamber; sterile distilled water, sterilized beaker, sterilized dissecting kits and vessels containing media as well as plant materials. Dissecting kits were placed in alcohol (70%), and flamed in spirit lamp to further sterilize them before being used for inoculation.

3.5 *In-vitro* Rapid Multiplication

To assess effect of transverse section (TS) and longitudinal section (LS) explants excision on rapid multiplication of *In-vitro* grown cultures, 0.5-0.8cm shoot cuttings (Plate 3.2A and B) from proliferated shoots were transferred and separately cultured on complete MS basal salts with

vitamins supplemented with 30g/l sucrose, 0.5mM NAA, 5.0mM BAP and 80mg/l $AdSO_4$ plus 0.8% purified (phytoblend) agar for rapid multiplication *In-vitro*. Culture vessels used were baby food jar with diameter 5.8cm Base Dia. × 4.8cm opening Dia. × 9.05cm, Height covered with plastic closure and sealed with parafilm to reduce infection and evaporation of the culture medium. The sealed cultures jars were incubated at 25±1°C, 16 hours photoperiod and 5000lux.

3.6 *In-vitro* Medium-Term Conservation

In-vitro established shoot proliferating cultures were used to set up medium-term conservation experiment. Individual shoots were separated and leaves and root were trimmed off. The length of the resultant explant used was approximately 0.8cm, as shown in Plate (3.2C). Isolated explants were cultured on complete MS basal salt with vitamins supplemented with 30g/l sucrose and different concentrations (0, 20, 25, and 30g/l) of mannitol, and also White medium for medium term conservation plus 0.8% purified (phytoblend) agar. Culture vessels used were sigmaware test tubes with diameter of 25mm × 150mm Height covered with plastic closure and incubated at 20±1°C, 12 hours photoperiod and light intensity of 3000lux.

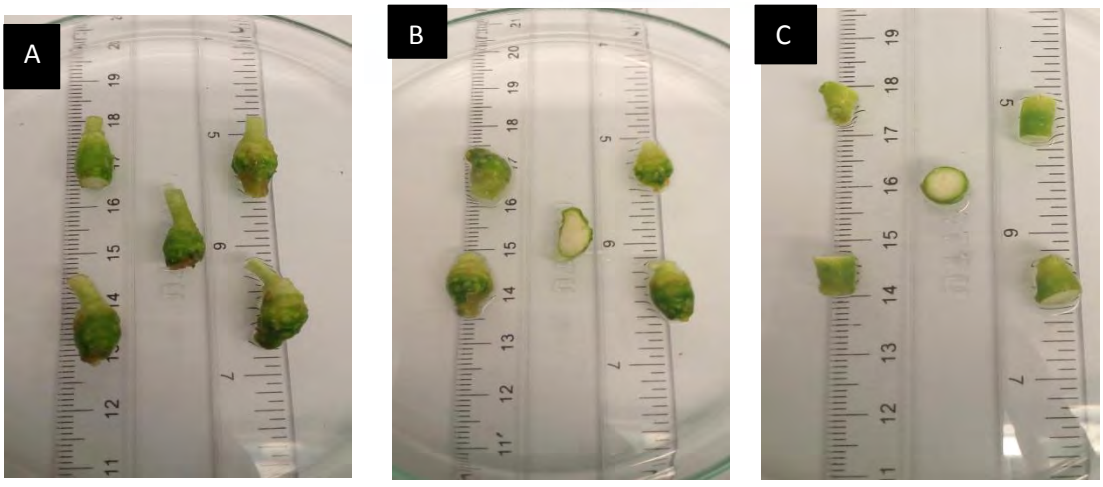


Plate 3.2 Multiplication shoot (F and G) and Conservation shoot explant (E)

E. *In-vitro* trimmed shoot explant length (0.8-1.0cm) for conservation.

F- G. *In-vitro* shoot explant length (0.5-0.8cm) for rapid multiplication.

F. LS excision of shoot length

G. TS excision of shoot length

3.7 *In-vitro* Recovery

The conservation experiments were conducted over a 6 month period, at the end of which surviving shoots were excised and cultured on complete MS basal salt with vitamins supplemented with 30g/l sucrose, 0.5mM NAA, 5.0mM BAP and 80mg/l $AdSO_4$ plus 0.8% purified (phytoblend) agar for growth *In-vitro*. Cultures vessels used were sigma ware test tubes with diameter of 25mm × 150mm Height covered with plastic closure and incubated at 25±1°C, 16 hours photoperiod and 5000lux.

3.8 FTIR analysis for concentration of Mannitol absorption content in *Colocasia* genotype

At the end of six months period of conservation, infra-red spectral analysis was carried out in the methanol extract of *Colocasia* genotypes using a scanning Michelson inferometer and Fourier Transformation (Shimadzu, Japan). The samples were weighed and ground in a mortar with 1:1 (w/v) ml of distilled water. The larger tissue was centrifuged at 4000rpm for 5 minutes and the supernatant solution was poured into various centrifuged tubes (10ml) with dimension of 1.5cm Base Dia. × 2cm opening Dia. × 9.5cm height. A micropipette was used to pipette the supernatant and drops onto an infra-red spectrophotometer for reading. This Fourier Transform is the infrared absorption spectrum of the sample and its wavelength; standards of 4.1, 8.3, 16.6, 20.75 and 25.0g/100g mannitol were read.

3.9 Biochemical analyses

A 20% homogenate of the different parts of *Colocasia* genotypes was prepared in phosphate buffer. The homogenate was centrifuged and the supernatant was used for the enzyme assay. H_2O_2-phosphate buffer (3.0ml) was taken in an experimental cuvette, followed by the rapid addition of 40µl of enzyme extract and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded at 240nm in a spectrophotometer (Genesys 10-S, USA). The enzyme solution containing H_2O_2-free phosphate buffer served as control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240nm by 0.05 units. Catalase and peroxidase enzymes were determined. Catalase activity was assayed following the method of Luck (1974).

Enzyme extract of 0.1ml was added to 3.0ml of pyrogallol solution and the spectrophotometer was adjusted to read zero at 430 nm. Hydrogen peroxide (H_2O_2) of 0.5ml was added and mixed in the test cuvette. The change in absorbance was recorded every 30 seconds up to 3 minutes in a spectrophotometer (Genesys 10-S, USA). One unit of peroxidase is defined as the change in absorbance/minute at 430nm. The method proposed by Reddy *et al*. (1995) was adopted for assaying the activity of peroxidase.

3.10 Statistical Analysis

The experiments were arranged in completely randomized design. Data were analyzed using one-way ANOVA tests and comparison of treatment means by LSD (least Significant Difference) using GENSTAT 9. Significance was determined at the 5% level (Gomez and Gomez, 1976) and the data presented in tables and figures are mean values of three replicates ± SD. Genotypes BL/SM/80 and KA 019 were used for rapid multiplication, whereas SAO 002,

SAO 006, CE/MAL/32, and CE/MAL/14 also for both medium term conservation and recovery experiment.

Data taken include; Number of shoots/culture, Number of leaves/culture, Height of shoot (cm) and its development. The analysis in the incipient phase of the conservation culture (i.e., 2 weeks intervals) and rapid multiplication (i.e., 1 week intervals) were made to determine if the used protocol ensured the multiplication and conservation with minimal growth and allowed the survival and adaptation of the taro explants in the restrictive condition. Percentage survival was also scored to enable comparison of survival and performance of mean number of leaves, shoots and commenting on the performance on Initiation medium.

CHAPTER FOUR

4.0 RESULTS

4.1 Initiation of *Colocasia* Genotypes

Results obtained when initiating the three taro varieties are presented in Table 4.1 below. Survival was at 68.75% for genotypes CE/MAL/32 and SAO 002. Genotype SAO 006 had a higher survival of 75.0%. Numbers of shoot developed ranged from 1.41- 1.75 was similar for all the genotypes. Similarly, the number of leaves was similar for all the genotypes and ranged from 1.40-1.94 leaves/shoot (Table 4.1).

However, SAO 006 produced about 82-95% taller plant height than CE/MAL/32 and SAO 002 (Table 4.1). Moreover, after the initiation period of two months, plantlets formed were sub-cultured onto multiplication medium which were later used for the medium conservation experiment.

Table 4.1 Growth of three genotype explants on initiation growth medium after 8weeks in culture

Colocasia genotype	% Survival	Number of Shoots/culture	Number of Leaves/shoot	Height of Shoot/culture(cm)
CE/MAL/32	68.75	1.51	1.40	1.63
SAO 002	68.75	1.41	1.50	1.75
SAO 006	75.00	1.75	1.94	3.18
Mean	70.83	1.56	1.61	2.19
Lsd(0.05)		NS	NS	NS

4.2 Transverse Section (TS) and Longitudinal (LS) Explant Excision Effect on Culture Growth

At 4 weeks, both the TS and LS excisions produced similar number of shoots (5.60) and number of leaves was higher in LS (6.06) than TS (5.60) for the genotype BL/SM/80 (Fig 4.1a and b). KA 019 genotype had the highest number of shoots occurring on LS (7.33) culture than TS (4.87) whereas highest number of leaves occurred on TS (8.87) than LS (6.53) culture (Fig 4.1a and b).

At 8 weeks (Fig 4.1a) shoot formation was higher in TS (16.07) culture than LS (13.27) excision of shoot in BL/SM/80. Higher leaves were formed in TS (33.67) than LS, 27.40. (Fig 4.1b). The highest shoot development at 8 weeks in KA 019 genotype occurred on LS (20.27) than TS, 19.60, (Fig 4.1a). Moreover, leaves formed were also high in LS (34.07) than that of TS (24.60) culture (Fig 4.1b) for KA 019 genotype.

At 12 weeks shoot formation was very high in TS (24.00) than LS (16.73) shoot culture in BL/SM/80 (Fig 4.1a). Leaf formation was also high in TS (39.67) than that of LS, 35.20. (Fig 4.1b). The highest number of shoots formed at 12 weeks in KA 019 occurred on TS (32.00) than LS (30.60) which had less number of shoots per culture (Fig 4.1a). The highest number of leaves occurred on LS (38.13) than TS (37.40) shoot culture (Fig 4.1b) for KA 019. Root and plantlet formation were highest at this period of rapid multiplication.

Among the two *Colocasia* genotypes, the highest number of shoots and leaves occurred on TS excision of shoot culture even though statistically there was no difference among treatment means (Table 4.1). Plantlets development was highest after 12 weeks period of rapid multiplication. (Plate 4.1 and 4.2).

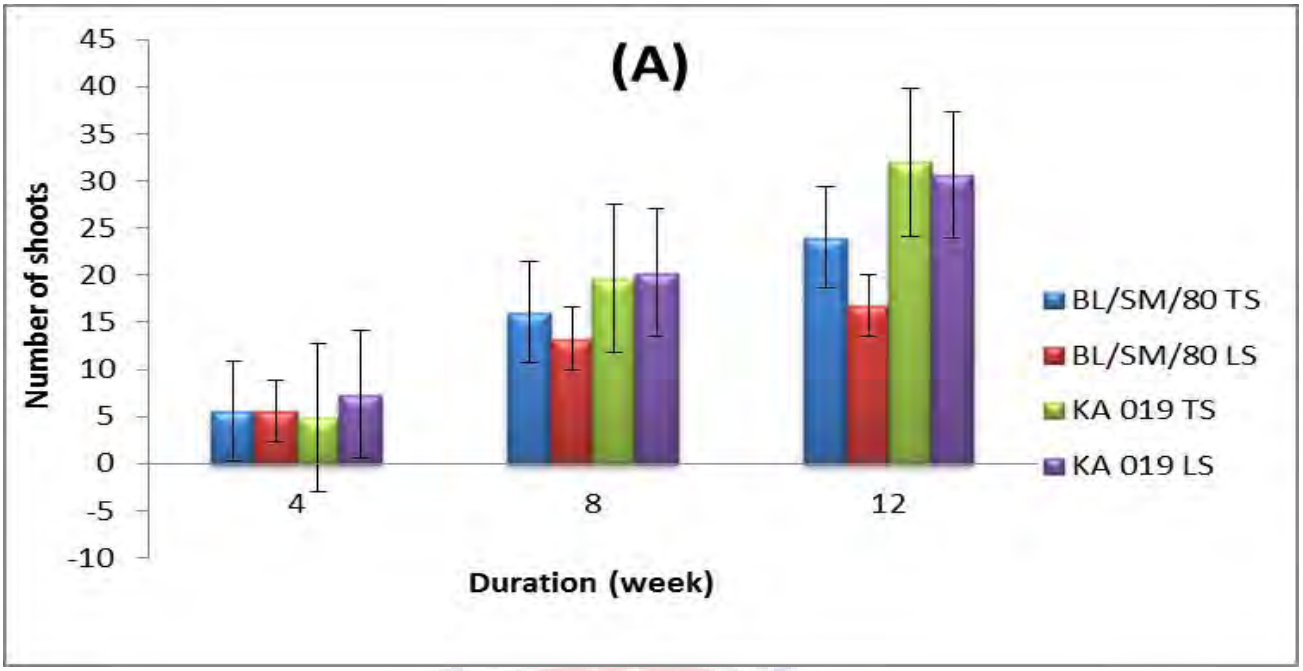


Fig 4.1 Transverse Section (TS) and Longitudinal Section (LS) explant excision effect on shoot development.

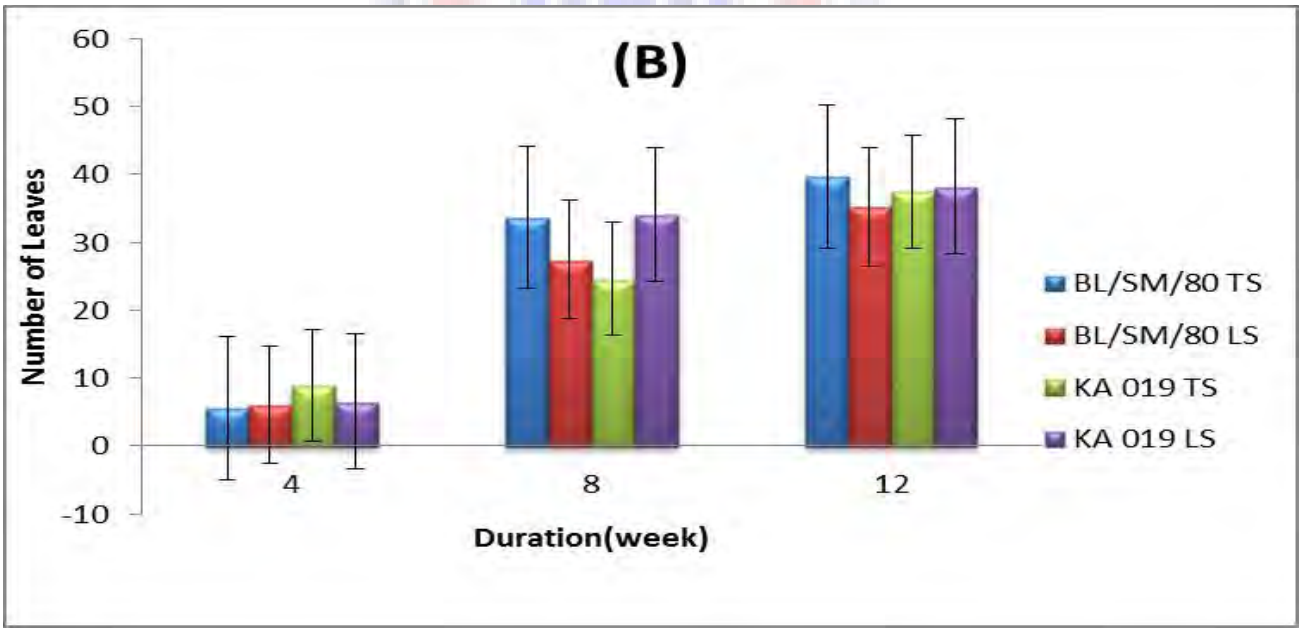


Fig 4.1 Transverse Section (TS) and Longitudinal Section (LS) explant excision effect on leave development

TS


LS

Plate 4.1 Taro exotic genotype BL/SM/80 growing *In-vitro*. Explants were excised as TS (A) and LS (B) cultures were grown on complete MS basal salt with vitamins supplemented with 30g/l sucrose, 0.5mM NAA, 5.0mM BAP and 80mg/l $AdSO_4$ plus 0.8% purified (phytoblend) agar.

TS


LS

Plate 4.2 Taro local genotype KA 019 growing *In-vitro*. Explants were excised as TS (C) and LS (D) cultures were grown on complete MS basal salt with vitamins supplemented with 30g/l sucrose, 0.5mM NAA, 5.0mM BAP and 80mg/l $AdSO_4$ plus 0.8% purified (phytoblend) agar

4.3 *In-vitro* Conservation of Taro Genotypes

4.3.1 Survival of *Colocasia* genotypes

In-vitro shoot survival and development was similar for both genotype SAO 002 and CE/MAL/32 at 100% and 2-3 shoot per culture were formed. This was observed on the White medium, 20g/l and control media (0g/l mannitol) after six month period, Plate 4.3 and 4.4(A-E)

There was also 100% survival of genotype SAO 006 and 6 shoots per culture were formed on control, 20g/l, 25g/l and 30g/l mannitol treatment (Plate 4.6A-4.6D). However, shoot culture on White medium could not survive at 6 months as there was no emergence of new shoots and such cultures became chlorotic and eventually died (Plate 4.6E).

Survival of genotype CE/MAL/14 and its shoot development was 100% with 3 shoot per cultures as this was also observed on the medium treatment for conservation (Plate 4.5A-4.5E)

4.3.2 Shoot development

Results presented in (Fig 4.2a-4.2e) showed the effect of different mannitol concentrations and White medium on shoot development from 1 to 6 months *In-vitro* conservation.

At 1 month, high shoot (2.00) formation was observed on White medium, 20g/l and control (0g/l mannitol) for both SAO 002 and CE/MAL/32 genotype, whereas higher shoot number (2.83) occurred on medium containing 25g/l mannitol for CE/MAL/14 genotype and shoots number (3.00) for genotype SAO 006 was highest on medium with 25 and 30g/l mannitol.

At 2 months, shoot number (2.00) was the same as observed on all media supplemented with and without mannitol and White medium for both SAO 002 and CE/MAL/32 genotype. The number of shoot (3.17) was higher for CE/MAL/14 genotype which occurred on medium with 25g/l

treatment only, whereas the highest shoot number (4.67) occurred on 25g/l treatment for SAO 006 genotype.

At 3 months, the same average number of shoot (2.00) occurred on all treatment media for SAO 002 genotype and high shoots number (2.50) which only occurred on 25g/l mannitol medium for genotype CE/MAL/32. However, there was higher number of shoot (3.33) for genotype CE/MAL/14 occurring on 25g/l mannitol medium, with the highest shoot (5.83) recorded on 20g/l mannitol only, for SAO 006 genotype.

At 4 months, same number of shoot (2.00) was observed on all treatment for SAO 002 genotype with an increase number of shoot from 2.50 (3month) to 2.67 (4month) which occurred on 25g/l mannitol medium for CE/MAL/32 genotype. The highest shoot number (6.17) occurred on medium containing 20g/l mannitol for SAO 006 genotype followed by CE/MAL/14 genotype with 2.83 shoot number per culture.

At 5 months, maximum number of shoot (2.00) were observed on media containing all treatment for SAO 002 genotype at this stage of development, whereas CE/MAL/32 genotype had high number of multiple shoot (2.67) on medium with 25g/l mannitol. However, genotype CE/MAL/14 had higher shoot number (3.67) on medium supplemented with 25g/l mannitol and highest multiple shoot (6.00) occurred on 20g/l mannitol medium for SAO 006 genotype. Average number of shoot also decreased from 2.00 (4month) to 1.00 (5month) on White medium for SAO 006 genotype.

At 6 months of conservation, number of shoot (2.00) was the same for SAO 002 genotype which occurred on all treatment from second month to six month, whereas maximum number of shoot

(2.83) was observed on medium containing 20g/l mannitol with the minimum (2.00) occurring on White medium for CE/MAL/32 genotype.

Lastly, with CE/MAL/14 genotype final stage of conservation, higher shoot number (3.67) occurred on medium containing 25g/l mannitol and highest multiple shoot (6.33) among all other three genotype was observed on 25g/l mannitol treatment and it decreased on White medium for genotype SAO 006.

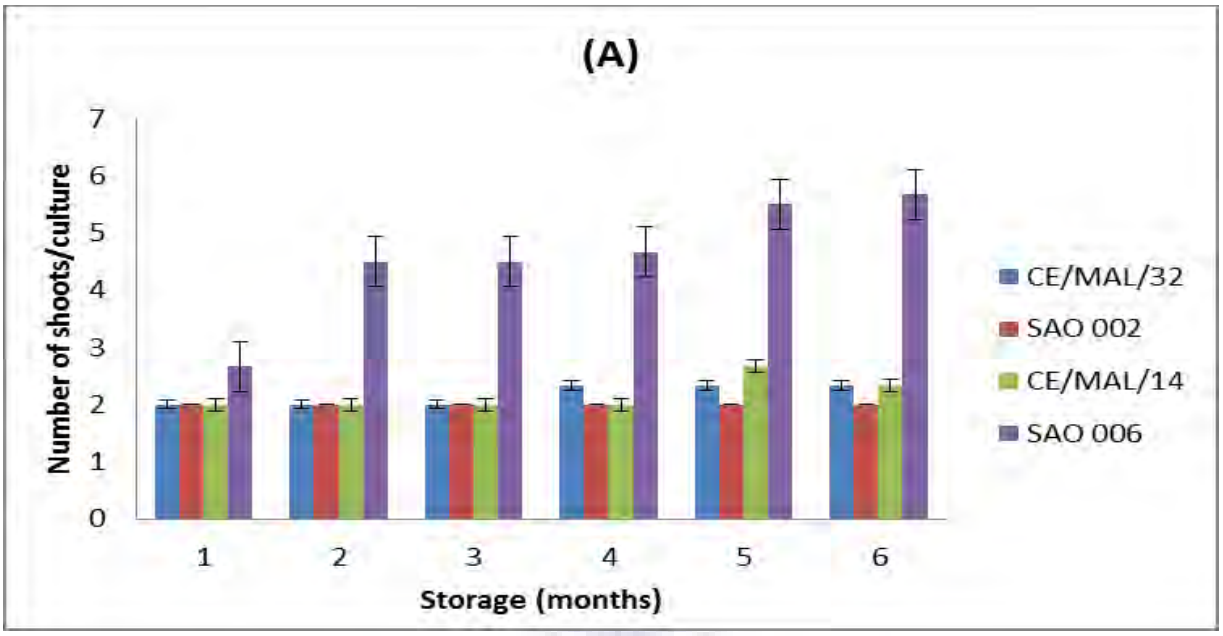


Fig 4.2 Effect of Control (0g/l mannitol) on shoot development

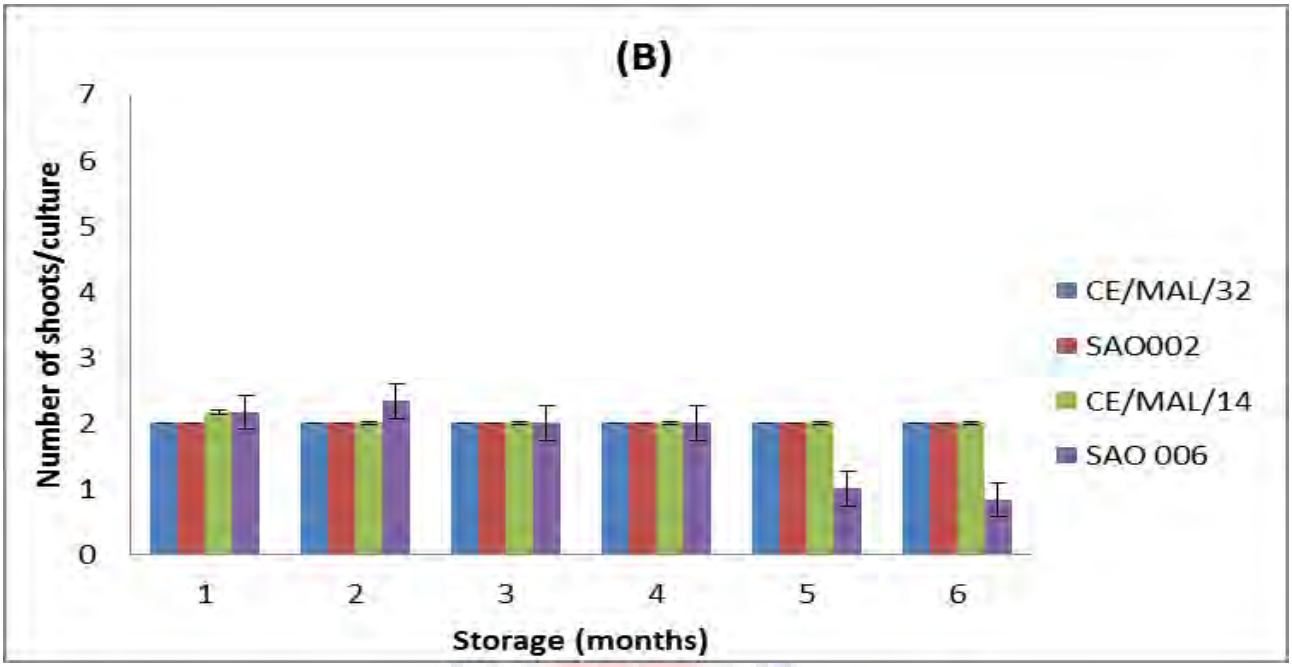


Fig 4.2 Effect of White medium on shoot development

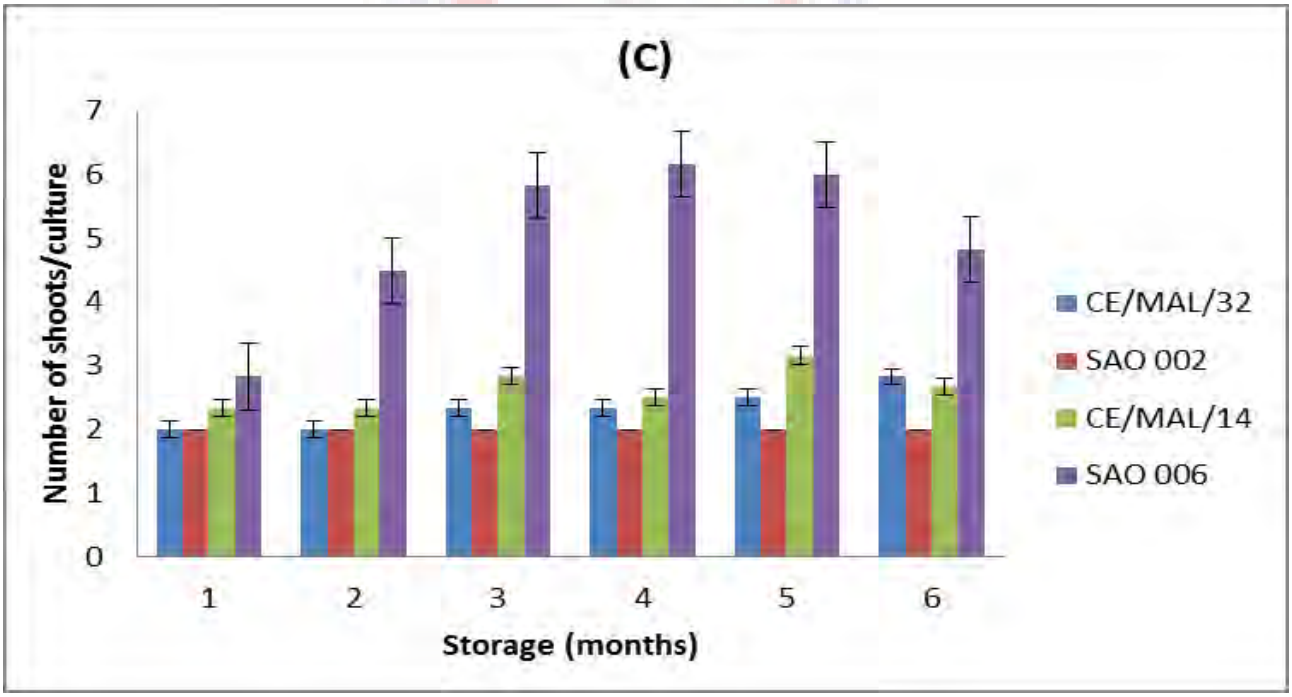


Fig 4.2 Effect of 20g/l mannitol on shoot development

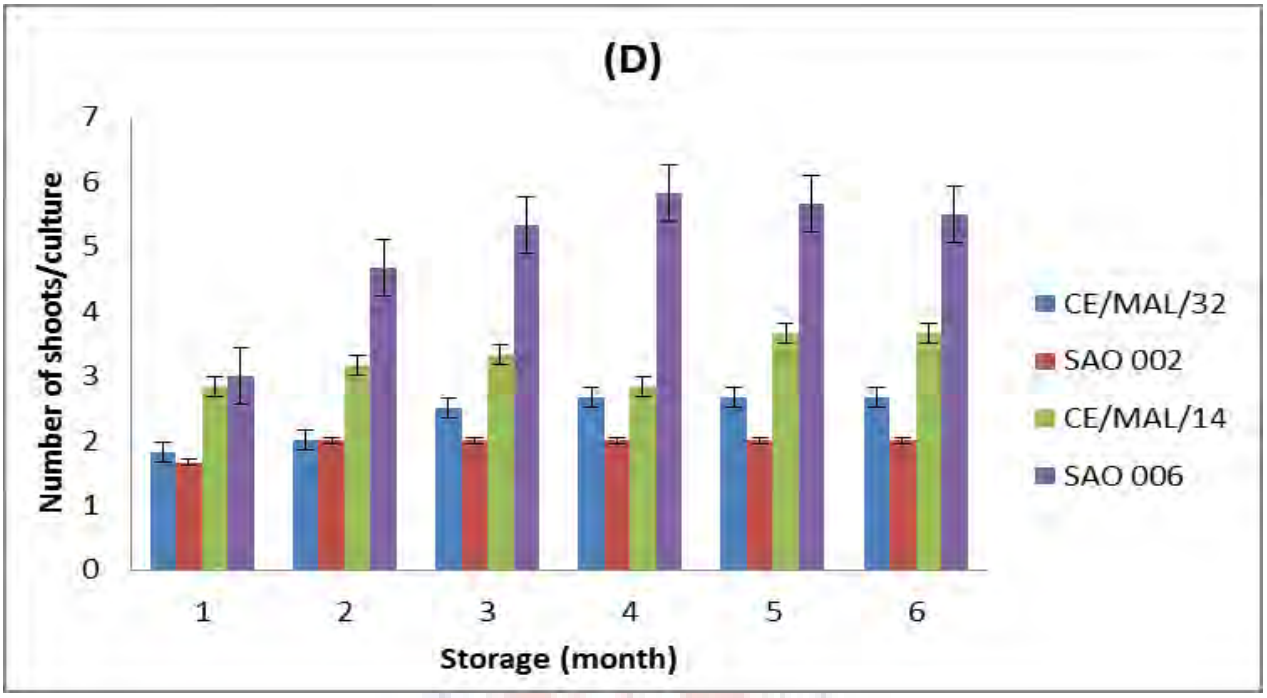


Fig 4.2 Effect of 25g/l mannitol on shoot development

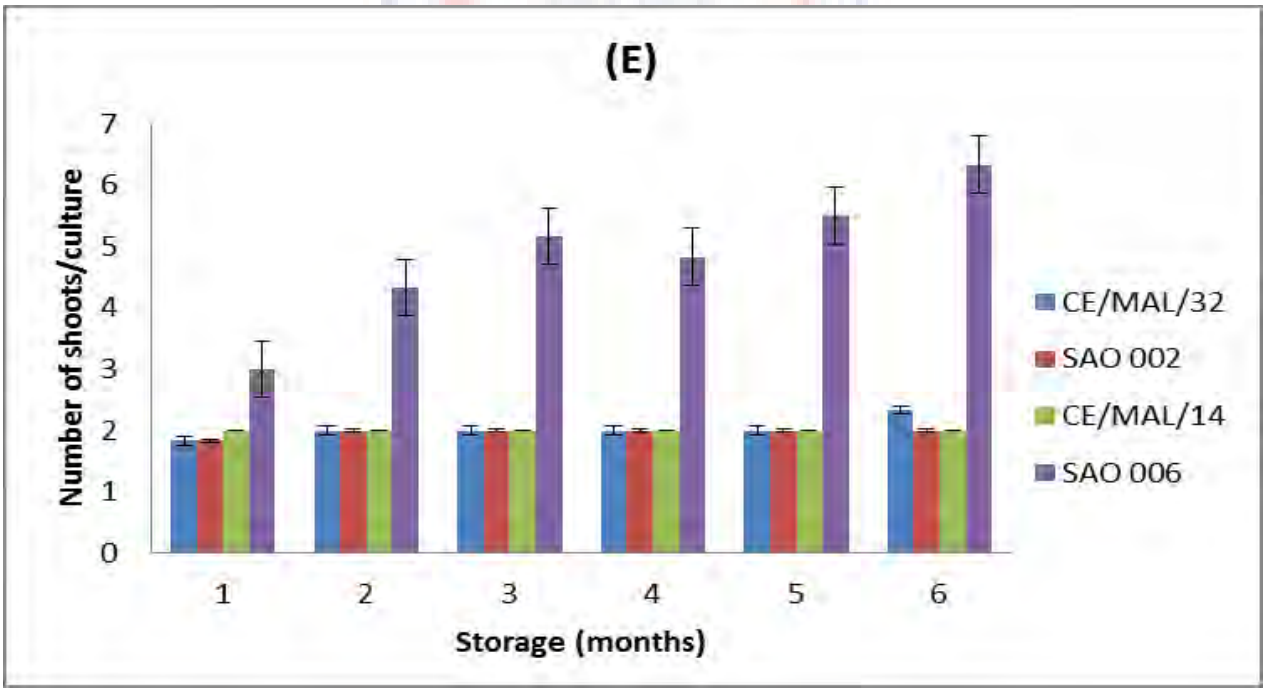


Fig 4.2 Effect of 30g/l mannitol on shoot development

4.3.3 Leave development

Results presented in (Fig 4.3a-4.3e) showed the effect of different mannitol concentrations and White medium on leave development from 1 to 6 months *In-vitro* conservation.

At 1 month, the number of leaves (5.67) developed for genotype SAO 002 was higher on the control medium, whereas CE/MAL/32 genotype recorded high number of leaves (4.50) on White medium only. Leave number was high (5.50) on 20 and 25g/l mannitol treated medium for CE/MA/14 with the highest number of leave (7.67) also occurring on 20 and 25g/l mannitol treatment for SAO 006 genotype.

At 2 months, there was an increase in leave number for genotype SAO 002 from 5.67 (1month) to 9.00 (2month) which was observed on the control medium (0g/l mannitol) and also an increase from 4.50 (1month) to 7.50 (2month) in the number of leave for CE/MAL/32 genotype on medium containing 25g/l mannitol. High number of leaves 11 and 15 occurred on 25.0g/l mannitol treatment only for CE/MAL/14 and SAO 006 respectively.

At 3 months, the average number of leaves (11.83) was recorded on control medium, with a decreased in leave number from 4.67 (2month) to 2.83 (3month) on White medium treatment for SAO 002 genotype. Genotype CE/MAL/32 had a high number of leave (8.67) which occurred on 25g/l mannitol treated medium. However, the number of leaves decreased from 4.50 (2month) to 3.83 (3month) on White medium. Average number of leave (14.33) for genotype CE/MAL/14 occurred on 25g/l mannitol treatment with a decrease from 7.83 (2month) to 7.17 (3month) and 5.67 (2month) to 5.50 (3month) on control and White medium respectively. The number of leaves (20.50) was highest which occurred on both 20 and 25g/l mannitol treatment for SAO 006 genotype.

At 4 months, an average number of leaves (14.67) was observed on control medium, with a decrease of leave number from 2.83 (3month) to 2.50 (4month) on White medium, whereas CE/MAL/32 had high average leave number (10.33) also occurring on control medium. The number of leaves continue to decrease (2.33) on White medium from the second month and also decrease from 7.67 (3month) to 6.33 (4month) on 20.0g/l mannitol treatment. Genotype CE/MAL/14 recorded a higher leave number (11.17) on 30g/l mannitol treatment, with highest number of leaves (21.00) on medium with 25g/l mannitol for genotype SAO 006.

At 5 months, there was an increase in number of leaves (16.67) on control medium with a decrease in both medium containing 20 and 25g/l mannitol from 10.17 (4month) to 9.00 (5month) for SAO 002 genotype. Genotype CE/MAL/32 had high number of leaves (13.50) occurring on control medium. Leave development was higher (12.50) for CE/MAL/14 genotype and highest (22.67) in SAO 006 genotype both occurring on medium with 25g/l mannitol treated medium. There was a decrease from 2.67 (4month) to 1.67 (5month) on White medium for SAO 006 genotype.

The number of leaves at 6 months of conservation was high (16.33) which occurred on medium without mannitol (control) and lowest (3.00) on White medium for genotype SAO 002. Few leaves were observed to be deteriorating on White medium that resulted in the decreased in number of leaves from 4.00 to 3.00. Genotype CE/MAL/32 had higher number of leaves (13.67) formed on control medium with a decrease from 4.50 (1month) to 2.67 (6 month) on White medium which was as a result of leave deterioration. Maximum number of leaves (11.67) and 22.67 occurred on medium containing 25g/l mannitol for CE/MAL/14 and SAO 006 respectively.

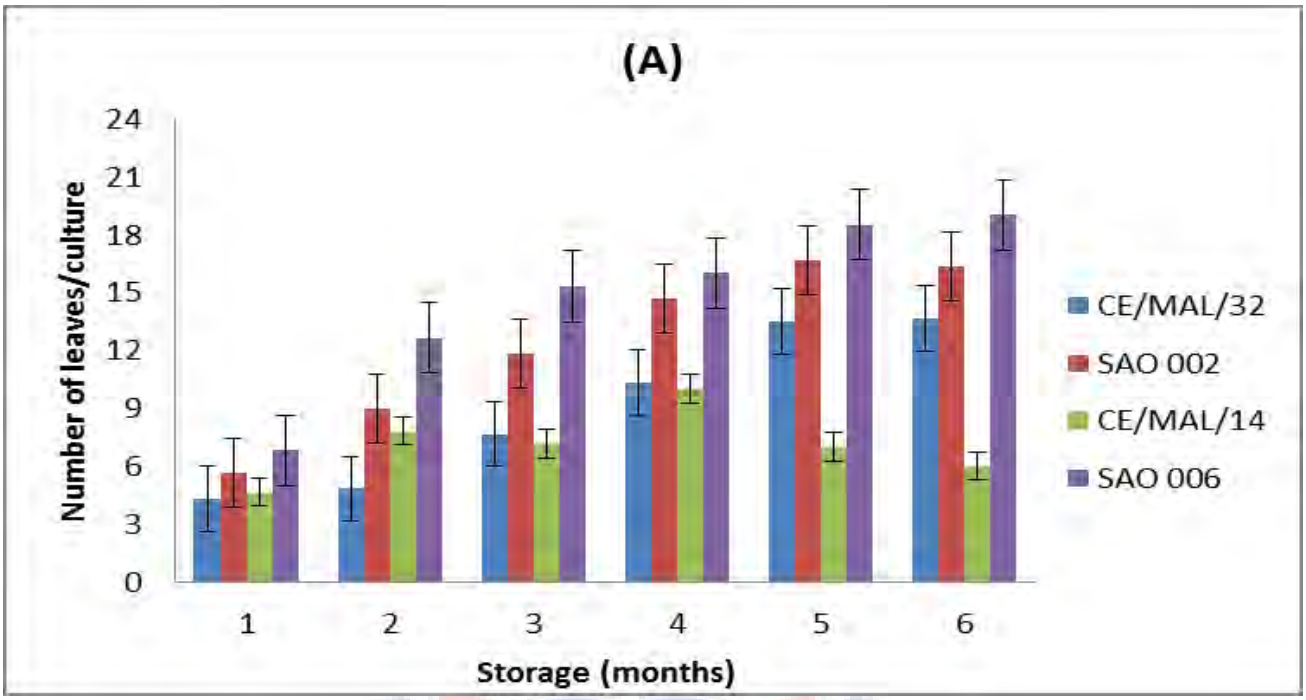


Fig 4.3 Effect of Control (0g/l mannitol) on leaves development.

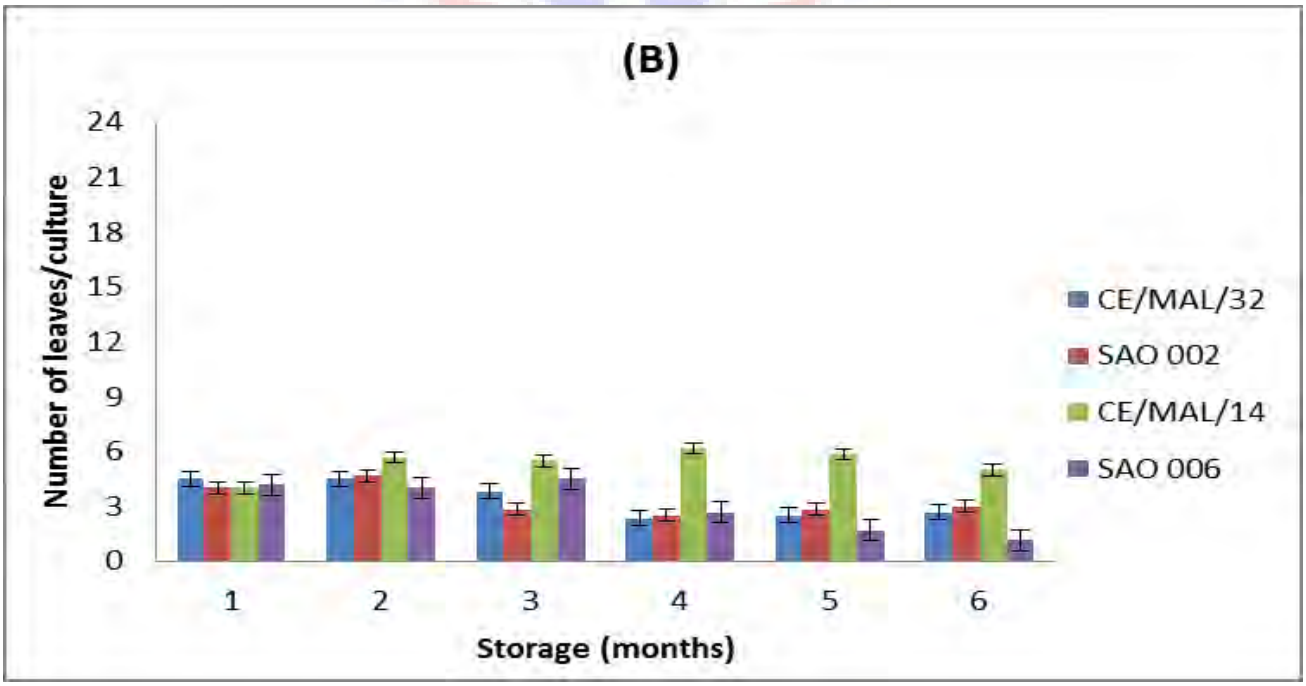


Fig 4.3 Effect of White medium on leaves development.

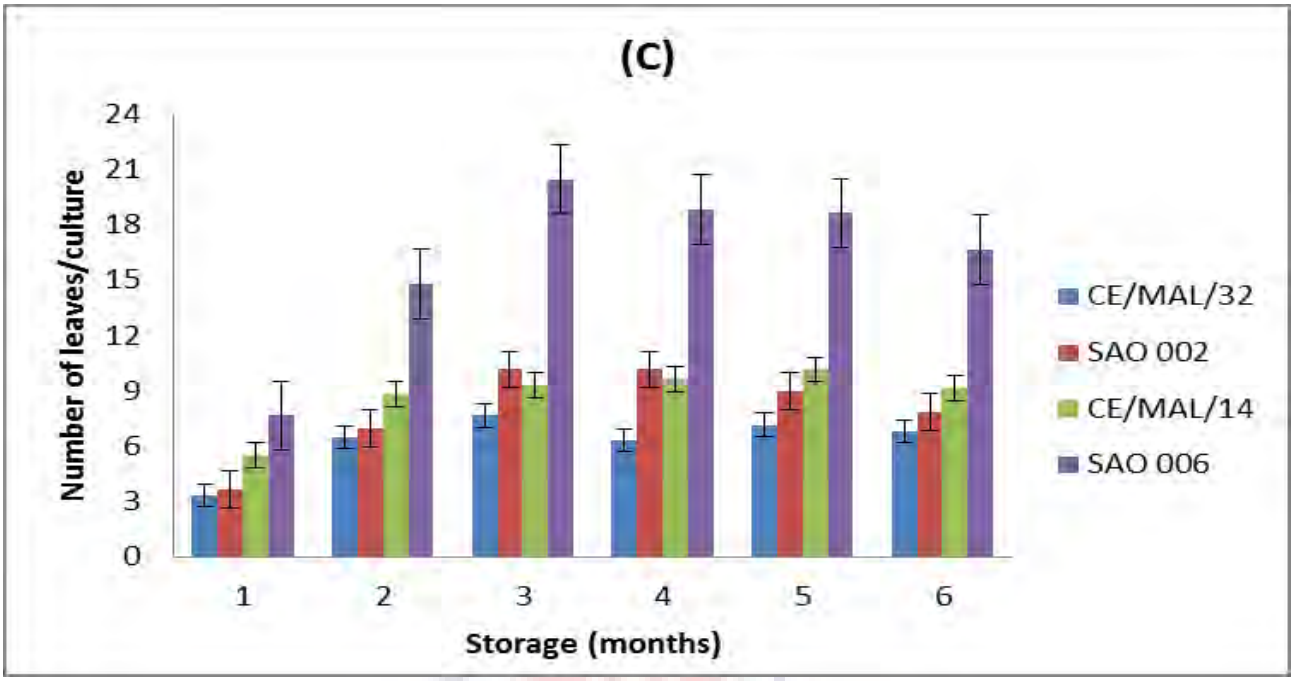


Fig 4.3 Effect of 20g/l mannitol on leaves development.

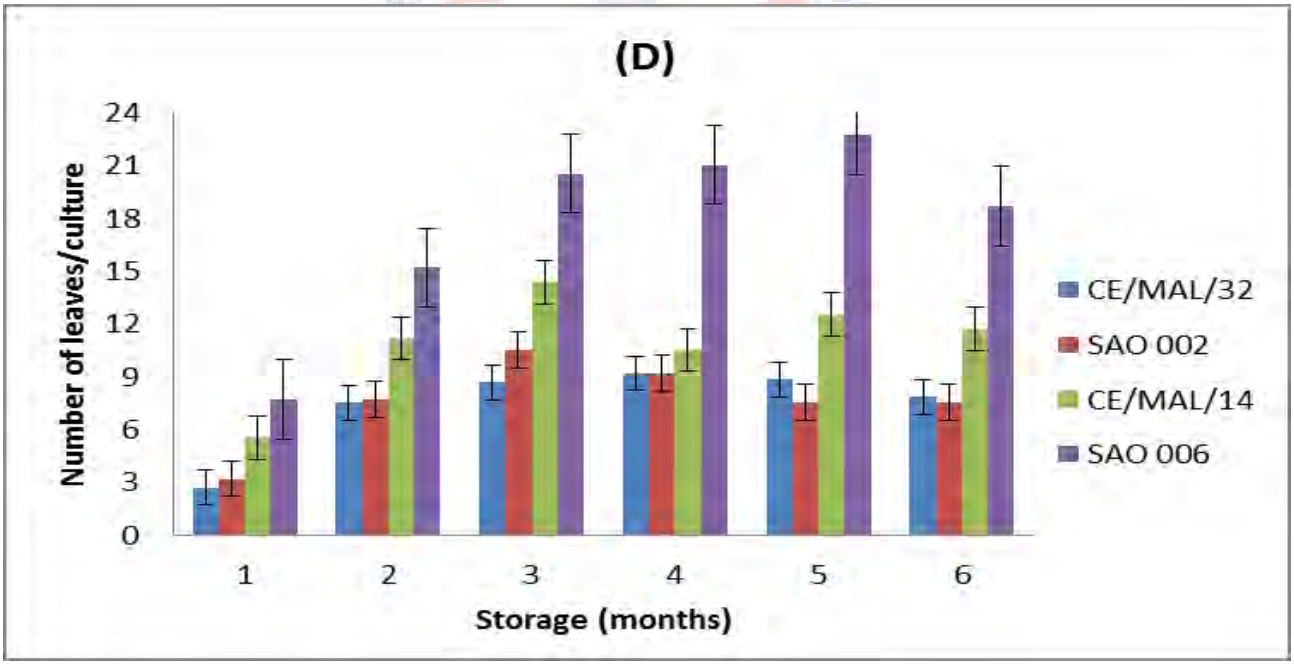


Fig 4.3 Effect of 25g/l mannitol on leaves development.

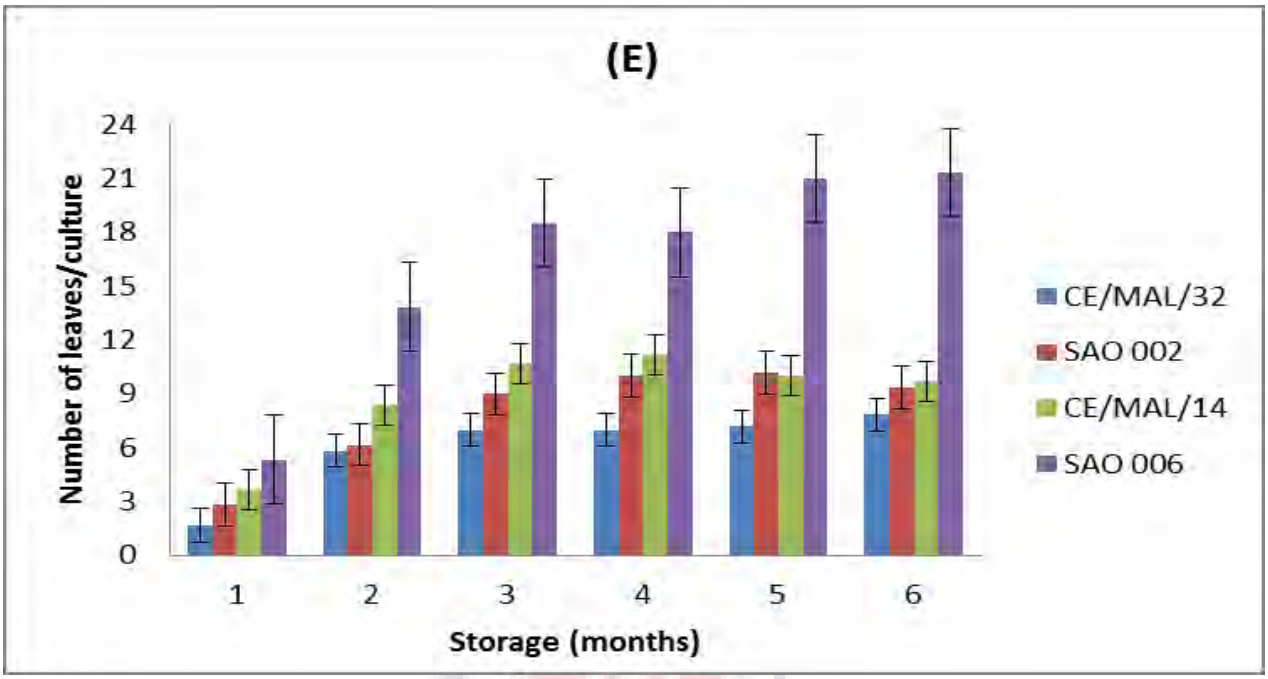


Fig 4.3 Effect of 30g/l mannitol on leaves development.

4.3.4 Shoot height

Results presented in (Fig 4.4a-4.4e) showed the effect of different mannitol concentrations and White medium on shoot height from 1 to 6 months *In-vitro* conservation.

At 1 month, height of shoot recorded was high (10.50cm) on White medium and low (3.23cm) on 30g/l mannitol treatment for SAO 002 genotype, whereas genotype CE/MAL/32 recorded was higher (11.20cm) on White medium with the lowest (3.80cm) on medium with 30g/l mannitol. Shoot height was also highest (14.67cm) on control medium and lowest (3.50cm) on 30g/l mannitol for CE/MAL/14, with shoot height recorded was high (10.85cm) on control medium and lower (2.70cm) on 30g/l mannitol for SAO 006 genotype.

At 2 months, shoot height was high (15.40cm) on control medium and lowest (5.40cm) observed on 30g/l mannitol for SAO 002 genotype, whereas CE/MAL/32 recorded high height of shoot (17.37cm) on control medium with low shoot height (6.28cm) which was observed on 30g/l mannitol. Genotype CE/MAL/14 had the highest shoot height (20.97cm) on control medium and lowest (9.75cm) on 30g/l mannitol, with shoot height higher (19.52cm) on control and lowest (5.90cm) on 30g/l mannitol for SAO 006 genotype.

At 3 months, there was high shoot height (20.08cm) on control medium and lowest (8.35cm) on 30g/l mannitol for both SAO 002 and CE/MAL/32 genotypes. However, height of shoot (28.78cm) was highest on control and lowest (14.37cm) on 30g/l mannitol for CE/MAL/14 genotype, with SAO 006 genotypes which had higher shoot height (23.70cm) on control medium and also lower (8.73cm) on 30g/l mannitol.

At 4 months, high shoot height (22.50cm) occurred on control medium and lowest height (10.60cm) on White medium for genotype SAO 002, whereas height of shoot (22.77cm) recorded was high also on control medium and low (12.80cm) on White medium for CE/MAL/32. Genotype CE/MAL/14 recorded higher shoot height (25.40cm) on control medium and lower (17.00cm) on 30g/l mannitol, with highest shoot height (25.77cm) on control medium and lowest (8.45cm) on White medium for SAO 006 genotype.

At 5 months, shoot height was high (25.02cm) on medium containing no mannitol (i.e., control) and low (10.65cm) on White medium for SAO 002, whereas genotype CE/MAL/32 had higher shoot height (24.85cm) on control medium and low (12.92cm) on White medium. However, shoot height was highest (27.67cm) on control medium and lowest (1683cm) on 30g/l mannitol

for genotype CE/MAL/14; with genotype SAO 006 had high shoot height (25.78cm) on control medium and low (3.08cm) on White medium.

At 6 months of conservation, for SAO 002 genotype, the maximum height of shoot (26.03cm) was observed on control medium and lowest (10.67cm) on White medium, whereas high shoot height (25.70cm) was observed on control medium and minimum length (11.03cm) occurred on White medium for CE/MAL/32 genotype. Genotype CE/MAL/14, height of shoot was highest (29.33cm) on control medium and lowest on (16.55cm) 30g/l mannitol medium. Lastly, SAO 006 genotype, shoot height was higher (25.52cm) on medium containing no mannitol (control) and low on (1.07cm) White medium which eventually died.

Among the four genotype, three genotypes (SAO 002, CE/MAL/32 and CE/MAL/14) had higher number of shoot (2.00-3.67), leave number (11.67-16.33) and shoot height (25.00-29.33cm) which occurred on control medium (0g/l), whereas SAO 006 genotype had the highest shoot number (6.33), leave number (22.67) and lowest shoot height of 16.82 occurring on 25 and 30g/l mannitol treatment medium Fig (4.3a-4.3e) Plate (4.3-4.5)

The best treatment that was able to restrict growth at the end of conservation period (6month) was 30g/l mannitol without any deteriorating in leaves or death of shoot, followed by 25g/l mannitol as compared to other treatment most especially White medium Plate (4.3-4.6(C-D).

Finally, the shoot cultured on White medium, 20 and control (0g/l mannitol) showed overgrowth, filling the culture vessel at the end of six month period conservation Plate (4.3-4.6).

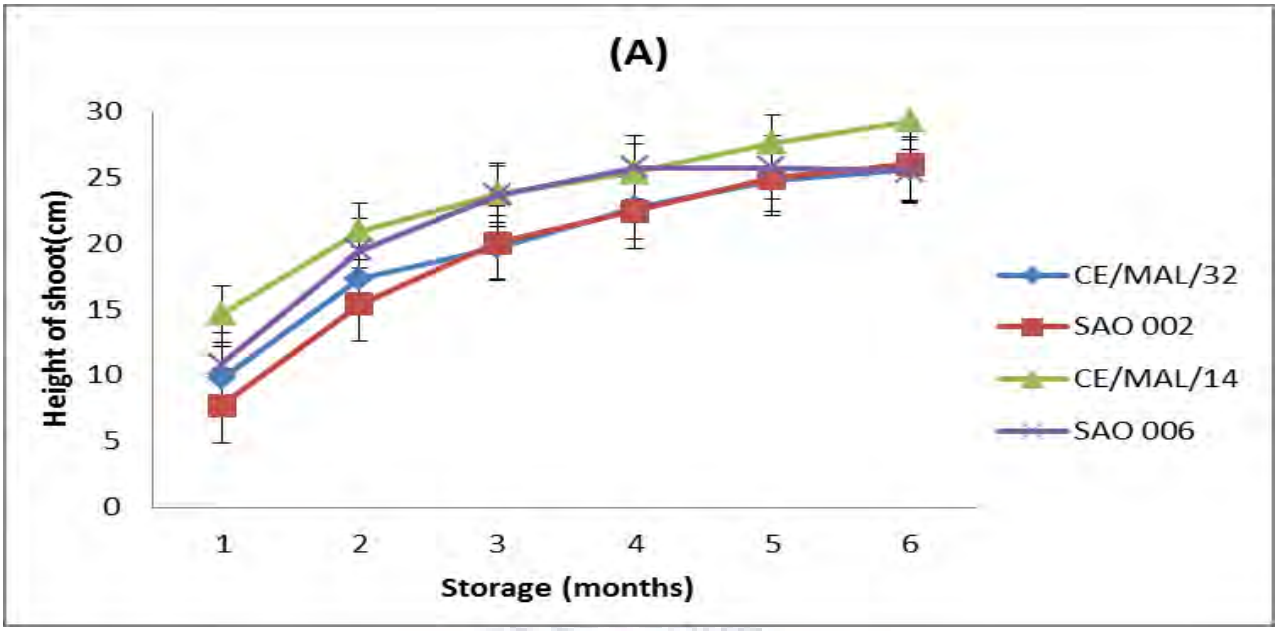


Fig 4.4 Effect of Control (0g/l mannitol) on height of shoot.

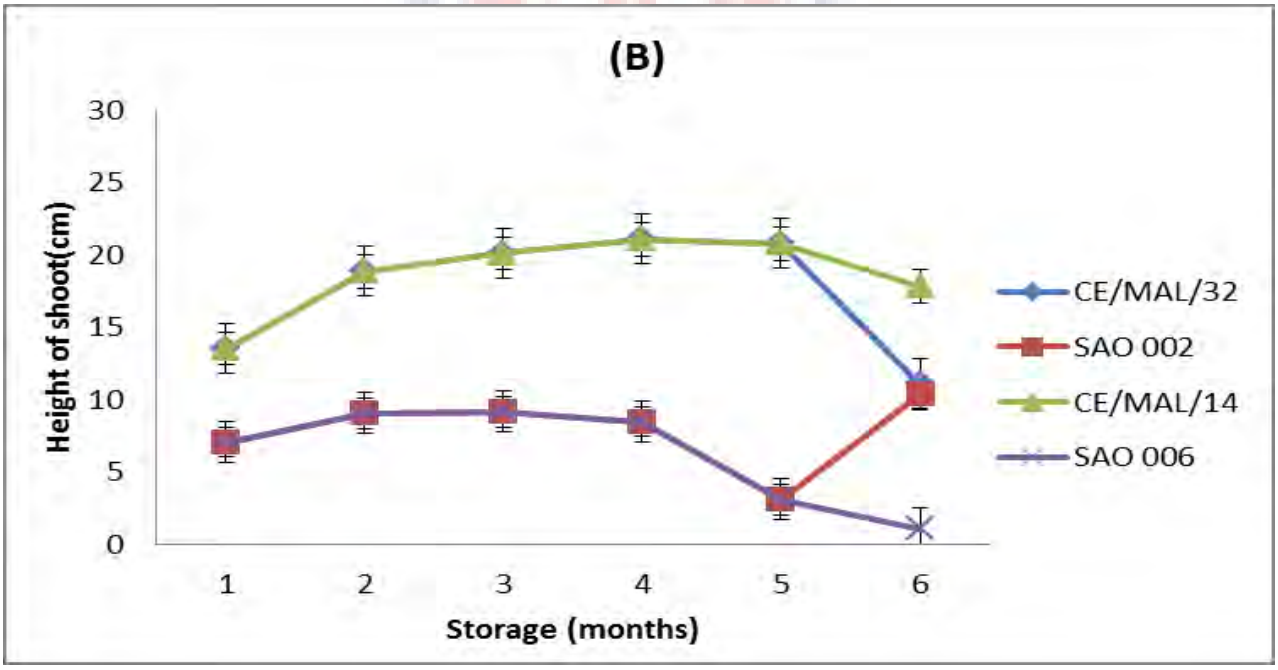


Fig 4.4 Effect of White medium on height of shoot.

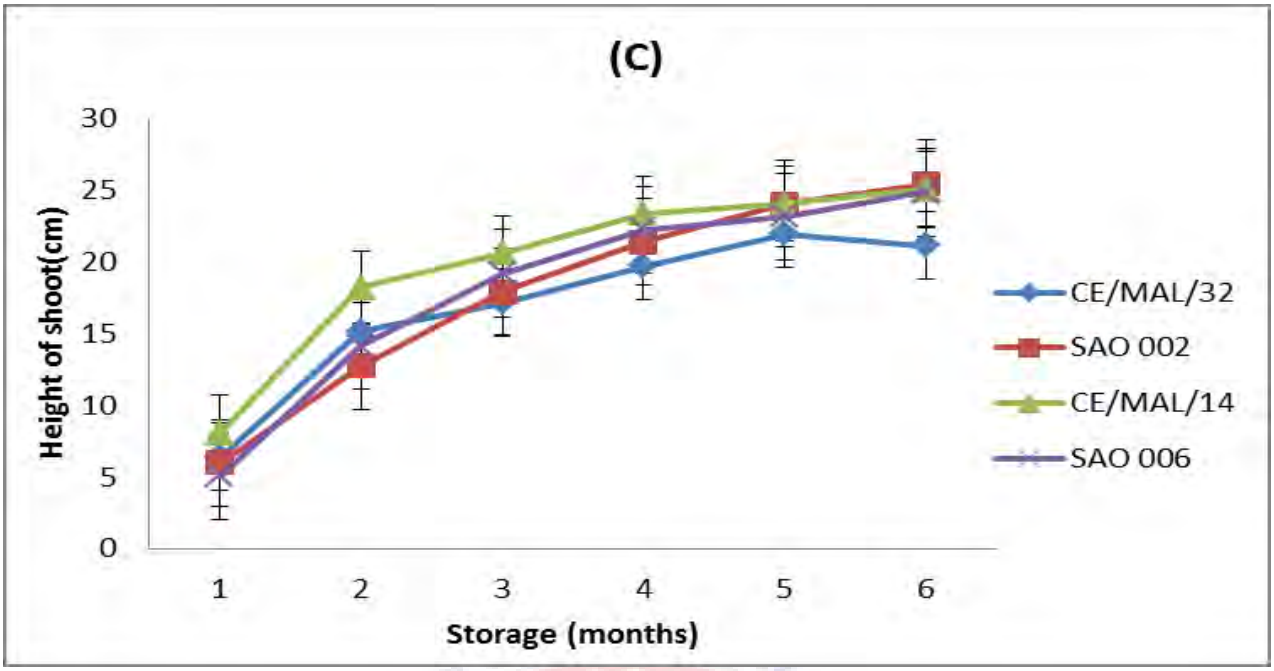


Fig 4.4 Effect of 20g/l mannitol on height of shoot.

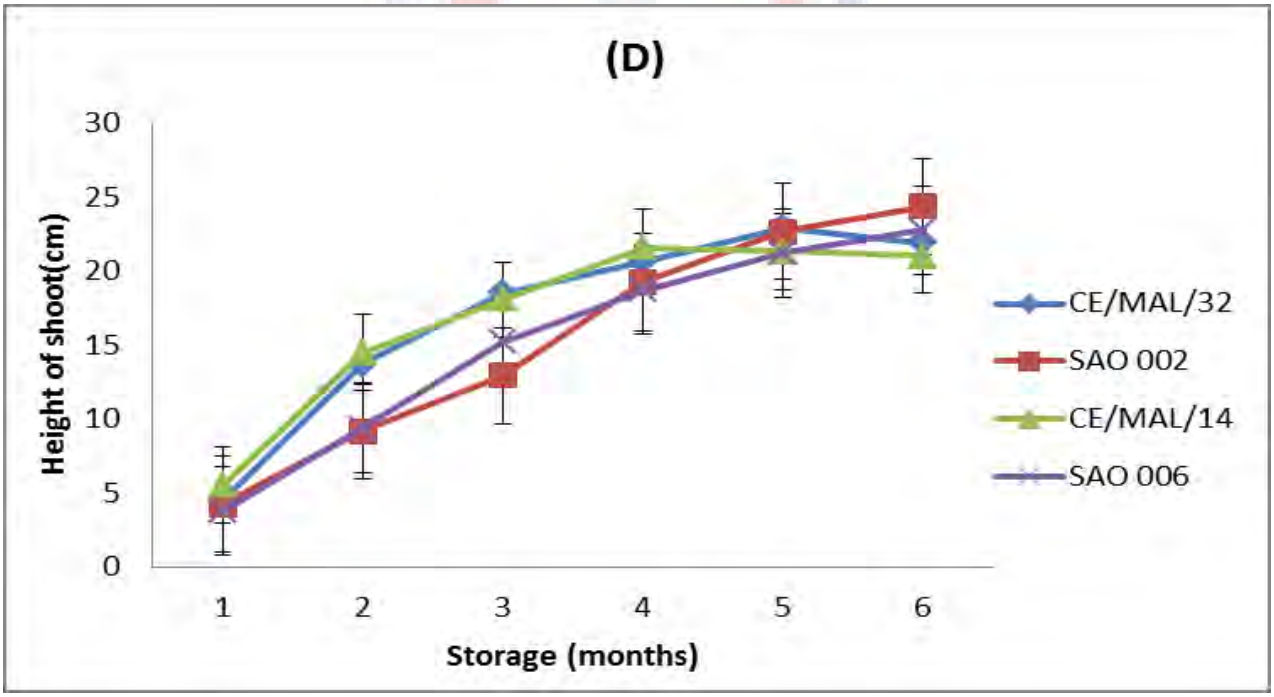


Fig 4.4 Effect of 25g/l mannitol on height of shoot.

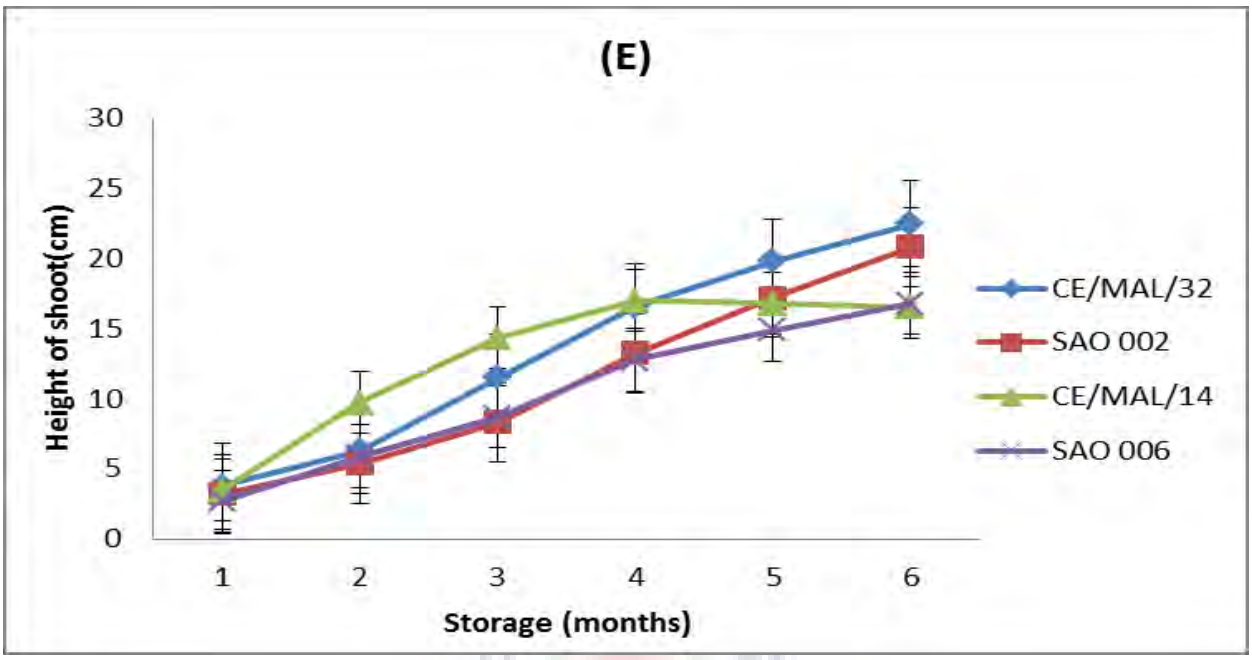
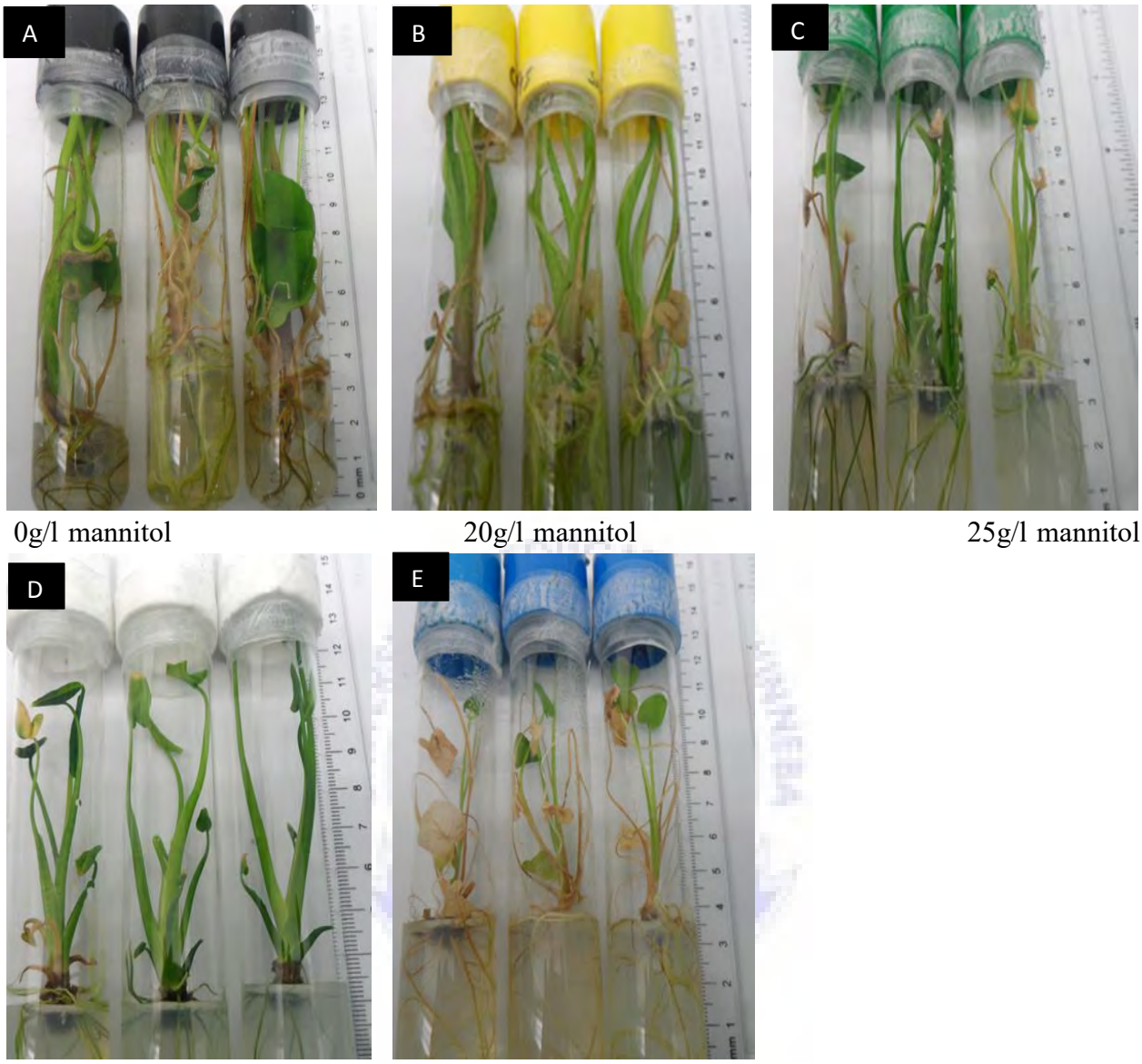


Fig 4.4 Effect of 30g/l mannitol on shoot height.

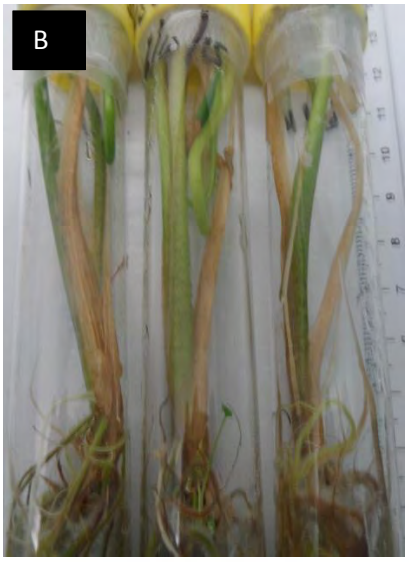


0g/l mannitol 20g/l mannitol 25g/l mannitol

30g/l mannitol White medium

Plate 4.3 Genotype SAO 002 development on complete MS medium supplemented with 0g/l (A), 20g/l (B), 25g/l (C), 30g/l (D) mannitol and on White medium (E).

0g/l mannitol



20g/l mannitol

25g/l mannitol

30g/l mannitol

White medium

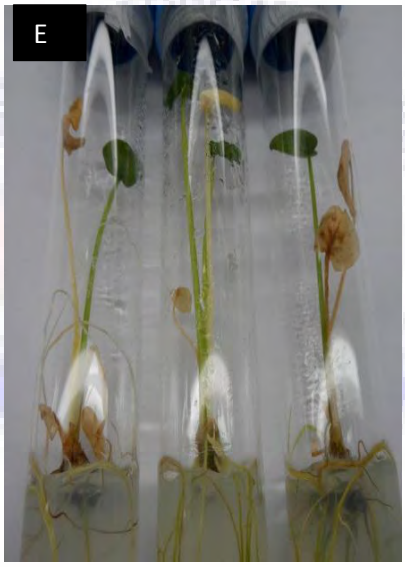
Plate 4.4 Genotype CE/MAL/32 development on complete MS medium supplemented with 0g/l (A), 20g/l (B), 25g/l (C), 30g/l (D) mannitol and on White medium (E).

0g/l mannitol

20g/l mannitol

25g/l mannitol

30g/l mannitol



White medium

Plate 4.5 Genotype CE/MAL/14 development on complete MS medium supplemented with 0g/l (A), 20g/l (B), 25g/l (C), 30g/l (D) mannitol and on White medium (E).

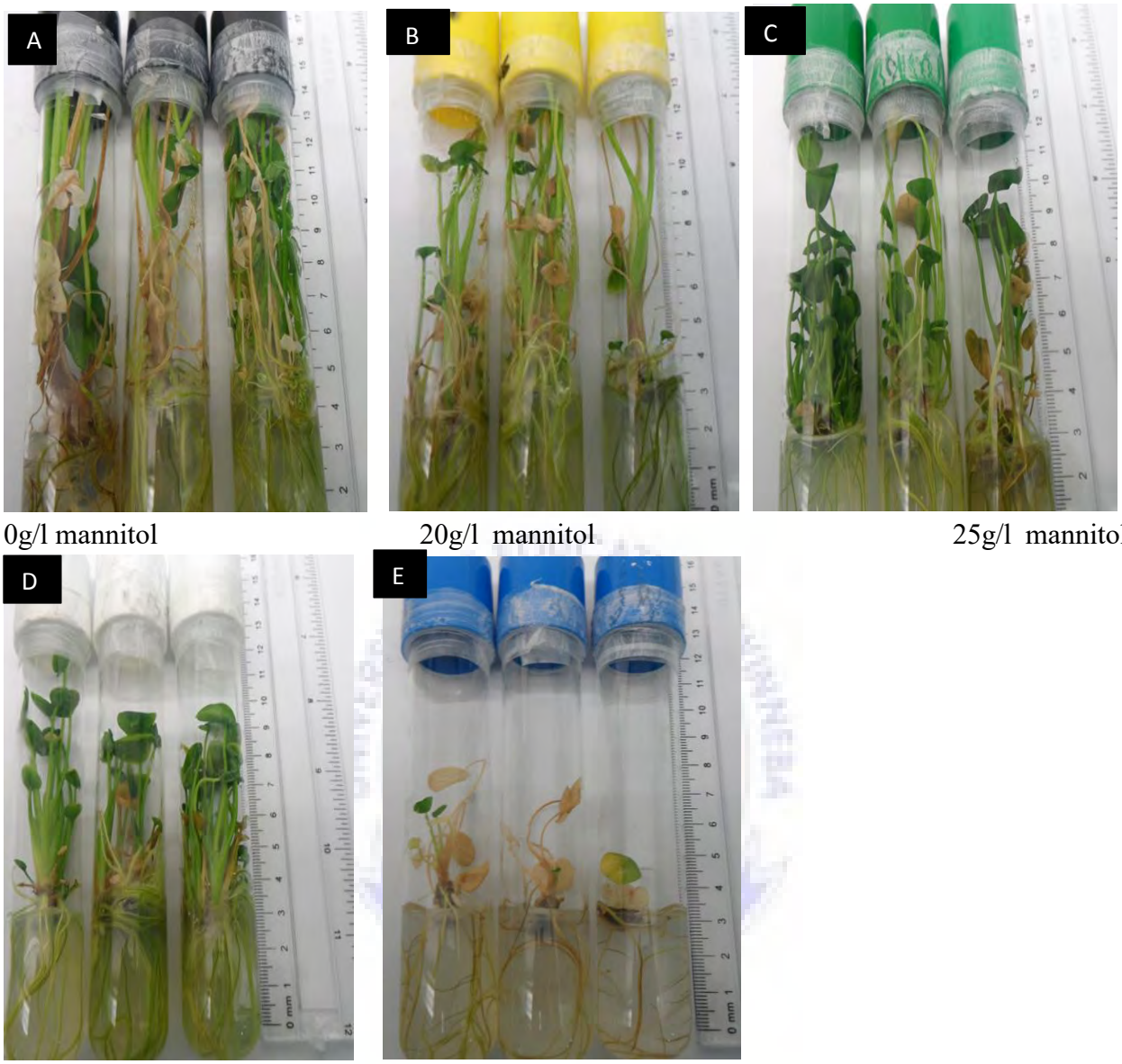


Plate 4.6 Genotype SAO 006 development on complete MS medium supplemented with 0g/l (A), 20g/l (B), 25g/l (C), 30g/l (D) mannitol and on White medium (E).

4.4 Performance of cultures following conservation

Results presented in Fig (4.5-4.7), Plate (4.7) showed that the ability of conserved explants to regenerated shoots after transferring onto recovery medium and incubating at 25± 1^0C, 5000lux light intensity and 16 hours photoperiod, as well as, the average number of proliferated shoots per culture, leaves per culture and their length (cm) increase significantly on all treatment media on reculture period from 1 to 4weeks. The highest regeneration percentage of shoots occurred in medium with 20 and 25g/l mannitol (91.67%), shoot number per explant on medium with 30g/l (1.75shoots/explant), leaves number per explant also on control medium (4.50 leaves/explant) and shoots length (cm) was recorded after on recovery medium for 4 weeks in White medium (6.46cm shoot length). Conservation medium with 30g/l mannitol resulted in the lowest regeneration percentage (66.67%).

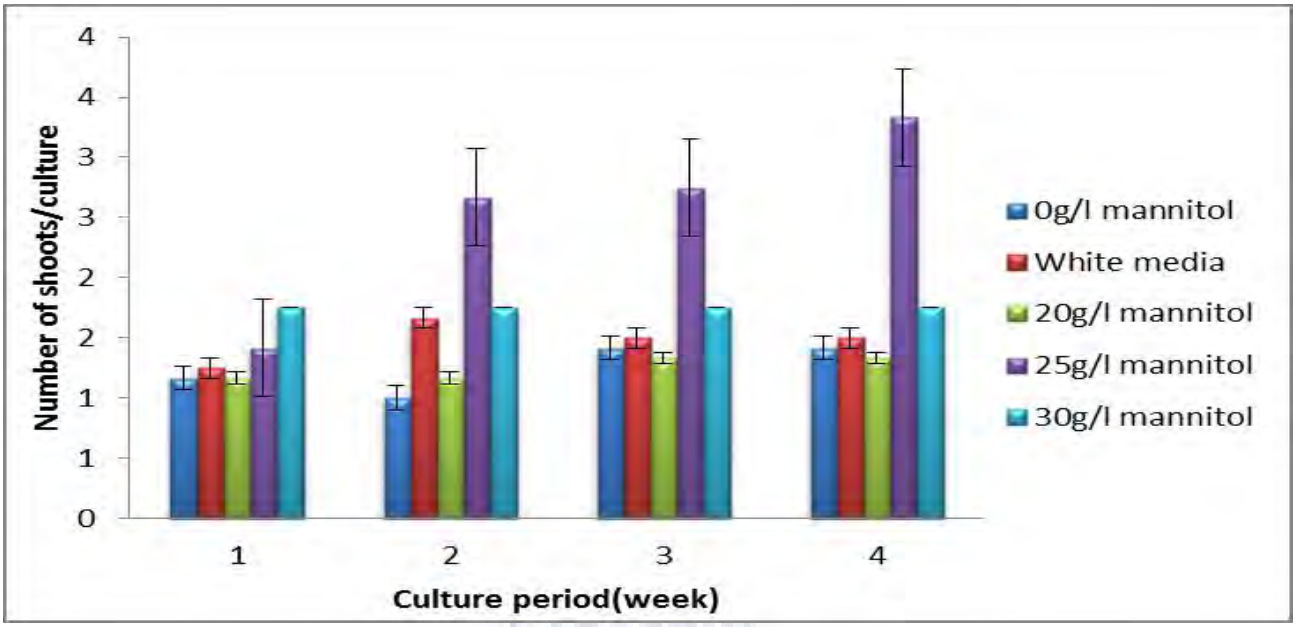


Fig 4.5 Culture growth following 6 months conservation on shoots development

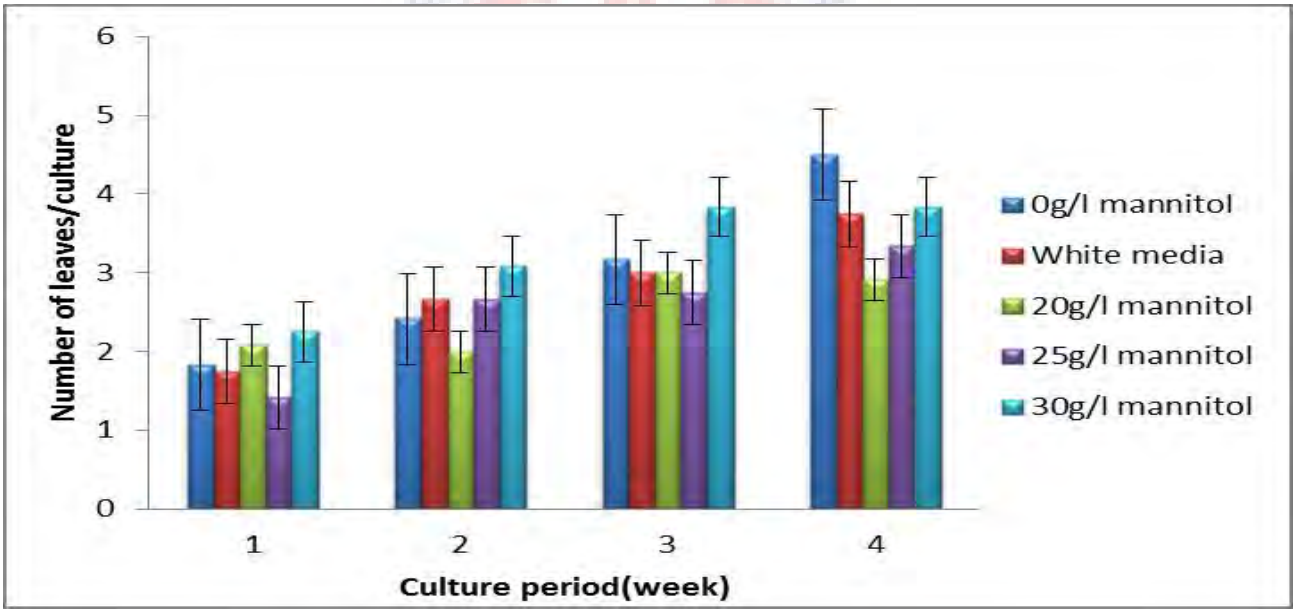


Fig 4.6 Culture growth following 6 months conservation on leaves development.

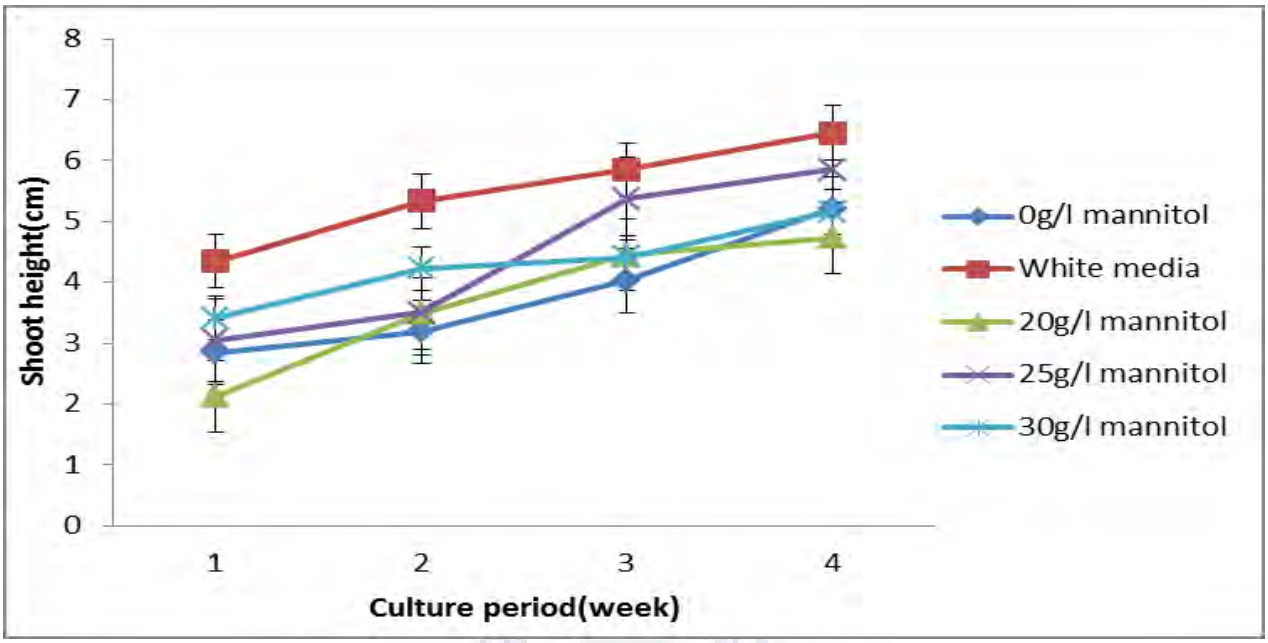


Fig 4.7 Culture growth following 6 months conservation on shoots≤ height.

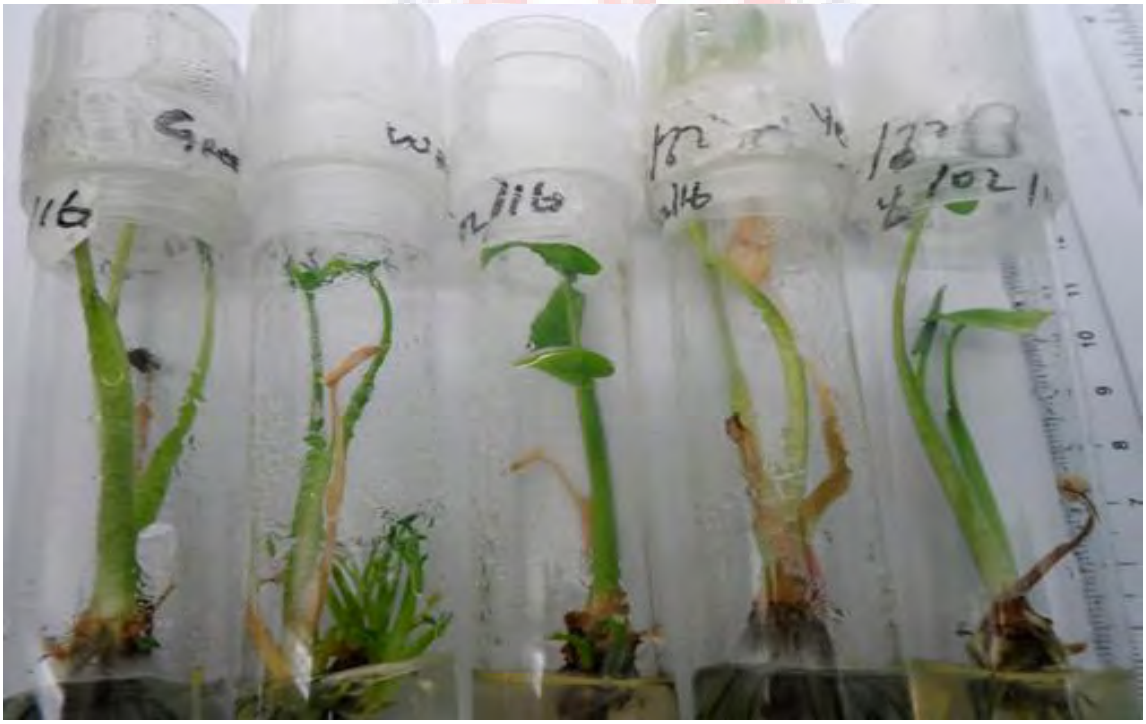


Plate 4.7 Plantlets developing (4week old) following 6 month conservation.

4.5 FTIR analysis

Determination of sugar alcohol

An infrared spectrum of four genotype samples of sugar alcohol is shown in Fig 4.8. The IR spectra obtained from *Colocasia* genotypes spectra showed at 1080 cm^{-1} C-O, C=C stretching with some C-H contributions; 1553, 1632, 1633, 1636, 1638, 1078, 1080, 1080 , 1080 and 1080 cm^{-1} (CO bending and C=O symmetrical stretching) and 2922, 2925, 2927, 2930, and 2938 cm^{-1} (C-H stretch). A band near of 1080.9 cm^{-1} is interpreted as a skeletal mode indicating the linkage of mannitol molecules in *Colocasia* genotypes as compare to the standard absorbance of the raw mannitol analysed with the band range of 1080-1081cm^{-1}. Functional group (Alkane, CH_2) asymmetric stretching, symmetric stretching, and bending were observed at 2922 cm^{-1}, 2925 cm^{-1}, and 2930. Strong bond of (C-O) in mannitol appears at 1080 cm^{-1}. The absorption band 1080 cm^{-1} formerly identified in *Colocasia* genotypes and FTIR spectra confirm the presence of mannitol.

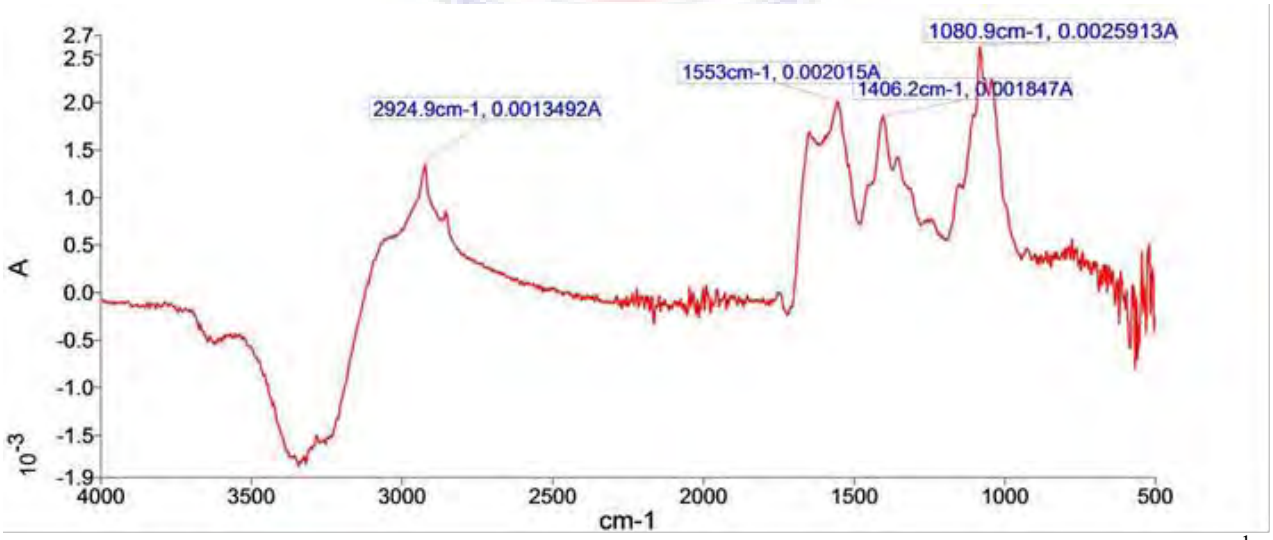


Fig 4.8 FTIR spectrum showing the absorbances of mannitol in colocasia genotype at 1080.9cm^{-1}

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Calibration curve represents the dependence of the absorbance (peak) area on the concentration of mannitol (Fig.4.9-4.12). Each sample was measured on an IR spectrophotometer of 4000-500 cm^{-1} and each spectrum was measured 32 scans accumulation.

Figure 4.8 shows that the FTIR spectra of mannitol are well defined. There were intense fingerprint bands in the wave number range 1000-500 cm^{-1}. The characteristic bands of mannitol have specific maximum at 1078, 1080, 1081 cm^{-1}, and the peak at 1080 cm^{-1} having the highest absorption. The most intense peak of mannitol (1080 cm^{-1}) is characteristic to the C-O stretch vibration.

The results constitute strong positive correlation between mannitol applied and uptake concentration for genotype CE/MAL/32 and CE/MAL/14, fig (4.10-4.11), indicating that, as the amount of mannitol applied increases, the uptake by *colocasia* plantlets also increases. There was also weak correlation between mannitol applied and uptake of concentration for genotype SAO 002 and SAO 006 fig (4.9 and 4.12), indicating that, as the various treatment of mannitol applied increases the uptake by *Colocasia* plantlets (SAO 002 and SAO 006) neither increase nor decrease.

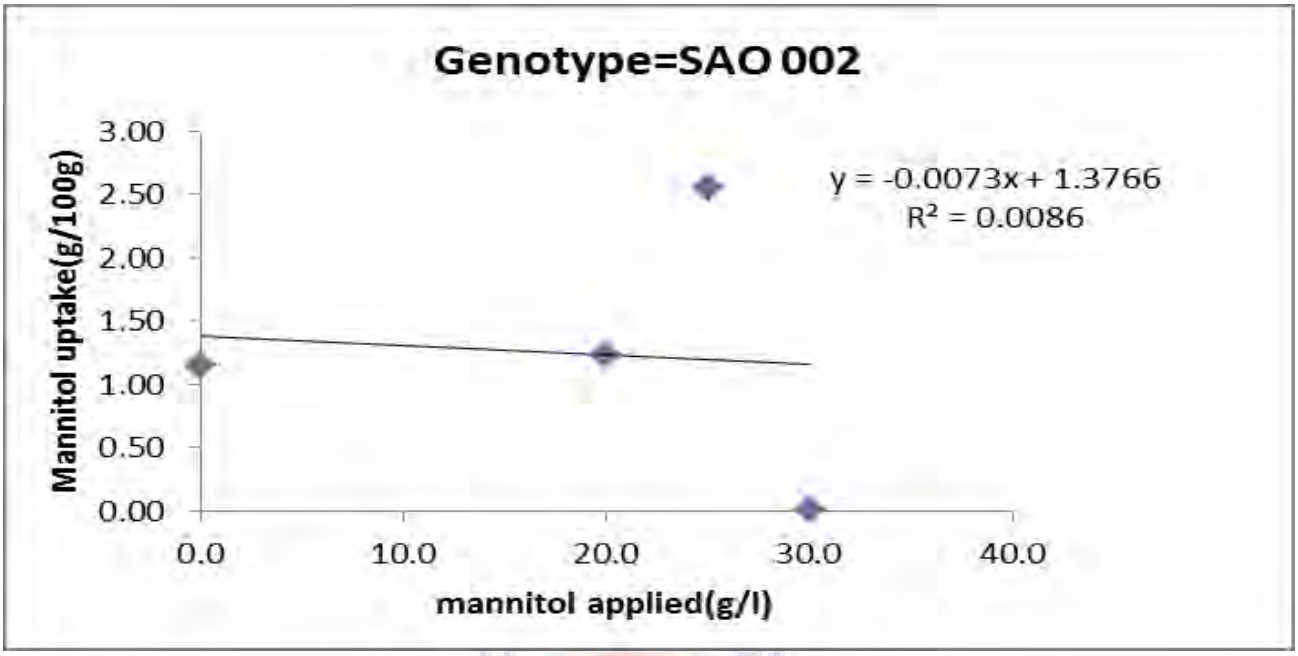

Fig 4.9 Calibration graph by mannitol applied and uptake.

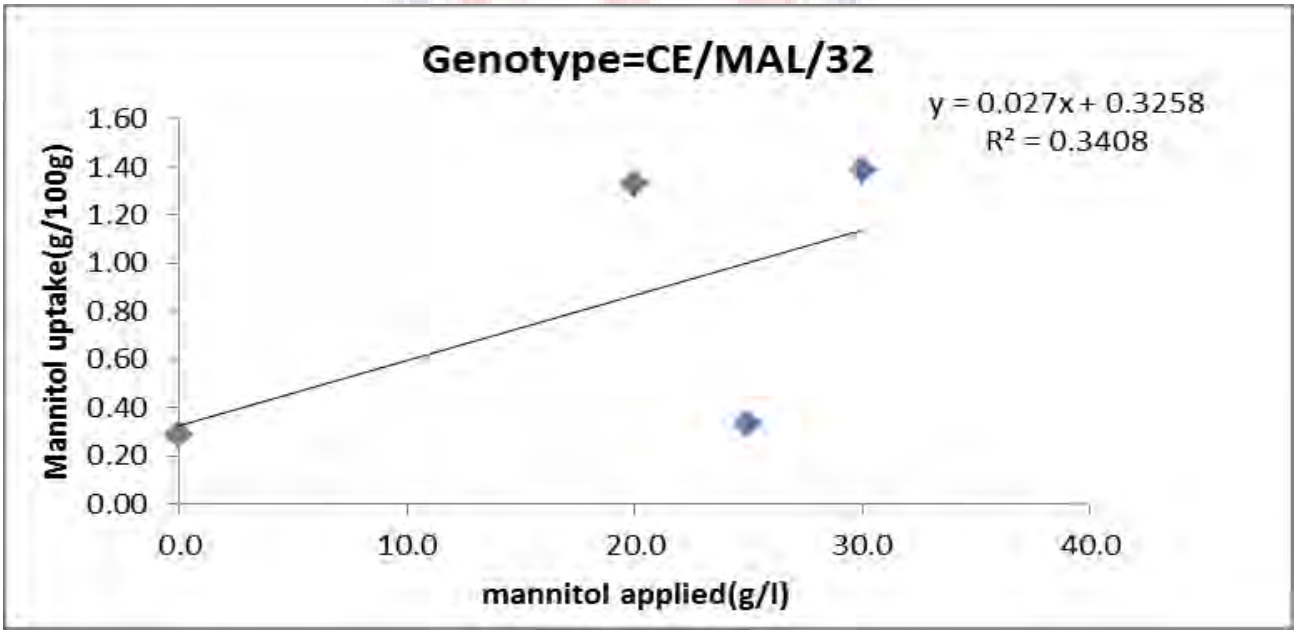

Fig 4.10 Calibration graph by mannitol applied and uptake.

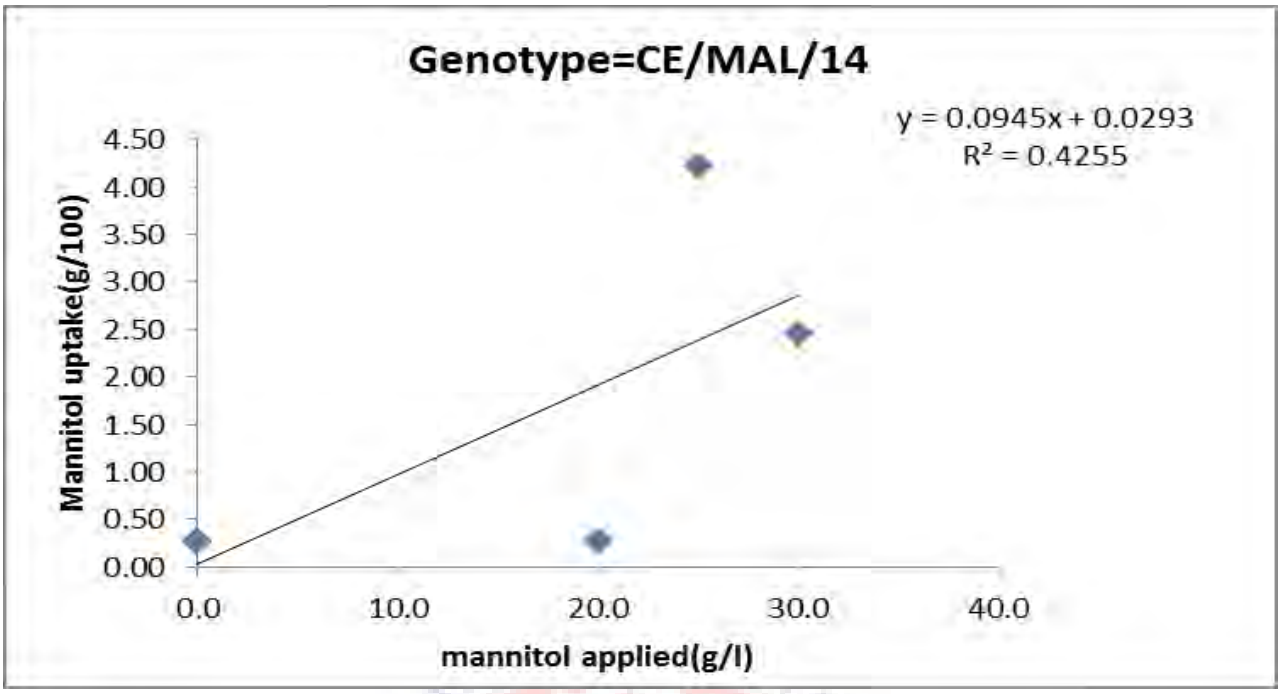

Fig 4.11 Calibration graph by mannitol applied and uptake.

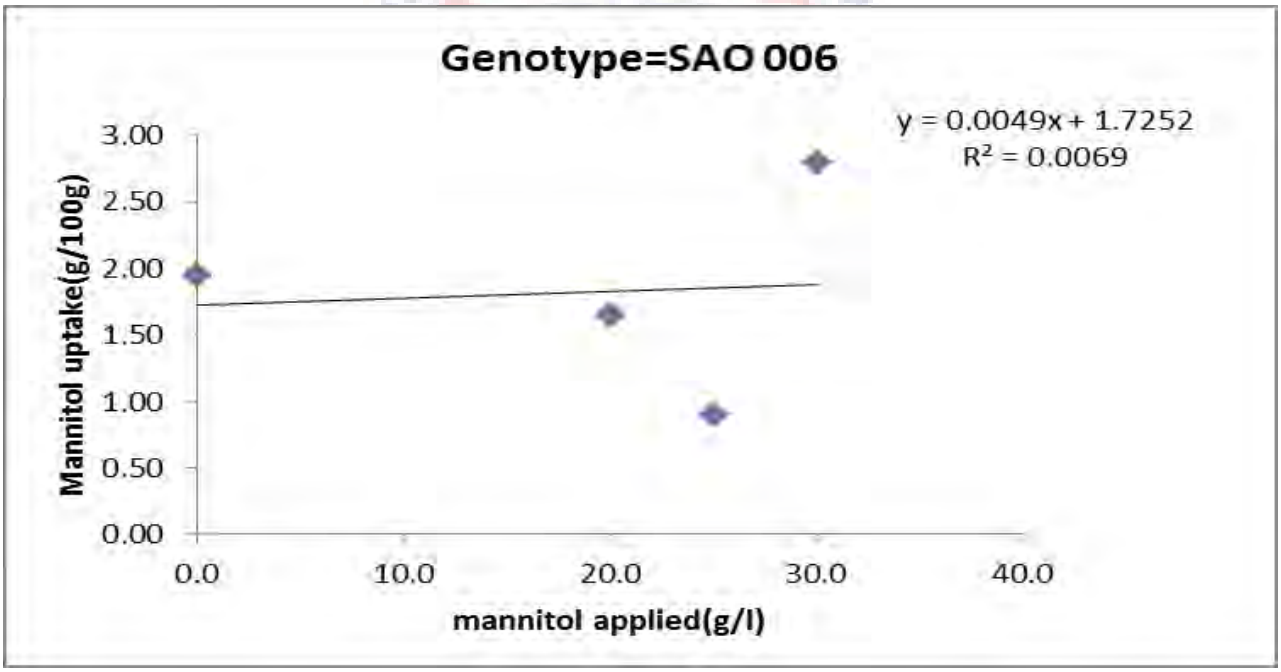

Fig 4.12 Calibration graph by mannitol applied and uptake.

4.6 Effect of mannitol on antioxidant enzymes activity (Biochemical analyses)

Mannitol is a non-reducing sugar normally synthesized in numerous species of vascular plants, which serve as an alternate metabolic reserve as well as an osmo-protectant. Mannitol accumulation increases when plants are exposed to a low water potential and the most obvious function of this compound is in osmotic adjustment.

Specific activity of peroxidase in *Colocasia* genotype showed a transient behavior (Fig 4.13). The peroxidase activity was highest on 25g/l mannitol treatment. At the control (0g/l mannitol) and White medium treatment, activities were 0.431 and 0.408 respectively. The 25g/l mannitol sample showed 0.781 of highest activity than all the treatments with lowest occurring on White medium treatment.

From the results (Fig 4.14), the highest activities of catalase occurred on control (0g/l mannitol), while in White medium treatment had the lowest (0.340) activities of catalase. Media containing 20, 25 and 30g/l mannitol had 0.468, 0.484 and 0.421 catalase activities, respectively.

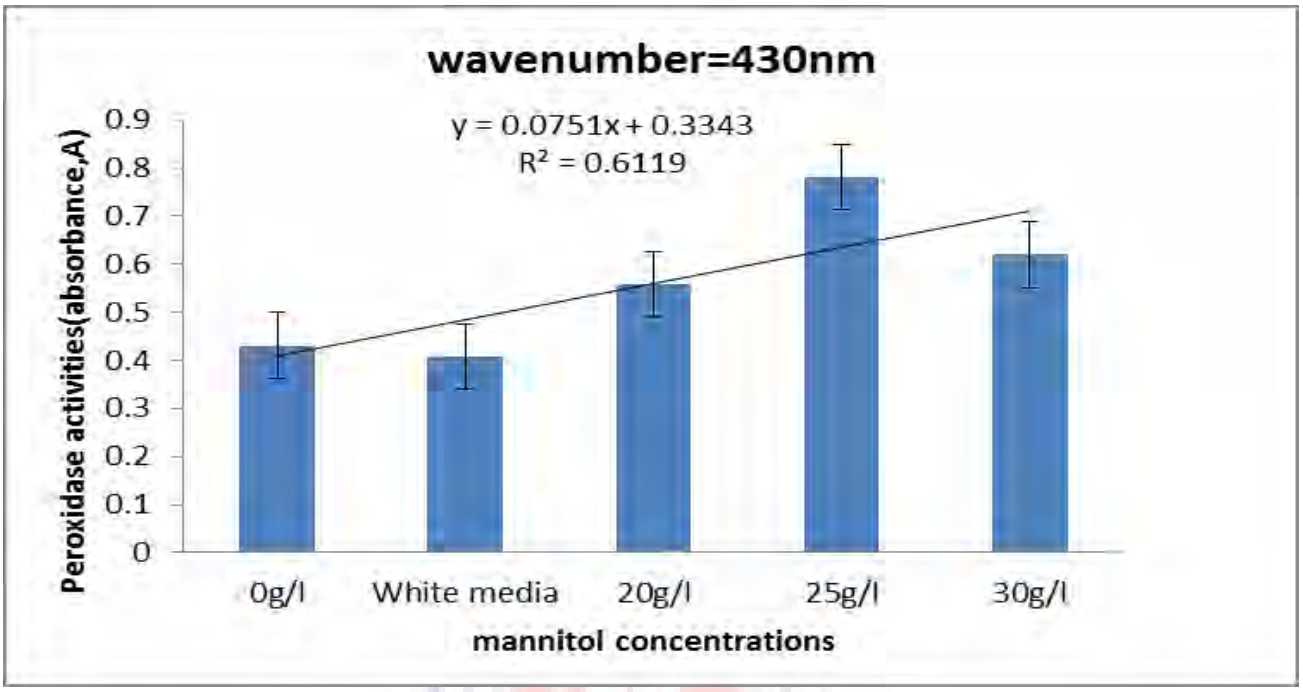

Fig 4.13 Peroxidase activities in *Colocasia* genotype after 6 month medium term conservation.

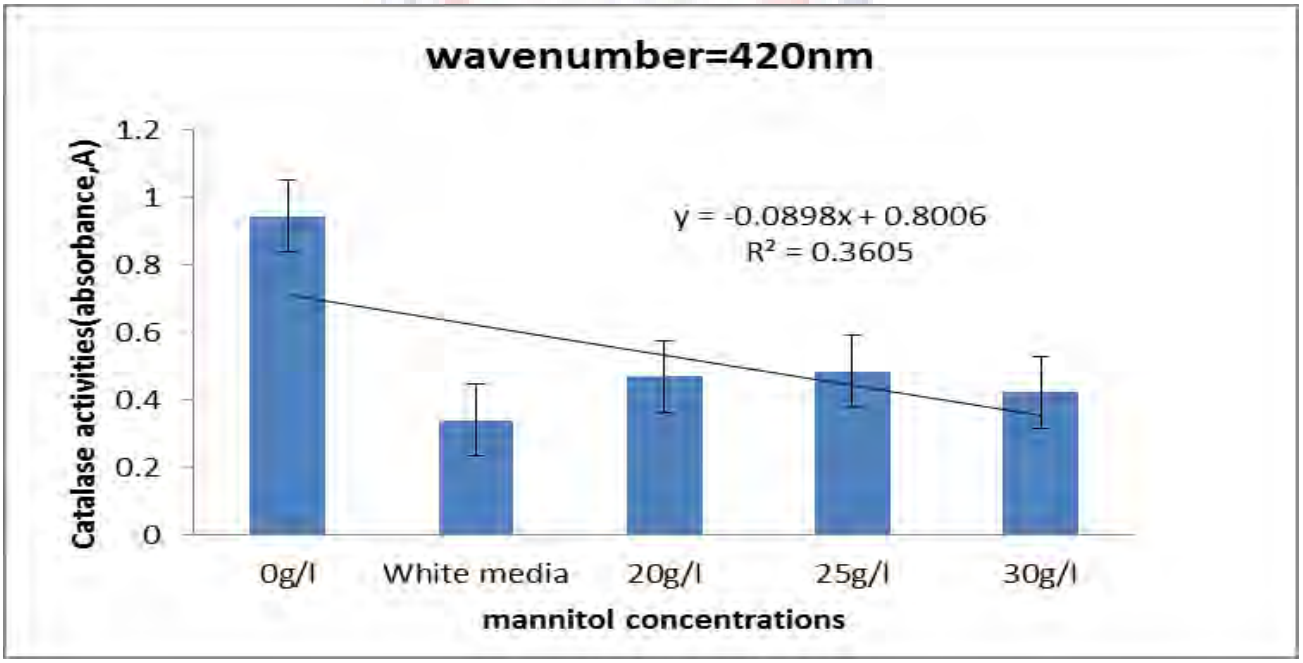

Fig 4.14 Catalase activities in *Colocasia* genotype after 6 month medium term conservation**.**

CHAPTER FIVE

5.0 DISCUSSION

5.1 *In-vitro* rapid multiplication of *Colocasia* genotypes

Transverse section (TS) and longitudinal section (LS) explants excision were used for rapid multiplication experiments. The results showed that shoot proliferation could be induced through transverse and longitudinal section excision on multiplication media with transverse excision section performed better for its effect on shoots and development per explant. Among excision modes, Duan *et al*. (2007) and Rattanpal *et al*. (2011) observed that transverse excision performed the best for its effect on the number and quality of regenerated shoots and its convenience to manipulate in sweet orange and rough lemon, respectively.

The frequency of shoots formation was 100% and the best responded explants producing highest number of shoots and leaves occurred on Transverse excision of shoot culture even though statistically there was no significant difference among treatment means. Some studies have shown that explant characteristics such as mode, type, source, genotype and history affect the success and commercial viability of tissue culture systems (Bhau and Waklu, 2001; Chan and Chang, 2002). The effect of explant mode of excision and type on successful tissue culture of various plants has been reported (Gubis *et al*., 2003; Blinstrubiene *et al*., 2004; Tsay *et al*., 2006; Gitonga *et al*., 2010).

Uniform sized plantlets with different mode of shoot excision could be used for rapid *In-vitro* multiplication experiments. However, the protocol developed in this study was efficient in the production of more multiple shoots and could be extendable to different species, hence can be used for large-scale multiplication of disease free plants in all these species. Rao *et al*. (2002)

reported the occurrence and micro-propagation of *Vanilla wightiana* Lindl., an endangered leafy species, endemic to Andaman Islands.

The results of higher shoot number in transverse section (TS) than longitudinal section (LS) explants excision may be due to the suppression of apical dominance in *Colocasia* genotype during subculture that induced basal dormant meristematic cells to form new shoots (Shukla *et al*., 2009). Hence, by adopting this procedure of shoot excision, a large number of shoots could be obtained per explants within few months (Asthana *et al*., 2011).

5.2 Medium term conservation of *Colocasia* genotypes

One advantage of slow growth conservation is that it does not require frequent subculture. Another advantage of slow growth conservation is after prolonged period of conservation; the culture can easily be retrieved for production of new plants with greater genetic integrity (Ahmad *et al*., 2011). Slow growth is generally achieved by adding osmotic agents in the form of varying concentrations of sucrose, sorbitol and mannitol (Gonçalves and Romano, 2007; Lata *et al*., 2010; Scherwinski-Pereira *et al*., 2010). Due to the addition of osmotic agents in culture media, there is a significant increase in the storage period of *In-vitro* tissues (Sharaf *et al*., 2012). To prolong the period of slow growth conservation, a set of experiments were planned by adjusting content of mineral elements by adding osmotic agents in culture media.

The effect of different mannitol concentrations, results in Fig (4.4d-4.4e), Plate (4.3-4.6D) showed that medium supplemented with 30g/l mannitol resulted in the lowest mean value of shoot height followed by 25 g/l mannitol. In this respect, Ahmad and Anjum, (2010) found that low concentrations of MS medium and the lower concentration of mannitol (2.5%) successfully increased subculture duration of pear genotypes. Almost all SAO 006 genotype died on White

medium at the end of six month period of conservation due to decline in growth, results in Fig (4.4b), Plate (4.6E).

The objective was to improve plantlet survival rate and prolong the subculture period. Addition of mannitol to the culture media could reduce the growth of taro plantlets in term of stem height. Reduced plant height is critical in culture conservation as plantlet do not get overgrown.

It has already been widely reported that sucrose in high concentration exhibited detrimental effects on general condition of plant due to osmotic stress (Withers, 1991). Using mannitol as an osmotic agent, the storage time of *Colocasia* could be significantly extended or the number of shoots be increased at the same storage period. Higher concentration mannitol (i.e., above 50g/l) also increased deterioration of shoots and complete necrosis as a results of high desiccation in the cultured plantlets. Similar results were also reported on plantlets of *Artemisia herba-alba* (Sharaf *et al*., 2012) when conserved via slow growth culture. Some other reports also supported that osmotic agent reduced growth of *In-vitro* grown cultures (Bajaj, 1995; Montalvo-Peniche, 2007; Du *et al*., 2012). Similar results considering the effect of mannitol on growth limitation, were reported in potato (Sarkar & Naik, 1998), in Ensete ventricosum (Negash *et al*., 2001), in the rare taxon *Veronica multifida* ssp. *Capselli carpa* (Holobiuc *et al*., 2006; Holobiuc *et al*., 2008).

Minerals dissolved in water are introduced into cells through differences in osmotic pressures of the cells. The inclusion of sugar alcohol in medium increases the osmotic potential, thus reducing the uptake of minerals from the medium by cells. As a consequence, plant growth is delayed (Golmirzaie and Toledo, 1999). High concentration of mannitol added to the media produced osmotic effect which resulted in the reduction of growth of taro plantlets. Mannitol can be

absorbed into plant cell (Thompson *et al.*, 1986). Too high concentration of mannitol added to the media might be harmful and caused death of the taro plantlets. Sarkar (1998) reported that 20 or 40 g/l mannitol in combination with sucrose could enhance survival of *In-vitro* conservation of potato but not with 60 g/l mannitol.

In this current study, mannitol inclusive in culture media induced a moderate osmotic stress which can activate the adaptation mechanisms that confer tolerance to more severe dehydration. Although, in some cases accumulation of high mannitol concentrations may have unfavorable effect on plant development (Abebe *et al*., 2003), in this current experiment, growth retardations, undesirable effects were not observed.

Alternatively, mannitol acts as a trigger in plant morphogenesis. Similarly, other studies reported that *In-vitro* application of moderate stress factors levels showed an inductive or stimulant role on plant regeneration (Xu *et al*., 1990; Kong & Yeung, 1995) especially in gymnosperms. Mannitol can be used as carbon source and was described as hydroxyl radical's scavenger, protecting some enzymes from Calvin Cycle against oxidative inactivation (Shen *et al*., 1997a). In transgenic tobacco, increased mannitol biosynthesis and catalase activity in chloroplasts determined an increase radical scavenging capacity and resistance to oxidative stress (Shen *et al.*, 1997b). The higher concentration of mannitol added to the medium, the higher reduction of shoot height as observed in fig4.4e, Plate (4.3d-4.6d) of taro genotypes as 30g/l mannitol was best to reduce shoot height followed by 25g/l mannitol treatment. Overgrowth of shoots occurred in medium containing 0g/l, 20g/l mannitol and White medium which could not sustain adequate growth, so plant subsequently died. Espinoza *et al*. (2002), stated that addition of mannitol reduced the growth of *Ipomoea batatas* plants.

5.3 *In-vitro* Recovery following conservation

With regard to the effect of different mannitol concentrations and White medium, results clearly revealed that conservation medium with 20g/l and 25g/l mannitol resulted in the highest regeneration percentage (91.67%) after culturing on recovery medium and incubation under normal conditions for 4 weeks. Also, medium containing 30g/l mannitol surpassed other treatment media in the number of new shoots (1.75 shoots/ explant). White medium showed the highest shoot length (6.46 cm), while conservation medium with 30g/l mannitol resulted in the lowest regeneration percentage (66.67%). These results are in agreement with those of Golmirzaie and Toledo (1997) who demonstrated that the use of sorbitol as an osmotic agent can be metabolized by the plantlets after few months of storage and exhibition an incremental growth rate. In the same line, Bekheet *et al.* (2001) showed that healthy shoot cultures of date palm were obtained after 6 months of storage in medium containing $40gl^{-1}$ sorbitol.

5.4 FTIR determination of mannitol uptake in *Colocasia* genotypes

Preservation of membrane integrity and cytosolic component during dehydration is of utmost importance in the survival of desiccation-tolerant tissues. Sugars, particularly, are thought to play a role in this preservation (Crowe *et al*., 1992). Studies on viviparous mutants have shown that an osmotic agent is important for suppressing precocious germination and for the acquisition of desiccation tolerance of seeds (Koornneef *et al*., 1989).

The use of mannitol was preferable for inducing growth as a gradient of water stress on plantlets grown in solid MS media, although concerns have been raised about its potential uptake by plantlet (Trip *et al*., 1964).

However, this uptake was observed in cases where mannitol was the only supply source of carbohydrates; the presence of sucrose in the medium is known to be used preferentially over mannitol (Brown *et al*., 1982) which makes it unlikely that mannitol directly affects growth of plantlets.

Mannitol is a product of mannose after reduction. Under the present study, the results showed strong positive correlation between mannitol applied and uptake concentration for genotype CE/MAL/32 and CE/MAL/14 (Fig 4.11-4.12), indicating that, as the amount of mannitol increases, and uptake by *Colocasia* plantlets also increases. There was also weak correlation between mannitol applied and uptake of concentration for SAO 002 and SAO 006 genotype (fig 4.10 and 4.13), indicating that, as the various mannitol treatment increases the uptake by *Colocasia* plantlets (SAO 002 and SAO 006) neither increase nor decrease. This shows that absorption of carbohydrates (i.e., mannitol) and other minerals have genetic influence.

5.5 Enzymes determination

The mannitol added to medium cultures causes a mild osmotic stress to cell cultures and, with time, this treatment may activate an adaptive mechanism that can render the cells tolerant to more severe treatments. Subjecting plants to medium supplemented with mannitol, the cells take up mannitol that can act as an osmoprotectant. For example, exposure of tobacco LT cells to mild osmotic stress appears to be sufficient to induce tolerance to vitrification, but it is conceivable that uptake of mannitol by the tobacco cells improves survival rates (Reinhoud, 1996).

However, accumulation of mannitol may have adverse effects, even though mannitol is a compatible solute, the target plant may not tolerate high levels of mannitol. The appropriateness

of the term compatible solute for osmolytes is questionable because marginal accumulation can induce pleiotropic effects (Hare *et al*., 1998). For instance, plants that accumulated higher levels of mannitol had severe abnormalities including sterility, stunted growth, twisted heads and curled leaves (Abebe *et al*., 2003). Exogenous application of Gly to non-accumulating plants was found to have osmotic-induced accumulation of Pro (Gibon *et al*., 1997) and stress-induced accumulation of Pro also results in reduced growth (Hare and Cress, 1998).

Therefore, mannitol has been proposed to enhance tolerance to water deficit stress primarily through osmotic adjustment (Loester *et al*., 1992). The amount of mannitol accumulated and its effect on osmotic adjustment was less than that of other carbohydrates, suggesting that the beneficial effect of mannitol resulted from other protective mechanisms than osmotic adjustment (Abebe *et al*., 2003).

Besides its function in osmotic adjustment, mannitol improves tolerance to stress through scavenging of hydroxyl radicals (OH^+) and stabilization of macromolecular structures (Crowe *et a*l., 1992; Shen *et al*., 1997). The importance of mannitol as a scavenger of the hydroxyl radical has been demonstrated *In-vitro* (Smirnoff and Cumbes, 1989) and *in-vivo* using transgenic tobacco (Shen *et al*., 1997). The mechanism, by which mannitol decreases damages by hydroxyl radicals, is still unknown.

In order to find a correlation between productions of ROS, the effect induced by the exogenous application of mannitol to antioxidant enzymes expression in medium term cultures was investigated. Oxidative stress is common in plants during water stress. Reactive oxygen species (ROS) including superoxide, peroxide and hydroxyl radicals, in general react aggressively with

biological molecules and can cause lipid peroxidation, breakdown of macromolecules and damage to nucleic acids (Smirnoff, 1998).

However, production of ROS is an unavoidable process in illuminated chloroplasts. Most peroxides are then produced through disproportionation of superoxide by superoxide dismutases (SOD). Additionally, hydroxyl radicals are produced in Haber-Weiss or Fenton reactions through the interaction of H_2O_2 and superoxide or directly from H_2O_2 in the presence of transitions metals, such as Fe $^{+'}$ and Cu^{+}~ (Halliwell and Gutteridge, 1990). The catalases (CAT) can consume H_2O_2 preventing the amino acid oxidation or eliminated H_2O_2 before it can be converted to hydroxyl radical. In contrast to the detoxification system for H_2O_2 and superoxide, an enzyme system that could scavenge the short-lived hydroxyl radicals has not been identified.

Results obtained showed changing of the activity expression of Catalase and Peroxidase and significant differences in response between mannitol treatments. There was significant difference in the enzymes expression in the case of mannitol treatments and White medium treatment *In-vitro* cultures. The expression of catalase and peroxidase was modified when the mannitol was added in medium term culture and at the same time with the increase of mannitol concentration.

The result constitutes a high positive correlation between all experimental treatments in peroxidase activities, which means as the concentration of mannitol increases in the plant, the peroxidase function against the reactive oxygen species increases, hence their activities in the plant increase. However, the activities of catalase was low positively correlated, indicating that as catalase activities increases with increase in mannitol rate in the plant, catalase function against the reactive oxygen species increases hence their activities in the plant increases at a low rate.

The increase of peroxidase and catalase activities depended on the concentration of mannitol, which induce osmotic potential in culture and also characteristic species. It is also possible that accumulation of mannitol at intercellular level, above a certain limit, to affect reactive oxygen species scavenger system, changes in the ROS conversion having as result of the increase in antioxidant enzyme activity (peroxidase and catalase). This idea was supported by the results obtained after 24 weeks of culture, when the two antioxidant activities varied.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

Multiple shoot and leaves formation was higher on culture media with Transverse (TS) explant excision as compared to culture media with Longitudinal (LS) excision of explant. This showed that Transverse (TS) section of explants has more axillary buds and accessory buds occurring at the base than Longitudinal (LS) section. Since there are more buds at the base, indicates the rapid multiplication rate that can be achieved.

The medium-term conservation for a *Colocasia* genotypes *In-vitro* establishment can be efficiently made using a growth retardation protocol, based on mannitol as a limiting factor. The study reveals that mannitol supplemented culture media could reduce the growth of *Colocasia* plantlets especially in stem height. Mannitol added medium enhanced shoot and leave multiplication, however, with White medium tended to reduce number of shoots and leaves per plantlet, leading to ultimate dead of cultures. Mannitol 30 g/l added to the culture medium could reduce the growth of plantlets and prolong subculture time to 6 months without any sign of culture deterioration or dead plantlet.

In general, results showed the ability of explant conserved generated from shoots growing after transferring and culturing on multiplication medium and incubating under regular usage conditions (i.e.,25±1^{0}C, 16hrs light, 5000lux) as well as, the average number of proliferated shoots per explant and their length (cm). The regeneration percentage decreased significantly with increasing mannitol concentrations.

In this study the FTIR spectroscopy as a non-destructive tool and useful method to determine mannitol after hydrolyses of *Colocasia* extract was applied. The time of FTIR analysis is considerably reduced compared to the classical methods. These results are promising and have a real analytical potential to supervise routine procedure for determining sugars in natural carbohydrate polymers.

The osmolite mannitol, at all concentrations tested, did not affect the viability of the whole explants. After a prolonged exposure, the accumulation of mannitol at intracellular level above certain limits, probably influenced ROS scavenger system, decreasing the antioxidant enzymes activity, owing to its protective role against ROS *In-vitro* generated.

The method of growth retardation tested for the *Colocasia* genotype is compatible with the survival of plant material in the condition of medium-term cultures, ensuring, besides the reduction of the growth of *In-vitro* plant material, the induction of the regeneration of small propagules in the absence of plant hormones. These advantages allow the maintenance of the *In-vitro* collection in a reduced space, having many regenerants in every replicate, ready to use for different purposes (conservative, for exchanges of plant material or for basic and applied research)

6.2 Recommendations

Any slow growth storage system will not work to the same extent for all varieties. There has to be allowances for this and this will impact on resources with the need to subculture different genotypes at different times.

Genetic stability is an important consideration of any *In-vitro* conservation strategy. Monitoring techniques for stability will depend on the history of the crop species regarding its trait.

Sophisticated, high-technology monitoring has value, when there is sufficient base to assume instability, either intrinsic, or due to the culture system and subculture frequency. In other instances, visual observation of morphological changes may be sufficient in comparison with appropriate controls. If variants are observed in the *In-vitro* system, the reason for the variation has to be determined and in this case the best option is to refer to the original field collection.

More studies are needed on genetic stability after relatively long periods of storage, to establish the safety of slow growth storage, when compared with other methods.

There is a need to include molecular genetic markers for initial identification as well as monitoring of the genetic stability of stored genotypes.

Cost analyses of *In-vitro* conservation are needed to allow comparison with other conservation methods, particularly the comparison between *In-vitro* storage and field genebanks.

Lastly, Broad spectrum activity of this osmotic agent should be determined *In-vitro* for both medium and long term conservation.

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