IN VITRO PROPAGATION OF ENSET (*ENSETE VENTRICOSUM* (WELW.) CHEESMAN)

By

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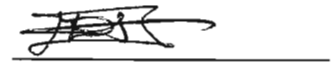
"Who has known the mind of the Lord or been able to give Him advice?"
Isaiah 40: 13

PREFACE

The experimental work described in this thesis was conducted in the Research Centre for Plant Growth and Development, School of Botany and Zoology, University of Natal, Pietermaritzburg, from January 2001 to August 2003 under the supervision of Professor J van Staden.

The results have not been submitted in any other form to another university. Except when the work of others is acknowledged in the text, the results are of my own investigation.



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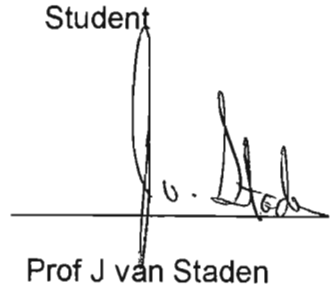

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ENSETE VENTRICOSUM **PLANTS AT AREKA AGRICULTURAL RESEARCH CENTRE, ETHIOPIA: AT DIFFERENT STAGES OF GROWTH (LEFT) AND A MATURE PLANT WITH ITS INFLORESCENCE (RIGHT)**

ABSTRACT

Enset (*Ensete ventricosum*) is an important food crop that is cultivated in Ethiopia. *In vitro* propagation: zygotic embryo culture, shoot tip culture, callus culture and somatic embryogenesis were investigated for this crop. Forty four percent germination of excised embryos of stored seeds of enset genotype Oniya was obtained when the embryos were placed horizontally on the medium that was supplemented with 0.5 mg l^{-1} BA and 0.2 mg l^{-1} IAA, after germination of intact seeds could not be achieved. Over 85% embryos, excised from seeds of two wild enset genotypes shortly after seed harvest, were germinated on MS medium with and without plant growth regulators (PGRs). Addition of 5 g l^{-1} activated charcoal (AC) prevented blackening of germinating zygotic embryos and improved *in vitro* growth of the seedlings.

Contamination of culture was reduced to a tolerable level (below 7%) when eight to ten mm long shoot tips from greenhouse-grown suckers were decontaminated for 15 min in 3.5% sodium hypochlorite and rinsed three times with sterile distilled water. However, this contamination method was not sufficient to decontaminate shoot tips from field-grown suckers. Avoiding injury to the apical domes of the shoot tips at the initiation stage, addition of 7 g l^{-1} AC to the medium and initiation of the shoot tips for two months before splitting for multiplication considerably decreased blackening and formation of callus for genotype Keberia and Mazia.

Three to five normal shoots per shoot tip were produced when halved shoot tips from *in vitro* germinated seedlings of enset genotype Oniya was cultured on gelled and in liquid medium and when halved shoot tips of greenhouse-grown genotype Mazia were cultured in a liquid medium. One to two shoots/buds per shoot tip were regenerated from halved shoot tips of greenhouse-grown suckers on gelled medium for genotypes Keberia, Oniya and Mazia. The presence of BA did not result in a significant increase in the number of shoots per shoot tip both with intact and halved shoot tips. Therefore, wounding the apical dome by splitting appears necessary to release lateral buds. Both blackening of explants in the presence of AC and contamination of culture *in vitro* were not observed with *in vitro* grown plant material.

Callus was produced on MS medium supplemented with 0.5 mg l^{-1} BA + 0.2 mg l^{-1} IAA from zygotic embryos of stored seeds of enset. Adventitious shoots from the callus were regenerated in the light on MS medium lacking PGRs. Embryogenic callus was obtained from shoot tips of genotype Mazia on MS medium with 0.5 mg l^{-1} BA + 0.2 mg l^{-1} IAA + 0.2 mg l^{-1} 2,4-D. A large number of somatic embryos were produced from the embryogenic callus. The results of these studies can be used in enset clonal multiplication, conservation of germplasm and breeding of the crop.

ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acids
AA	Ascorbic acid
ABA	Abscisic acid
AC	Activated charcoal
BA	Benzyladenine
Bl	Blackening (of explants)
Cl	Callusing (of explants)
CRD	Completely randomised design
D	Decontamination method
Dicamba	3,6-dichloro-2-methoxybenzoic acid
EC	Emulsifiable Concentration
ES	Explant source
GA_3	Gibberellic acid
Gt	Genotype (of enset)
HCl	Hydrochloric acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butric acid
KOH	Potassium hydroxide
LR	Light regime
MS	Murashige and Skoog (1962) medium
MSF	Multiple shoot formation
NAA	α-Naphthaleneacetic acid
NL/S	Number of leaves per shoot
NNS	Number of normal shoots
NR/E	Number of roots per explant
NS/E	Number of shoot per embryo (explant)
NSHB	Number of small and hyperhydric buds
PD	Pseudostem diameter
PGRs	Plant growth regulators
S	Size of explant
Sh	Shaking (of liquid medium)
SL	Shoot length
ST	Shoot tip
TDZ	Thidiazuron
TNSB	Total number of shoots and buds

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CHAPTER ONE

LITERATURE REVIEW
IN VITRO PROPAGATION OF *ENSETE*

1.1 Introduction

Enset (*Ensete ventricosum* (Welw.) Cheesman) is a diploid (2n= 18) herbaceous perennial. The genera *Ensete* and *Musa*, belonging to the Musaceae, are monocotyledons. Enset is the vernacular name used in the Amharic language in Ethiopia for *Ensete ventricosum*, which is a staple food crop and is part of a successful and sustainable indigenous farming system in the south and southwestern parts of the country. Enset produces seeds only after a long juvenile period, five to ten years depending on the altitude and management practices (TSEGAYE 2002) and seed dormancy is also a problem. As a result, it is usually multiplied by vegetative means and grown as clones. Conventional vegetative propagation mostly involves the use of corms of two to six year old plants. This is a slow process especially for new clones. Enset germplasm is currently conserved in a field genebank where it is exposed to both biological and physical constraints.

The use of tissue culture techniques to propagate plants *in vitro* is an extension of conventional propagation. Tissue culture is commonly used as a collective term to describe all types of *in vitro* plant cultures although strictly it should refer only to those of unorganised aggregates of cells (GEORGE and SHERRINGTON 1984). In tissue culture techniques, the plant cultures are contained within glass or plastic vessels, hence the term *in vitro* plant propagation. The term micropropagation is also used to describe the *in vitro* techniques because cultures are started with very small pieces of plants and small shoots are thereafter propagated. The foundation of micropropagation is the so-called totipotency theory, which states that cells are autonomic and, in principle, capable of regenerating to give a complete new plant (PIERIK 1993).

Plant regeneration by tissue culture can be achieved by zygotic embryo culture, somatic embryogenesis or organogenesis (DODDS and ROBERTS 1995, SMITH

and DREW 1990). Somatic embryos, which resemble the seed embryos, are formed *in vitro* and can grow into seedlings. Organogenesis is employed for the regeneration of shoots from existing meristems and regeneration from *de novo* (adventitious) meristems. Various combinations of nutrients, hormones and environmental factors for different species or genotypes may stimulate the micropropagation of plants by tissue culture. Manipulation of these factors may enable plant breeders and propagators to control plant cell morphogenesis and to develop reliable cell to plant regeneration systems.

There are two areas in which plant tissue culture methodology is important in plant production and breeding (SHORT 1990). The first, comprises current technologies such as clonal multiplication, pathogen elimination, embryo rescue, haploid production and genetic conservation. The second, concerns situations in which genetic modification of plants can be induced by mutagenesis, somaclonal techniques, somatic hybridisation and recombinant DNA technology. These techniques for genetic modification depend upon micropropagation for the regeneration and multiplication of new characteristics. For instance, to use recombinant DNA technology in plant breeding a whole plant must be regenerated from transformed cells. Micropropagation offers many advantages over conventional methods for the multiplication of large numbers of plants independent of climatic conditions saving both space and time (SHORT 1990). In addition, *in vitro* derived plants are frequently more vigorous and of superior quality compared to those produced by *in vivo* methods. Micropropagated banana and plantain establish more quickly, grow more vigorously and taller, have a shorter and more uniform production cycle and produce higher yields than conventional propagules (DREW and SMITH 1990, ROBINSON *et al.* 1993, VUYLSTEKE 1998). Only limited information is available on *in vitro* plant regeneration of *E. ventricosum*. Rate of multiplication for this species was reported to be 2-3 shoots per explant (corm and leaf tissues) per four week subculture (NEGASH *et al.* 2000). In other studies, shoots were regenerated from callus (AFZA *et al.* 1996, MORPURGO *et al.* 1996, ZEWELDU 1997).

1.1.1 *Origin, distribution and morphology of* Ensete

Centres of origin of *Ensete* are the lowland and mountain areas of Uganda, Tanzania and the Sudan (SMEDS 1955) and Ethiopia (KULS in WESTPHAL 1975). CHEESMAN (1947) revised the genus *Ensete* reporting 25 species. BAKER and SIMMONDS (1953) identified the synonyms whereas SIMMONDS (1960) with further work reported only six species, *Ensete gilletii*, *E. homblei*, *E. perrieri* and *E. ventricosum* with an African distribution and *E. glaucum* and *E. superbum* with an Asian distribution. *E. ventricosum* is widely spread in a wild state in Africa from Cameroon to East Africa and Transvaal (South Africa), and cultivated in Ethiopia (PURSEGLOVE 1972). Enset is found in its wild state in the south and southwestern parts of Ethiopia. It is widely cultivated in Ethiopia where it adapts to altitudes between 1500 and 3000 m with annual precipitation of 1100 to 1500 mm (BEZUNEH and FELEKE 1966, WESTPHAL 1975).

Enset resembles a banana plant in that both have underground stems (corms), a concentric bundle of leaf sheaths (pseudostem) and big leaves with conspicuous midribs. However, the pseudostem of enset dilates at the base and usually is thicker than that of the banana (CHEESMAN 1947). The corm of enset is upright while the rhizome of banana is slightly horizontal. Enset corms have nodes and internodes, which are prominent at sucker and early developmental stages of the plant. As the corm advances in growth and age, the internodes become compact. The apical portion of the corm contains meristematic tissues that produce both underground and aerial parts of the plant. The apical meristematic area is a growing point from which the apical bud is removed upon propagation to release lateral buds to grow (SIMMONDS 1959, BEZUNEH and FELEKE 1966, DIRO *et al.* 1996). BEZUNEH (1984) described growth and other morphological characteristics of enset. Depending on type of clone, environmental conditions and management practices, enset plants attain a height of 4 to 11 m, a pseudostem height of 2 to 5 m with a circumference of 1.5 to 3.0 m. The corm is 0.7 to 1.8 m long and 1.5 to 2.5 m in circumference at maturity. Pigmentation of the plant body is always amongst the first recognizable characteristic for the identification of landraces (SHIGETA 1996). Some are purple to dark red, but most are light green with variegated brown patches (BEZUNEH 1984).

1.1.2 Importance of enset

Ensete is of considerable local importance in Ethiopia, where it is the foundation of unique agriculture (SIMMONDS 1986). Enset is a source of food, mainly carbohydrate. About 15 million Ethiopians are enset growers and consumers (SPRING 1996) while the number of enset growers was estimated to be 9.8 million (CSA 1997). A mixture of scraped leaf sheath and pulverized corms, after fermentation in a pit, results in production of kocho. Kocho is the main product consumed after making a pancake-like food. Bulla is another important food product from enset produced from solidified liquid after dehydrating a fresh mixture of scraped leaf sheath and pulverized corms. Bulla is consumed mainly as porridge, in gruel and as crumbled forms. Corms of some clones are cooked and consumed similar to roots and tubers of other crops.

In addition, parts of some clones are used as traditional medicines. Cooked corms are consumed to heal bone fractures or breakage; a semisolid bulla, shortly after squeezing, is applied to wounds for healing; the corm of a selected clone is cooked and consumed to facilitate discharge of a placenta after birth; and pancake-like food from kocho or crumbled food from bulla is consumed as a treatment against diarrhoea (UNDP/ECA 1996). A novel phenylphenalenone was detected and isolated from *Ensete ventricosum* (HOLSCHER and SCHNEIDER 1998). There is also a potential for use of enset starch as a binder and disintegrant for compressed tablets (GEBRE and NIKOLAYEV 1993). A starch that can be used for paper, textile and adhesive industries is produced from enset (http://www. capitalethiopia. com 2003). Fibre, a by-product of enset in food processing, is a valuable raw material for household usage. Local fibre factories use this as an import substitute because the quality of enset fibre is equal to that of abaca and better than sisal (BEZUNEH 1996). Almost all parts of enset are sold in markets as a source of income. Processed products such as kocho and bulla are sold in small town markets and also transported to the cities. Leaves, as a wrapping material, and fibre are additional sources of income.

Enset is a valuable security crop as it tolerates transient drought. It saved the lives of many people during the past recurrent drought in Ethiopia. There are optimal

times and stages for the harvest of enset, but it can be harvested all the year round and at different growth stages as needed. WOLDETENSAYE (1997) reported that drought has never caused a serious problem for the cultivation of enset in the districts she studied. Enset leaves are fed to livestock and are extremely important during prolonged dry spells. Enset has a very large leaf area and the canopy is closed after plants are established. Thus, it protects rainfall from splashing the soil. Leaf litter checks runoff and also improves nutrient recycling. According to ELIAS (1998) soil fertility is being maintained, and even increased, in farm components such as the enset-garden, *darkua* (area near to the homestead planted usually with maize) and taro (*Colocasia esculenta*) fields. It was also emphasized that erosion does not occur in these fields, probably because of high organic matter and a more stable soil structure, the presence of mulch material and greater care provided by the farmers. WOLDETENSAYE (1997) also reported that higher levels of nutrients are present in enset fields than in non-enset fields. Therefore, enset contributes to sustainable agriculture and food security.

1.2 Enset Propagation

Plant propagation is the multiplication of plants by seeds and vegetative means involving the control of two developmental cycles, vegetative and reproductive. In the vegetative stage the plant grows by elongation of terminal and lateral shoots producing a series of nodes and internodes. As the shoots shift to the reproductive stage, vegetative growing points develop into flowers (HARTMANN and KESTER 1990). Enset is commonly propagated by vegetative means while it is in its vegetative phase, before the inflorescence begins elongating from the base of the pseudostem.

1.2.1 Seed propagation

Propagation by seeds is the major method by which plants reproduce in nature and one of the most efficient and widely used propagation methods for cultivated crops (HARTMANN and KESTER 1990). Basal flowers of enset are usually hermaphrodite and produce 5-15 seeds per fruit, 10-18 fruits per hand with 15-20 hands per bunch (BEZUNEH 1996). The seeds are 6 mm or more in diameter

(CHEESMAN 1947, PURSEGLOVE 1972). Enset seeds are enclosed by hard seed coats. The hard seed coat of the Musaceae offers protection to the embryo during maturation, dispersal and dormancy. However, it hampers germination because the embryo requires strong forces to rupture the seed coat (GRAVEN *et al.* 1996). The onset of dormancy is part of the normal developmental pathway for seed formation and accompanied by the differentiation of protective structures such as the seed coat (FOSKET 1994). FOSKET (1994) further stated that the development of dormancy progressively shuts down the cellular metabolic processes or reduces them greatly, which includes most gene transcription and the translocation of mRNA into proteins. Thus, the preparation for dormancy is an active process that involves transcription and formation of the specialized structures of the seed coat.

The seed coat causes dormancy in two ways, physical and mechanical (HARTMANN and KESTER 1990). Seed coverings that are impervious to water produce physical dormancy. Softening or scarifying the covering structures can induce germination in this type of dormancy. Mechanical dormancy is when seed enclosing structures are too strong to allow embryo expansion during germination though water may be absorbed. Embryo dormancy is another aspect of seed dormancy. Evidence for a dormant embryo is that the excised embryo usually will not germinate normally and the seedling produced may be abnormal (HARTMANN and KESTER 1990). GRAVEN *et al.* (1996) reported a degree of embryo-imposed dormancy in *Musa*. However, in *Musa balbisiana* the presence of factors affecting germination in the integuments, chalazal mass, and/or the endosperm was suggested (STOTZKY and COX 1962) because excised embryos were not dormant and could easily be cultured aseptically (COX *et al.* 1960).

Wild species of enset propagate from seed (ALEMU and SANDFORD 1991, BEZUNEH 1996, SHIGETA 1996). Enset growers rarely use seed propagation, as germination of intact seed is very poor (BEZUNEH 1971, TESFAYE 1992). TESFAYE (1992) reported that poor enset seed germination is attributed to the physical properties of the testa and size and physiology of the embryo. Moreover, if harvesting is delayed after flowering and fruit set, carbohydrates from the pseudostem are translocated to the growing inflorescence and finally the plant

dries up resulting in total loss of kocho yield (HUFFNAGEL 1961). Consequently, seed setting in enset under cultivation is of a rare occurrence. Propagation by seed can however play an important role in enset breeding for variability and germplasm conservation.

Germination of intact seeds of wild banana differed between harvest lots depending on maturity of the fruit at the time of harvest, post harvest age of the seed and method of storage (SIMMONDS 1952). Improved germination of intact enset seeds occurred when exposed to daily alternating temperatures (BEZUNEH 1971) and when seeds were treated with hot water (40 °C) for 24-48 hours and scarified around micropylar opening (TESFAYE 1992). The first evidence of seed germination in banana is displacement of the micropylar plug by the elongating radical-hypocotyl axis and the first conspicuous organ of the seedling is the primary root (McGAHAN 1961b).

1.2.2 Conventional vegetative propagation

Vegetative, or asexual, propagation is used to produce identical genotypes as the mother plant. Clonal propagation is a highly efficient method to fix genetic variation, in contrast to the sequence of generations required for seedling populations (HARTMAN and KESTER 1990). New side shoots of bananas and plantains arise from a sympodial rhizome, whereas *Ensete* do not produce new side shoots; *Ensete* is thus monopodial (PRICE 1995). Vegetative propagation, using corms, is a common practice in enset cultivation. A whole corm (BEZUNEH and FELEKE 1966) is planted or it is longitudinally split into two or four parts through the apex and each part is planted separately (ALEMU and SANDFORD 1991, BELHU *et al.* 1994, DIRO *et al.* 2002). The largest number of suckers, 35 suckers per half corm, was obtained from a three-year-old Halla clone when the mother plant was left undisturbed for one year, after removal of the apical bud (DIRO *et al.* 2002).

The apical buds should be removed from whole or split enset corms to induce sucker production because if planted without removing, only one sucker emerges per whole corm (BELHU *et al.* 1994, DIRO *et al.* 1996) and a few suckers per half

corm because the apical buds inhibit growth of lateral buds. Inhibition of lateral bud growth due to chemicals released by the terminal bud (apical dominance) is one of the limiting factors for the perennial production of AAB plantains that originated from *Musa acuminata* (AA) and *Musa balbisiana* (B) (ORTIZ 1995). A phenomenon where development of lateral buds is partially or completely inhibited by an actively growing apical region is termed as correlative inhibition (HILLMAN 1984). Apical dominance is also maintained by interaction of the two growth regulators, auxin and cytokinin (WICKSON and THIMANN 1958, BERRIE *et al.* 1987). The exceptions to complete inhibition of growth of lateral buds in *E. ventricosum* are the clones Awsako and Welgala, which send out few voluntary suckers (HSIU 1972). In addition, an unusual specimen of *Ensete*, which produces side shoots, was collected and maintained at the Phu Ho field germplasm bank in Viet Nam (KHOI and VALMAYOR 1995).

Some enset growers use a mother corm of four to six-year-old (BEZUNEH and FELEKE 1966) while others use two to three-year-old plants to produce suckers. Under mid-altitude Ethiopian conditions it was found that two to three-year-old mother plants of Halla clone gave better sucker emergence and growth (DIRO *et al.* 1999). These results indicate that the conventional vegetative propagation cycle of enset generally takes a long time.

1.2.3 In vitro *propagation*

In *Ensete ventricosum in vitro* culture, zygotic embryo culture was reported by BEZUNEH (1980). Different investigators (AFZA *et al.* 1996, MORPURGO *et al.* 1996, ZEWELDU 1997) carried out experiments on enset shoot tip culture where regeneration of plants was achieved through a callus phase but not from existing meristems. These authors also reported the extensive blackening of shoot tips at the initiation stage that led to necrosis and difficulty to regenerate plants. NEGASH *et al.* (2000) reported regeneration of plants from corm and leaf explants of *E. ventricosum* but the pathway was not indicated whether or not the callus phase was involved. Investigations were undertaken on *E. superbum* in relation to shoot tip culture (MATHEW and PHILIP 1996), use of male flower apices for regeneration of multiple shoots (KULKARNI *et al.* 1997), callus culture and somatic

embryogenesis (MATHEW *et al.* 2000) and ontogeny of somatic and zygotic embryos (MATHEW and PHILIP 2003).

MA and SHII (1972) reported the first *in vitro* clonal propagation of *Musa* (in ISRAELI *et al.* 1995), the genus related to *Ensete*. Since then significant progress has been made and practical applications have been introduced for the management and improvement of *Musa* (KRIKORIAN and CRONAUER 1984, CRONAUER and KRIKORIAN 1984a, b, CRONAUER and KRIKORIAN 1986, VUYLSTEKE *et al.* 1998). This includes, micropropagation by shoot tip culture (ISRAELI *et al.* 1995, VUYLSTEKE *et al.* 1998), virus elimination (GUPTA 1986, DREW *et al.* 1989) and germplasm conservation (DE LANGHE 1984, BHAT and CHANDEL 1993, PANIS *et al.* 1998). The technique is also used in genetic improvement (SASSON and COSTARINI 1989, ISRAELI *et al.* 1995). Along with the conventional breeding somatic embryogenesis, protoplast culture and transformation techniques are being used to improve Cavendish and other banana plants (SASSON 1997).

In enset cultivation, enset wilt caused by *Xanthomonas campestris* pv *musacearum* (YIRGOU and BRADBURY 1968) Xcm (DYE *et al.* in QUIMIO and TESSERA 1996) is one of the limiting factors. It is destructive as it kills enset plants at all stages (ASHAGARI 1985, QUIMIO and TESSERA 1996). Enset root mealy bug infest enset at the sucker and early stages of plant growth and development and kills the plants. The root lesion nematode, *Pratylenchus goodeyi* and the root knot nematode, *Meloidogyne* sp., are widely distributed in association with enset (QUIMIO and TESSERA 1996). Mosaic and chlorotic streaks, both of viral nature, were observed and are considered as potential threats to enset farming. More than 400 enset accessions (both cultivated and wild types) have been collected from different growing areas and are maintained in a field genebank at Areka Agricultural Research Centre, Ethiopia. A procedure for *in vitro* screening of *Musa* spp for resistance to burrowing nematode (*Radopholus similis*) was developed (ELSEN *et al.* 2002). This shows the potential of using *in vitro* technique to screen enset genotypes against the root knot and root lesion nematodes. In general, considering the challenges to enset production, development and

application of *in vitro* techniques can play a big role in future breeding of desirable clones.

1.2.3.1 Zygotic embryo culture

In this technique, mature or immature seed embryos are dissected from seeds and cultured *in vitro* to raise seedlings. Embryo culture has been used to explore the nutritional and physical requirements for embryonic development (HU and WANG 1986), to by pass seed dormancy, which may shorten the breeding cycle, to test seed viability, to provide microcloning source material and to rescue immature hybrid embryos from incomparable crosses (HU and WANG 1986, PIERIK 1987). Because of their juvenile nature with high regenerative potential, embryos provide excellent material for *in vitro* clonal propagation, for example in the Gramineae and Coniferae (HU and WANG 1986).

Since the embryos of seed plants are enclosed within a sterile environment, direct decontamination of the embryo surface is not necessary unless the seed coats are cracked or pathogens are known to exist within the seed coats. If so then, the entire ovules, seeds, or fruits are surface decontaminated and thereafter the embryos are aseptically excised from the surrounding tissues (HU and WANG 1986). Thus, the extent of culture contamination in embryo cultures is usually lower than other types of *in vitro* culture. Although immature embryos are frequently more easily cultured than mature ones, their dissection requires much skill and the embryos require more complex media (GEORGE and SHERRINGTON 1984, GEORGE 1993). These authors also stated that in general, mature embryos require only inorganic salts supplemented with sucrose, whereas immature embryos have an additional requirement for vitamins, amino acids, growth regulators and sometimes endosperm extract.

BEZUNEH (1980) cultured embryos of enset on a modified semi-solid medium of MURASHIGE and SKOOG (1962). Better results were reported when 5 g l^{-1} sugar and agar were used, whereas embryos that were preincubated for 15 to 20 minutes in 4 mg l^{-1} of the sodium salt of GA_3 (10%) showed additional swelling and elongation. NEGASH *et al.* (2000) cultured enset embryos on BA and IAA-

containing MS medium. *In vitro* culture of mature banana embryos, which were stored for three to 78 weeks after harvest, was reported (COX *et al.* 1960). Maturity of embryos at excision and the composition of the culture medium influence germination of excised embryos (JOHRI and RAO 1984). AFELE and DE LANGHE (1991) reported improved germination of excised embryos when seeds of *Musa balbisiana* were soaked in water for five days prior to embryo isolation and when the longitudinal axis of the embryo was placed flat half way embedded on the medium. Embryo rescue increased banana seed germination rates by a factor of three to ten (ORTIZ *et al.* 1995).

1.2.3.2 Shoot tip culture

Shoot tip culture is the use of a lateral or main shoot apex (apical dome plus a few subjacent leaf primordial), which may be up to 20 mm in length, to produce multiple shoots, whereas in meristem tip cultures much smaller explants are used with the aim to produce a single virus-free plantlet from each explant (GEORGE and SHERRINGTON 1984). Researchers and nursery personnel in both the public and private sectors routinely and increasingly use banana micropropagation by shoot tip culture (VUYLSTEKE *et al.* 1998).

ZEWELDU (1997) reported that *Musa* multiplication medium with cytokinins (5 mg l^{-1} BA combined with 1 mg l^{-1} TDZ) was used for *Ensete* shoot tip initiation but was not effective because of very high phenolic oxidation: the culture and medium turned brown within a shorter period of time compared to that observed in plantains or bananas. Although several protocorm-like bodies were observed, there was no further regeneration and subsequent shoot formation from the cultured shoot tip.

MURASHIGE (1974) subdivided the sequential stages of micropropagation into three (Stages 1, 2 and 3). Since then Stage 0 and Stage 4 were added (GEORGE and SHERRINGTON 1984). Stage 0: preparation of the mother plant, Stage 1: establishment of the aseptic culture, Stage 2: multiplication of propagules, Stage 3: regeneration of whole plant and Stage 4: hardening for subsequent field planting.

Stage 0: Here, preparation of the mother plant is an important activity. Several buds may be taken from a single mother plant as a source for explants and these are multiplied to several thousand plants; therefore, the careful selection of the source plant is extremely important considering such characteristics as trueness-to-type, vigour and rate of growth (ISRAELI *et al.* 1995). Healthy, vigorously growing plants will render suitable explants (CONSTABEL and SHYLUK 1994). To yield more hygienic explants, stock plants can be grown in greenhouses (DEBERGH and READ 1991).

According to MURASHIGE (1978) the most regenerative organ or tissue may be different for each plant, often materials that serve well in a traditional propagation practice serve also as excellent explant source. For *in vitro* propagation of bananas, shoot tips (meristem plus a few attached leaf primordia), harvested from vegetative buds of suckers of various sizes, have been used successfully to establish cultures. The terminal buds produced only one plantlet, whereas a larger explant with axillary buds can produce multiple plants in tissue culture propagation of bananas (DORE SWAMY *et al.* 1983). Banana floral apices cultured *in vitro* reverted and produced vegetative shoots (FITCHET 1987, COTE *et al.* 1996).

Stage 1: At this Stage, decontamination procedure, size of explant, medium composition and culture environment are factors that determine success in establishment (initiation) of the aseptic culture.

Decontamination: For banana *in vitro* propagation, the outer leaves, leaf bases and corm tissue of a selected explants are trimmed and surface decontaminated with sodium hypochlorite with a surfactant under aseptic conditions (KRIKORIAN and CRONAUER 1984, ISRAELI *et al.* 1995). HAMILL *et al.* (1993) modified a double decontamination method for banana shoot tip culture. That is, a block of tissue (20 mm x 40 mm) was rapidly excised, decontaminated in 3.5% NaOCl with Tween 80 for 15 min. Bleached tissue was removed leaving a block (15 mm x 30 mm) with an intact apex, leaf primordia and corm material, which was re-decontaminated as before for 5 min. The bleached tissue was again removed without rinsing, to leave a block of tissue (5 mm x 8 mm). Shoot tips of *Ensete superbum* from a botanical garden were decontaminated with 0.1% mercuric

chloride solution for 5 min followed by three washings by sterile water (MATHEW and PHILIP 1996). The corm tissues of *Ensete ventricosum*, about 2 cm^2 in size, were decontaminated in 1.5% (w/v) NaOCl solution with some drops of Tween 20 for 10 to 15 min then rinsed three times with sterile distilled water. However, endogenous contaminants were reported (ZEWELDU 1997).

Explant size: The size of explant is an important factor for successful establishment (VUYLSTEKE and DE LANGE 1985). Very small explants increase the likelihood of producing virus free plants but the mortality is high and they grow slowly. Nevertheless, the size of the explant has to be empirically determined for each species keeping in mind the objectives of the study (CONSTABEL and SHYLUK 1994). In bananas, multiplying shoot cultures have been established by culturing explants of 0.5 cm^2 (DE GUZMAN in CRONAUER and KRIKORIAN 1986). A larger initial shoot cube (one cm^3 that contains the apex) was also used, by cutting the cube into quarters (DE GUZMAN *et al.* in CRONAUER and KRIKORIAN 1986). A better survival rate was reported from shoot tip explants with an apical dome than from shoot tip explants without an apical dome (WONG 1986). It was also reported (ISRAELI *et al.* 1995) that in some cases the shoot apex is wounded by a series of cuts or split longitudinally by two cuts, yielding four explants that can be cultured separately.

When plant tissues are exposed to stress situations such as mechanical injury, which is the case with isolation of explant from the stock plant, metabolism of phenolic compounds is stimulated (DEBERGH and READ 1991). In general, phenolics are very labile products that are very easily oxidized. Many of these compounds are phytotoxic and will lead to death of plant tissue if released into cells (COLLIN and EDWARDS 1998). Different ways to prevent blackening of tissues and medium were reported (GEORGE and SHERRINGTON 1984, COLLIN and EDWARDS 1998). These include, avoiding or minimizing stress to the stock plants, adsorption of phenolic compounds by activated charcoal or polyvinylpyrrolidone, polymerisation of phenolic quinones by reducing agents (antioxidants such as ascorbate, citrate, dithiothreitol and glutathione), thereby removing one of the substrates that lead to blackening of the tissues. In banana, citric acid, ascorbic acid and activated charcoal were added separately to the

medium and it was found that ascorbic acid was the most effective and that 25 mg l^{-1} prevented oxidation (GUPTA 1986). Lower temperatures and shorter time of illumination reduce blackening (ISRAELI et al. 1995). Liquid medium can also wash away the cell components from the surface of the explants.

Medium composition: Success in plant cell culture is largely determined by the quality of nutrient media (CONSTABEL and SHYLUK 1994). Formulations designed by MURASHIGE and SKOOG (MS) (1962), (GAMBORG *et al.* (GB5) (1968) and SCHENK and HILDEBRANDT (SH) (1972) can be regarded as standard. As reported by CONSTABEL and SHYLUK (1994) nine out of ten laboratories prefer the medium designed by MURASHIGE and SKOOG (1962). There are eight major groups of components in media required for plant cell tissue culture (COLLIN and EDWARDS 1998). These include, major inorganic nutrients, microelements, iron, vitamins, carbon source, organic nitrogen, plant growth regulators and agar. Major inorganic nutrients are nitrogen (as NO^{-}_{3} and NH^{+}_{4}), phosphorus (as $PO^{\equiv}_{4}$), potassium, sulphur (as $SO^{=}_{4}$), calcium and magnesium, all of which are usually present in mM quantities. These elements have both structural and functional roles in protein synthesis (particularly N and S), nucleotide synthesis (P, N, S) cell wall synthesis (Ca^{++}), enzyme cofactors (Mg^{++}) and membrane integrity (Mg^{++}). Microelements include manganese, zinc, boron, copper, cobalt and molybdenum. Many of these have important functions in enzyme function as cofactors. Iron is an important enzyme cofactor.

Vitamins include thiamine-HCl, nicotinic acid, pyridoxine-HCl, myo-inositol, pantothenate, biotin, para-benzoic acid, cholate and choline chloride. These vitamins all generally function as important coenzymes in reactions crucial to primary plant metabolism. An adequate carbon source is one of the important constituents of the culture medium used for meristem and shoot tip culture (CONSTABEL and SHYLUK 1994). CONSTABEL and SHYLUK (1994) explained that typically all media contain sucrose in the range of 1-3% as a carbon source. Sucrose can be replaced with glucose while other sources of C are not as effective as sucrose (e.g., fructose, lactose, maltose, and starch) (COLLIN and EDWARDS 1998). In addition, carbohydrates have an osmotic role in organogenesis.

Auxins and cytokinins stimulate cell division and control cell differentiation and morphogenesis (COLLIN and EDWARDS 1998). Both naturally occurring auxins such as indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) and synthetic auxins such as 2, 4-dichlorophenoxyacetic acid (2,4-D), α-Naphthalene acetic acid (NAA) and p-chlorophenoxyacetic acid (pCPA) are used. Likewise, naturally occurring cytokinins, zeatin (Z), isopentenylaminopurine (iP) and benzyladenine (BA) and synthetic ones like kinetin (Kn) are available. To solidify media, agar (0.6 to 1%), agarose (0.6 to 0.8%), or gelrite (0.1 to 0.3%) are added to nutrient media (CONSTABEL and SHYLUK 1994).

The most widely used medium contains the MURASHIGE and SKOOG (1962) salts for banana *in vitro* culture (CRONAUER and KRIKORIAN 1986). Other supplements discussed by ISRAELI *et al.* (1995) include myo-inositol (100 mg l^{-1}), L-tyrosine (200 mg l^{-1}), thiamine-HCl (0.5 mg l^{-1}), adenine sulphate (160 mg l^{-1}), benzyladenine (BA) (5 mg l^{-1}), indole-3-acetic acid (IAA) (2 mg l^{-1}), and sucrose (30 g l^{-1}). The pH is adjusted to 5.8 and the medium is solidified with agar (7 g l^{-1}). Test tubes (25 x 150 mm) with the medium are autoclaved at 121 °C and 103.4 kPa for 15 min. Cultures are maintained at 28 ± 2 °C and 60-70% relative humidity in a 16 h light/8 h dark with fluorescent light of 1000-3000 lux (ISRAELI *et al.* 1995). After a few days on the medium, the banana explants swell and turn green, shoots appear two to three weeks later and subculturing is done after four to six weeks; or earlier if blackening occurs (ISRAELI *et al.*1995). The termination of Stage one with the method of axillary shoot increase, the stem tip or the bud of nodal segments should have elongated perhaps to 1 cm; lateral bud explants should reveal emerging shoots (MURASHIGE 1978). Development of one or more adventitious shoots, each 1 cm long, would indicate the culmination of Stage one for the method that relies on adventitious shoot production.

Culture environment: Since there exists a strong and complex interaction between growth regulators and culture conditions adequate attention has to be given to optimisation of culture environment, along with hormonal regimes, in a strategy aimed at developing efficient meristem and shoot tip culture techniques (HARTMANN and KESTER 1990). Many different kinds of container can be used for banana and plantain shoot tip cultures. The volume of the container can

sometimes affect *in vitro* growth; therefore, good practice is to use progressively larger vessels at the different stages of micropropagation (VUYLSTEKE 1998). Cultures should be grown in a separate, lighted facility where both day length and irradiance can be controlled (HARTMANN and KESTER 1990). Most shoot tip cultures are kept under artificial lightening provided by cool, white, fluorescent tubes. A photoperiod of between 12 and 16 h light is widely used and is adequate for prolific growth and rooting of banana plants (VUYLSTEKE 1998). Generally, an average light irradiance of 50 μmol m^{-2} s^{-1} is achieved at plant tissue level, which is about 2.5% of full sunlight intensity (COTE *et al.* in VUYLSTEKE 1998). Temperatures of 21 °C to 30 °C are generally adequate, although some kinds of plants may need lower temperatures. The relative humidity at the temperatures given is about 30 to 50%; if too low, dehydration might occur; if too high, contamination may occur.

Stage 2: The function of Stage two is to increase the number of propagules for later rooting to the plantlet stage (HARTMANN and KESTER 1990, DODDS and ROBERTS 1995). The most critical component of Stage two culture medium is a high level of cytokinin to promote axillary shoot proliferation (NEHRA and KARTHA 1994). Among various cytokinins used, BA is the most commonly employed at this Stage of culture for different species. Variation in multiplication rate of *Musa* is due to different cultivar-dependent responses to the cytokinin concentration in the medium (VUYLSTEKE 1998). Multiple banana shoot cultures were produced on agar medium by splitting a small shoot longitudinally through the apex, by culturing apices in liquid medium and also on MS basal medium plus BA (5 mg l^{-1}) (CRONAUER and KRIKORIAN 1984b). Increasing the BA concentration from 4.5 to 11.26 mg l^{-1} significantly increased the proliferation rate in two-year-old subcultures of the ABB cooking banana cv. Nzizi but further increasing the BA concentration to 22.52 mg l^{-1} did not result in any significant improvement on the 11.26 mg l^{-1} BA treatment (VUYLSTEKE 1998). However, multiple shoots, up to 28 per explant of cv. Basrai, were produced on MS medium with 100 mg l^{-1} BA + 3 mg l^{-1} kinetin + 0.5 mg l^{-1} IAA (NANDWANI *et al.* 2000). Differences in sucker production *in vivo* of *Musa* may be exaggerated, or even overcome under *in vitro* conditions (KRIKORIAN and CRONAUER 1984b). The phenylurea cytokinin,

thidiazuron (TDZ) was reported to have a powerful cytokinin effect on *Musa* shoot tip multiplication (ZEWELDU 1997).

Stage 3: The major change in Stage 3 is the shift to conditions that favour root initiation and shoot elongation (HARTMANN and KESTER 1990). At this Stage, steps are taken to induce development of individual plants to such a size that they will be able to survive in soil (ISRAELI *et al.* 1995). These authors also noted that subculturing a propagule to a medium without BA or with a low BA concentration, or replacing BA with kinetin, which reduces axillary bud and shoot formation, induces development. The medium, in the case of bananas, is supplemented with 2 mg l^{-1} IAA and 5 mg l^{-1} kinetin. Other auxins, NAA (GUPTA 1986, FITCHET 1987) or IBA (DORE SWAMY *et al.* 1983) were also used. Moreover, CRONAUER and KRIKORIAN (1984b) reported that basal medium devoid of any growth regulators resulted in rooting. The plantlets from enset showed high rooting capacity in hormone-free MS medium (ZEWELDU 1997).

Stage 4: A post regenerative acclimatization period is required to enable the survival of the tissue cultured plants *in vivo* (RICE *et al.* 1992), which is nurturing of the plantlets in a potting mix. It involves adaptation from heterotrophic to autotrophic conditions, increase in light intensity and decrease in humidity and exposure to diurnal temperature change and to pathogens (ISRAELI *et al.* 1995). *In vitro* plants, as compared with *ex vitro*, have a less developed cuticle, limited stomatal activity, limited mesophyll development and many intercellular cavities. The culture environment defines organs initiated in culture and they have the same characteristics as they did *in vitro* after the transfer to the greenhouse (DEBERGH and ZIMMERMAN 1991). A gradual return to normal characteristics occurs during acclimatization in the greenhouse.

The occurrence of somaclonal variations in *Musa* has been reported on different occasions, the frequency ranging from 0% to 90% (DREW and SMITH 1990, ISRAELI *et al.* 1991). The dwarf characteristic was retained over five generations and is therefore likely to be a stable genetic trait rather than an epigenetic change that will reverse with time (DREW and SMITH 1990). It was explained that by contrast the thin-leafed characteristic produced *in vitro* was an example of a

transient (epigenetic) change that was reversible. In general, it is supposed that the more organised the explant, the less the variation; and the less organized the starting material, the more the variation (KRIKORIAN 1989). Using this view, shoot tips would vary least; callus, cell suspension or protoplast procedures would progressively generate the most varied plantlets.

1.2.3.3 Callus culture and somatic embryogenesis

Callus is a coherent and amorphous tissue, formed when plant cells multiply in a disorganized way (GEORGE and SHERRINGTON 1984, GEORGE 1993). Establishment of a callus from an explant can be divided roughly into three developmental stages: induction, cell division and differentiation (DODDS and ROBERTS 1995). During the initial induction phase metabolism is stimulated prior to mitotic activity (DODDS and ROBERTS 1995). This results in de-differentiation of cells where adult cells are able to revert from the adult to juvenile state (PIERIK 1987). Monocotyledons are generally less likely to form callus tissue than dicotyledons; and thus with monocotyledons, embryos, young leaves, seedlings and very young flower initials are often preferred as starting material (PIERIK 1987, RAZDAN 1993). Subsequently, there is a phase of active cell division as the explant cells revert to a meristematic state (PIERIK 1987, DODDS and ROBERTS 1995) under the influence of plant growth regulators. The third phase involves the appearance of cellular differentiation and the expression of certain metabolic pathways that leads to the formation of secondary metabolites (DODDS and ROBERTS 1995). Although callus remains unorganised, as growth proceeds, some kinds of specialized cells may again be formed (GEORGE 1993). Such differentiation can appear to take place at random, but may be associated with centres of morphogenesis, which can give rise to organs such as roots, shoots and embryos. Root-shoot differentiation is a function of quantitative interaction between auxin and cytokinin (RAZDAN 1993). Root formation generally takes place in a medium with a relatively high auxin and low cytokinin concentration while formation of adventitious shoots takes place if there is a low auxin and high cytokinin concentration (PIERIK 1987). Mostly, whole plant regeneration from cultured cells may occur either through shoot-bud differentiation or somatic embryogenesis (RAZDAN 1993).

Organogenetic events of *Ensete* callus are influenced by the ratio of growth regulators, namely auxins and cytokinins, and the light regime (ZEWELDU 1997). Under the same hormonal treatment, light promoted differentiation of callus with subsequent organ formation, whereas the callus that was maintained in darkness demonstrated predominantly further growth without differentiation. The highest adventitious shoot formation was observed, in organogenesis of enset callus, when 4.5 mg l^{-1} BA was used with either 0.017 mg l^{-1} IAA or 0.19 mg l^{-1} NAA (ZEWELDU 1997). MORPURGO *et al.* (1996) reported that callus cultures could be easily established starting from corm explants and adventitious buds of *Ensete ventricosum*. CRONAUER and KRIKORIAN (1987) reported the production of adventitious shoots from calloid cultures of triploid dessert banana. The indirect production of adventitious shoots from explants via a callus stage may accentuate the problem of genetic instability of the progeny and although the large scale multiplication of callus may confer commercial advantage, the repeated subculture of callus from many plant species may reduce its morphogenic potential (RICE *et al.* 1992).

Somatic embryogenesis is a process by which embryos regenerate from somatic cells or tissues (PIERIK 1987). Somatic embryos of *E. ventricosum* could be obtained by lowering the level of cytokinins in the medium (AFZA *et al.* 1996, MORPURGO *et al.* 1996). In *E. superbum*, the shoot tips from *in vivo* conditions cultured on MS medium supplemented with 10 mg l^{-1} NAA, 2.5 mg l^{-1} kinetin and 1000 mg l^{-1} L-glutamine produced a proliferating callus in 120 days of culture from which adventitious shoots were regenerated but somatic embryos were not formed (MATHEW *et al.* 2000). The presence of 2 mg l^{-1} 2,4-D and 1.5 mg l^{-1} BA was crucial for induction of a proliferating embryogenic callus of *E. superbum* from the corm tissue at the base of *in vitro* generated plants. However, the differentiation and maturation of somatic embryos happened only in hormone-free medium (MATHEW and PHILIP 2003). These authors also compared the ontogenetic stages of the somatic embryogenesis to that of zygotic embryogenesis of *E. superbum* and found remarkable similarities. In earlier works, the production of somatic embryos of bananas, which were derived from shoot tissues, was reported but the produced embryos failed to germinate (CRONAUR and KRIKORIAN 1983, BANERJEE *et al.* 1987). Somatic embryos were produced from callus of zygotic

embryos of diploid *Musa* species and germinated (CRONAUER-MITRE and KRIKORIAN 1988, ESCALANT and TEISSON 1989). NOVAK *et al.* (1989) initiated proembryogenic callus from basal leaf sheath and rhizome tissue of diploid bananas on modified SCHENK and HILDEBRANDT (SH) (1972) medium with 6.63 mg l^{-1} dicamba and converted a maximum of 12% embryos into plantlets.

Embryos can be distinguished from adventitious shoots because they are bipolar, having a shoot and root pole, a shoot axis and cotyledons (or a coleoptile and a scutellum in monocotyledons) of a similar venation to that in zygotic embryos; they also have no vascular connections with the underlying parental tissue (GEORGE 1993). The embryonic cells from which somatic embryos are derived show a number of common features, which are characteristics of rapidly dividing cells (PIERIK 1987, GEORGE 1993). These include, small size, dense cytoplasmic contents, large nuclei with prominent large nucleoli, small vacuoles and many starch grains. Somatic embryos can be differentiated directly from a cell or tissue without previous callus formation or indirectly from callus (PIERIK 1987, RICE *et al.* 1992, GEORGE 1993). In banana, in addition to the bipolarity, the shoot and root primordia of somatic embryos form a hook-like embryonal axis, which is a characteristic feature of the members of the Musaceae (BANERJEE *et al.* 1987).

1.3 Conclusions

Although enset (*Ensete ventricosum*) is important in the Ethiopian economy, it is little investigated and remains an undervalued commodity crop. Recently it started appearing as one of the nationally important crops in the country's agricultural research agenda. Generally, little research work has been done in the past on *in vitro* propagation of *Ensete ventricosum* where shoots were mainly generated through a callus phase and blackening at culture initiation stage was detrimental to cultured shoot tips (AFZA *et al.* 1996, MORPURGO *et al.* 1996, ZEWELDU 1997). The rate of multiplication for this species was reported to be two to three shoots per explant (corm and leaf tissues) per four-week interval (NEGASH *et al.* 2000). In the latter work it is not really clear whether or not corm and leaf tissue explants were used to initiate shoot tips and also the involvement or absence of callus in the regeneration pathways was not reported. Information on somatic embryogenesis of

this species is also scanty (AFZA *et al.* 1996, MORPURGO *et al.* 1996). However, *in vitro* propagation of this crop can play a role in clonal multiplication, pathogen elimination and germplasm conservation. The use of somaclones and *in vitro* mutants in the long term may be beneficial to create variability for enset improvement. Biotechnologies in the area of somatic embryogenesis, protoplast culture and transformation techniques are potential areas for enset improvement.

It should be noted that there are no inviolable rules for the successful propagation of a particular species by tissue culture, and it is often necessary to adjust and readjust the composition of the ambient medium and even the environment so that cultures may be induced to grow and to organize (RICE *et al.* 1992). HARTMANN and KESTER (1990) also explained the existence of a strong and complex interaction between growth regulators and culture conditions and thus adequate attention has to be given to optimisation of the culture environment, along with hormonal regimes, in a strategy aimed at developing efficient meristem and shoot tip culture techniques. Therefore, to exploit the applications of tissue culture techniques in enset production and breeding, *in vitro* plant regeneration systems such as zygotic embryo culture, callus culture and somatic embryogenesis and shoot tip culture of *Ensete ventricosum* were investigated in this study and hence this literature review.

1.4 Aims and Objectives of the Study

The aims and objectives of the study were: To improve procedures of *in vitro* germination of zygotic embryos as germination of intact seeds is low and erratic, to develop a protocol for rapid propagation of enset addressing problems associated with blackening of explant and formation of unwanted callus *in vitro* and to investigate callus culture and somatic embryogenesis in enset.

CHAPTER TWO

IN VITRO CULTURE OF ZYGOTIC EMBRYOS OF *ENSETE VENTRICOSUM*

2.1 Introduction

Enset (*Ensete ventricosum* (Welw.) Cheesman) is a diploid (2n=18) herbaceous perennial belonging to the Musaceae. It produces seeds with hard seed coats. The hard seed coat offers protection to the embryo during maturation, dispersal and dormancy, which is characteristic of the Musaceae. However, it hampers germination because the embryo requires strong forces to rupture the seed coat (GRAVEN *et al.* 1996). According to McGAHAN (1961a) the seeds of *Ensete* differ from those of *Musa balbisiana* by being considerably larger and by having a conspicuous hilum cavity, otherwise the differences appear to be ones of degree rather than kind.

Germination of intact seeds of wild banana differed between harvest lots depending on maturity of the fruit at the time of harvest, post harvest age of the seed and method of storage (SIMMONDS 1952). *In vitro* culture of mature banana embryos, which were stored for 3 to 78 weeks after harvest, was reported (COX *et al.* 1960). Maturity of embryos at excision and the composition of the culture medium influence germination of excised embryos (JOHRI and RAO 1984). AFELE and DE LANGHE (1991) reported improved germination of excised embryos when seeds of *Musa balbisiana* were soaked in water for five days prior to embryo isolation and when the longitudinal axis of the embryo was placed flat, half way embedded into the medium.

Intact enset seed germination is very slow and generally poor under natural conditions. It was suggested that poor enset seed germination is due to the physical properties of the testa and size and physiology of the embryo (TESFAYE 1992). A degree of embryo-imposed dormancy in *Musa* was also reported (GRAVEN *et al.* 1996). However, germination of intact enset seeds was improved when the seeds were exposed to daily alternating temperatures (BEZUNEH 1971) and when treated with hot water and scarified (TESFAYE 1992). The germination

of excised enset embryos on simple semi-solid medium following pre-incubation of the embryos in gibberellic acid (GA_3) (BEZUNEH 1980) and on BA + IAA-containing medium (NEGASH *et al.* 2000) was reported. Failure of seedlings of *Ensete* from excised embryo to produce adventitious roots, blackening of the medium because of release of cell components and formation of unwanted callus from cultured embryos are known (BEZUNEH 1980). Owing to various factors, seed propagation is not a common practice in enset cultivation. Propagation by seed can however play an important role in enset breeding for variability and germplasm conservation. At present, enset accessions are conserved in a field genebank and are exposed to many adverse biological and physical factors. Detailed information on seed or excised embryo germination would be useful to complement the field genebank, as the seeds can be stored for long periods without loss of viability. Furthermore, *in vitro* seedlings could also be good starting material for micropropagation, avoiding the difficulties in decontaminating vegetative material and juvenility. While only limited work has been done generally on embryo culture of *E. ventricosum*, there is no information on *in vitro* regeneration of plants from stored seeds of different genotypes.

In the present studies, the embryos were aseptically excised from seeds of two cultivated genotypes (Mariya and Oniya) of *E. ventricosum* that had been stored for about six years and from seeds of two wild types, three to four months after harvest, and cultured. Enset genotypes Mariya and Oniya are widely cultivated in southern Ethiopia. However, their seeds were used in this study because of availability. Methods of seed decontamination prior to embryo excision, *in vitro* germination of the excised embryos and growth of the seedlings were investigated.

2.2 Materials and Methods

2.2.1 Plant material

The seeds used in this study were obtained from Areka Agricultural Research Centre, Ethiopia. The stored seeds were collected in February and March 1996 from two cultivated genotypes (Mariya and Oniya) of *E. ventricosum*, sun dried and stored in brown paper bags at room temperature until used. The studies were

carried out between September 2001 and August 2002. Seeds of two wild types (W01 and W02) of *E. ventricosum* were collected in January 2003 and the studies on *in vitro* culture of embryos from these seeds were executed in March 2003. As these seeds were only kept for about 3 months before use they were not considered as having been stored and are referred to as seeds from wild enset types.

2.2.2 Germination of intact stored seeds

Before the *in vitro* culture of embryos, germination of intact stored seeds was examined. Intact seeds were soaked in hot water (40 °C) for 30 h, as described by TESFAYE (1992) without scarification. Their germination was tested under three different sets of conditions: seeds were planted in pots with sand as medium; then placed in petri dishes on wet filter paper; and thereafter in jars on MS medium supplemented with sucrose (30 g l^{-1}) and gelled with agar (8 g l^{-1}).

2.2.3 In vitro *culture of embryos*

Different experiments on *in vitro* zygotic embryo germination were conducted. In all the experiments, only seeds that sunk when placed in water were used. The seed coat (Figures 2.1 and 2.2) was ruptured using sterile pliers, holding seeds between thumb and forefinger. The embryos, which usually occur in the micropylar area (Figure 2.1), were removed with a scalpel and inoculated onto the medium. The basal medium of MURASHIGE and SKOOG (1962) was used supplemented with sucrose (30 g l^{-1}) and gelled with agar-agar powder (8 g l^{-1}). The medium, glassware and instruments were autoclaved at 121 °C for 20 min. In all embryo germination experiments, the seeds were decontaminated for 15 min in 3.5% sodium hypochlorite, and then rinsed three times in sterile distilled water.

2.2.3.1 Decontamination of explants

To compare decontamination procedures, the stored seeds of Mariya and Oniya clones were decontaminated after they were soaked in distilled water for 30 min (water pretreatment) or without prior soaking in water (without water pretreatment).

Decontamination was done for 15 min in 3.5% sodium hypochlorite then rinsed three times in sterile distilled water. Embryos were then excised and inoculated onto MS medium without plant growth regulators. A 2 x 2 factorial experiment was carried out in a completely randomized design (CRD). Thirty test tubes per treatment in two replications with one embryo per test tube were used. Further studies on methods of decontamination of seeds and/or embryos were carried out using seeds of wild enset type W02. In this case, seeds were first decontaminated in 3.5% sodium hypochlorite for 30 min and rinsed three times with sterile distilled water. Then embryos were excised and divided into two groups, each group consisting of 24 embryos. Embryos of the first group were inoculated onto medium without further decontamination, while embryos in the second group were decontaminated in 3.5% sodium hypochlorite for 5 min, rinsed three times with sterile distilled water and then inoculated onto the medium. All embryos were placed horizontally on MS medium containing 0.5 mg l^{-1} BA + 0.2 mg l^{-1} IAA and 5 g l^{-1} AC.

2.2.3.2 In vitro *embryo germination*

For experiments on *in vitro* germination of embryos from stored seeds of Mariya and Oniya genotypes, factorial combinations of two types of embryo orientations on the three different medium compositions were used in a CRD. Embryo orientations on the medium were vertical (haustorium embedded in medium with the meristematic region exposed) or horizontal (longitudinal axis of the embryo was placed flat halfway embedded into the medium). The composition of the media were: MS without plant growth regulators (PGRs) and MS supplemented with (mg l^{-1}) 0.5 BA + 0.2 IAA or 0.5 BA + 0.2 2,4-D. After inoculation, the cultured embryos were transferred to a growth room and incubated in the dark at 24 °C. Seedlings were then transferred to irradiances of 4-6 μmol m^{-2} s^{-1} for a week and thereafter to 43 μmol m^{-2} s^{-1}. *In vitro* germination of embryos from seeds of two wild enset types (W01 and W02) was investigated on six media compositions with and without activated charcoal (AC) in factorial treatment combinations. After seed decontamination, the embryos were aseptically excised and inoculated horizontally onto the medium. The medium compositions were: MS without PGRs and MS supplemented with (mg l^{-1}) 0.5 BA + 0.2 IAA; 0.5 BA + 0.2 2,4-D; 0.5 BA + 0.2 IAA

+ 0.2 2,4-D; 1.5 BA + 2 2,4-D or 1.5 BA + 2 2,4-D + 0.2 IAA. Based on the treatments, 5 g l^{-1} AC was used. Twenty-four embryos per treatment, one embryo per test tube in two replications, were used. Explanted embryos were incubated in a growth room with a 16 h light/8 h dark and irradiances of 43 µmol m^{-2} s^{-1}.

2.2.4 Data collection and statistical analysis

Data on germination and contamination, number of shoots per embryo, shoot and root length and number of roots and leaves per shoot were collected. Percentage of explants without blackening was used to assess the effect of AC on blackening of cultured embryos. Callus formation was considered when explanted embryos produced clearly observable callus usually at the base of the shoots but sometimes embryos formed callus without producing radicles and epicotyls, which was used as indications of germination. Percentages of callus formation and of explants without blackening were computed based on growing embryos. Embryos were considered growing when they gave rise to shoots and/or callus. GenStat 5 Release 4.2 (McCONWAY *et al.* 1999) was used to analyze the data. Significant means were separated by least significant differences (LSD) at a 5% probability. Standard errors of means (SE) were also computed. A Correlation matrix was run to explain associations of growth parameters.

2.3 Results

2.3.1 Germination of intact stored seeds

Hot water treatment of intact seeds did not result in germination. However, by rupturing the seed coat physically, two months after application of the hot water treatment, elongation of embryos was observed in some seeds of the Oniya clone placed on wet filter paper in petri dishes or on sucrose medium in jars. This suggested that the elongating radicle-epicotyl parts of the embryos could not push through the micropylar collar of the seed coat (Figure 2.1) and as a result the embryos died. There was a slight dark blue coloration of the medium indicating release of seed components.

2.3.2 Methods of decontamination

Seeds were first pretreated with water for four days prior to decontamination to test the effect of imbibition of water on embryo germination. Most of the embryos were found wet when they were excised after soaking in water. However, germination could not be observed because of extensive contamination of the embryos particularly of the Oniya genotype. Seeds were then pretreated with water for only 30 min prior to decontamination to determine if the water pretreatment caused the contamination. Different levels of contamination of cultured embryos were observed depending on enset genotype and water pretreatment before decontamination (Figure 2.3). Embryos from seeds of Oniya pretreated with water and then decontaminated were highly contaminated (95%) while embryos from the same genotype and seed lot, decontaminated without prior water treatment, gave only 5% contamination. This was the only treatment where embryo germination occurred (18%). While the embryos of Mariya were only slightly contaminated irrespective of treatment no germination occurred. The seeds of the Mariya clone were clean around the hilum and the micropylar plug was exposed (Figure 2.2a). In some seeds the plugs (seed lids) were dislodged, possibly during seed harvesting and storage. In the case of Oniya seeds the hilum was covered with dry tissues and the micropylar plugs were not exposed (Figure 2.2b). From the results it seems that exposing and/or removing the seed plug during storage had an adverse effect on embryo viability. While dead tissue around the hilum harboured contaminants the seeds remained viable.

Decontamination of seeds in 3.5% sodium hypochlorite for 15 min kept contamination levels on embryos of Oniya genotype to below 5%. But when seeds of W01 and W02 genotypes were decontaminated using the same procedure contamination levels up to 20% were encountered. In another decontamination experiment using seeds of W02, where embryos were excised from seeds that were decontaminated for 30 min and cultured, 83% of the embryos germinated without being contaminated while 17% of the embryos were contaminated without germination. When excised embryos were decontaminated for 5 min, in addition to the decontamination of the seeds, there was no contamination but all of the embryos failed to germinate.

2.3.3 *Germination* in vitro *of embryos and growth of shoots*

2.3.3.1 Germination and shoot growth of embryos from stored seeds of Oniya

The interaction effects between genotype and orientation of embryos and between genotype and medium composition influenced *in vitro* germination of the embryos of Mariya and Oniya (Tables 2.1 and 2.2). This would be because the embryos from the seeds of the Mariya clone failed to germinate under all treatment combinations. For Oniya, orienting the embryos horizontally on the medium resulted in a higher germination percentage than when placed vertically. MS medium supplemented with BA and IAA resulted in higher germination of enset embryos than MS medium without PGRs and with BA + 2,4-D.

Table 2.1: Effect of embryo orientation, medium composition and enset genotype (Mariya and Oniya) on *in vitro* germination (%) of the embryos. MS medium (MS) supplemented with (mg l^{-1}) 0.5 BA + 0.2 IAA or 0.5 BA + 0.2 2,4-D

Treatment	Percent embryo germination	
	Mariya	Oniya
Vertical	0	29
Horizontal	0	38
SE	0.5	
LSD (5%)	1.4	
Medium composition		
MS	0	25
MS + BA + IAA	0	44
MS + BA + 2,4-D	0	32
SE	0.6	
LSD (5%)	1.8	

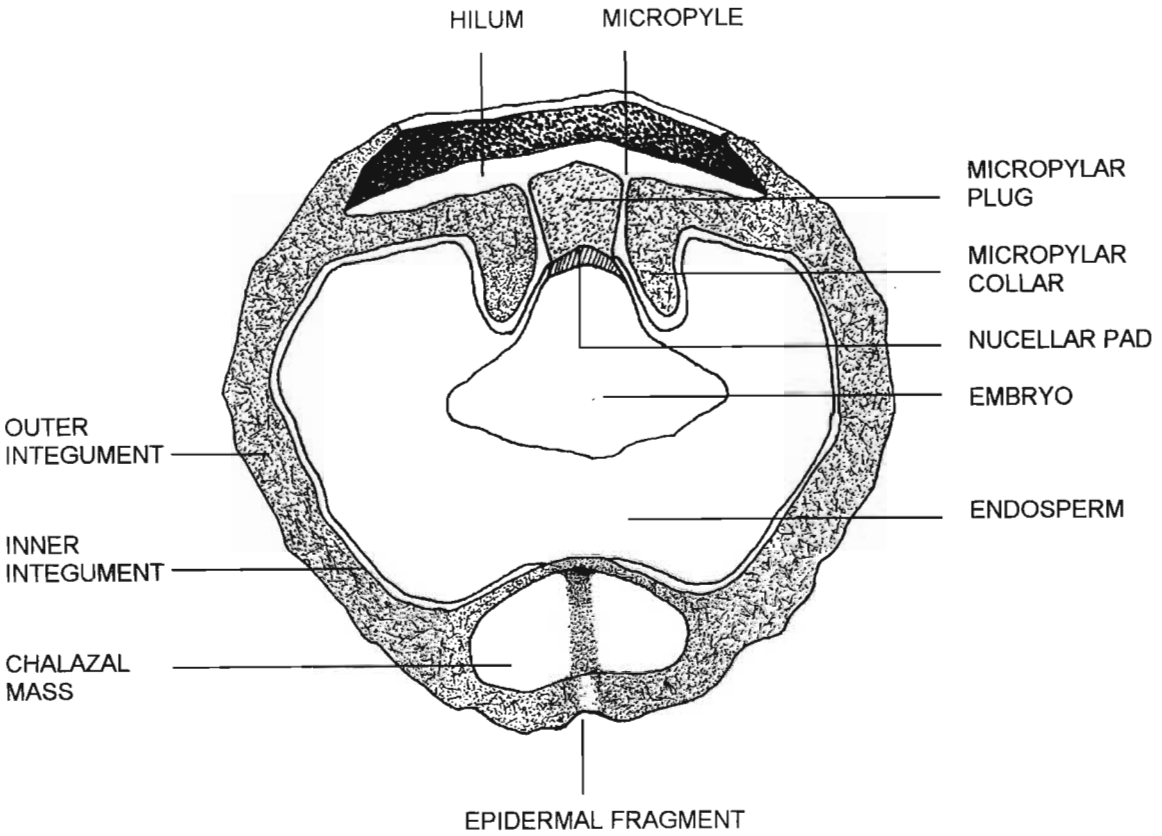

Figure 2.1: Longitudinal cross section of the seed of *Ensete ventricosum*

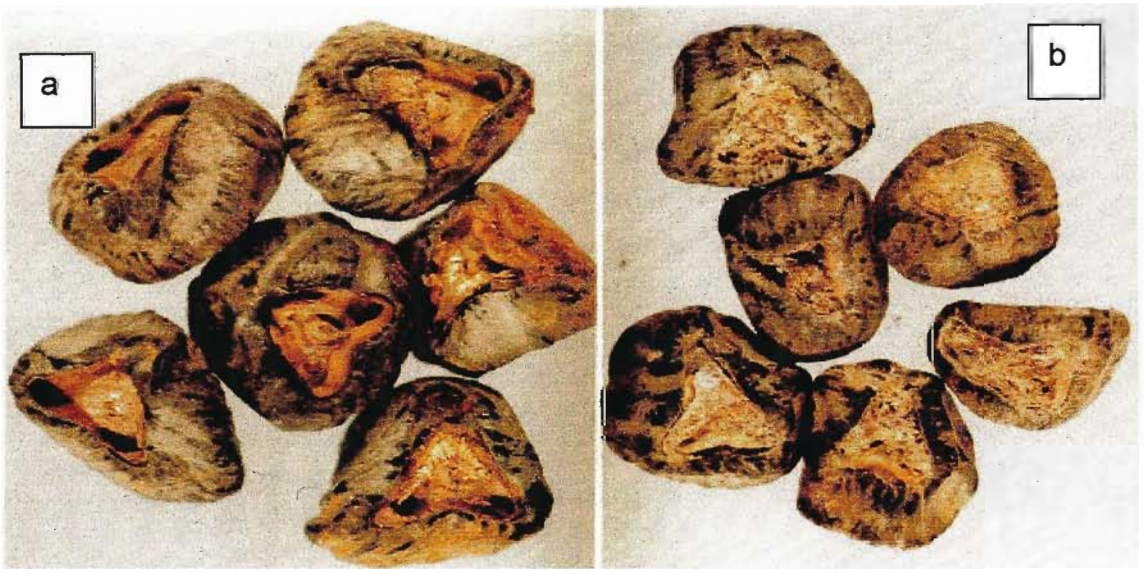

Figure 2.2: Seeds of the two genotypes of *E. ventricosum*, used in the study after six years storage: (a) Seeds of genotype Mariya with exposed hilum; and b) Seeds of genotype Oniya where the hilum was not exposed

Table 2.2: Statistical significance of treatment effects on *in vitro* embryo germination of the two enset genotypes, Mariya and Oniya. MS medium supplemented with (mg l^{-1}) 0.5 BA + 0.2 IAA or 0.5 BA + 0.2 2,4-D

Treatment	F-probability level
Enset genotype (Gt)	<0.001 *
Embryo orientation (EO)	<0.001 *
Medium composition (MC)	<0.001 *
Gt x EO	<0.001 *
Gt x MC	<0.001 *
EO x MC	0.599 ns
Gt x EO x MC	0.599 ns

*- indicates significant treatment effect

ns- indicates non-significant treatment effect

F-probability level ≤ 0.05

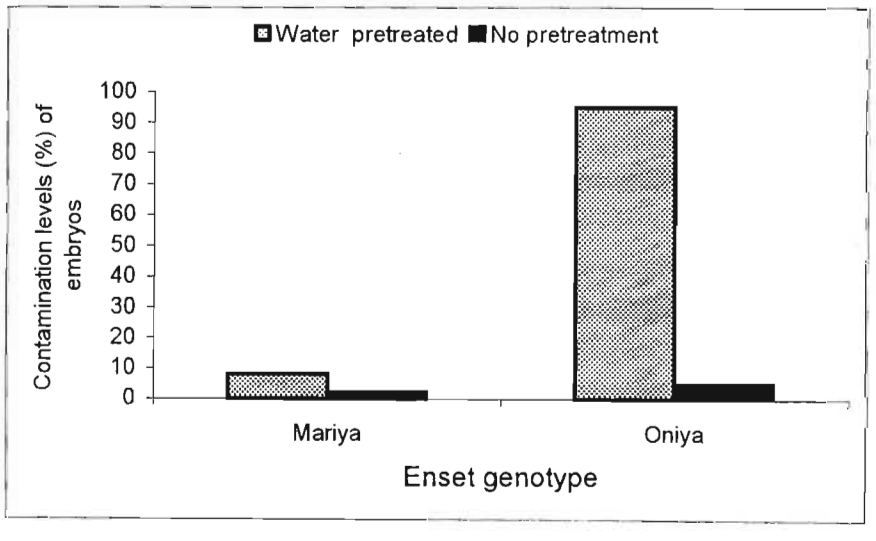

Figure 2.3: Contamination levels (%) of zygotic embryos cultured *in vitro* as influenced by water pretreatment of seeds for 30 min before seed decontamination for the two enset genotypes, Mariya and Oniya. SE= 3.0; and LSD (5%)= 6.9

The number of shoots per embryo, formation of multiple shoots per treatment, and vegetative growth of the seedlings (shoot height, number of leaves per shoot and length of root) were significantly influenced by medium composition (Table 2.3). Shorter shoots and roots and fewer leaves were recorded in the presence of BA + IAA, with on average 38% of the embryos producing multiple shoots (Table 2.4). Multiple shoots were also formed when medium without PGRs was used. The maximum number of shoots per embryo was three (average 1.7) from MS medium and eight (average 4.5) from BA + IAA-containing medium. There was an inverse relationship between formation of multiple shoots and growth of the seedlings (Table 2.5). As the number of shoots per embryo increased, shorter plants and roots and fewer leaves per shoot were obtained. Taller shoots produced more leaves and roots than the shorter ones. Formation of the multiple shoots resulted in reduced shoot size. However, about 90% of the multiple shoots grew into complete seedlings after separating and subculturing them individually. Inclusion of activated charcoal at a later stage to the medium improved growth of the shoots.

Table 2.3: Growth of shoots from embryos of enset genotype Oniya as affected by *in vitro* treatments (MC= medium composition, EO= embryo orientation and MC x EO). MS medium supplemented with (mg l^{-1}) 0.5 BA + 0.2 IAA or 0.5 BA + 0.2 2,4-D. NS/E= number of shoots per embryo; MSF= multiple shoot formation; SL= shoot length; NL/S= number of leaves per shoot; NR/E= number of roots per embryo; and RL= root length

Treatment	F-probability level					
	NS/E	MSF	SL	NL/S	NR/E	RL
MC	0.010 *	0.002 *	0.019 *	0.010 *	0.208 ns	0.021 *
EO	0.121 ns	0.056 *	0.231 ns	0.710 ns	0.157 ns	0.435 ns
MC x EO	0.055 ns	0.230 ns	0.230 ns	0.581 ns	0.593 ns	0.375 ns

* - indicates significant treatment effect

ns- indicates non-significant treatment effect

F-probability level ≤ 0.05

Table 2.4: Effect of medium composition on *in vitro* growth of shoots from embryos of genotype Oniya, three months after embryo culture. MS medium (MS) supplemented with (mg l^{-1}) 0.5 BA + 0.2 IAA or 0.5 BA + 0.2 2,4-D

Medium Composition (MC)	Number of shoots embryo^{-1}	Multiple shoot (%)	Shoot length (cm)	Number of leaves shoot^{-1}	Number of roots embryo^{-1}	Root length (cm)
MC1	1.7	15	3.1	3.2	2.7	1.6
MC2	4.5	37	1.8	2.2	2.7	0.5
MC3	1.0	0	7.7	4.7	4.2	4.0
SE	0.6	4	1.1	0.4	0.8	0.6
LSD (5%)	1.9	14	3.7	1.3	ns	2.2

ns- indicates non-significant difference between treatment means

Table 2.5: Associations of number of shoots per embryo and other shoot growth parameters for enset genotype Oniya. NS/E= number of shoots per embryo; SL= shoot length; NL/S= number of leaves per shoot; NR/E= number of roots per embryo; and RL= root length. n= 24

NS/E	1.000				
SL	-0.652 *	1.000			
NL/S	-0.616 *	0.666 *	1.000		
NR/E	-0.227 ns	0.823 *	0.348 ns	1.000	
RL	-0.565 *	0.699 *	0.785 *	0.322 ns	1.000
	NS/E	SL	NL/S	NR/E	RL

* - indicates significant correlations between growth parameters

ns- indicates non-significant correlations

t-probability≤ 0.05

2.3.3.2 Germination and shoot growth of embryos from seeds of W01 and W02 wild enset types

Medium composition with and without activated charcoal (AC) influenced *in vitro* germination of zygotic embryos of two wild types (W01 and W02) of *E. ventricosum* (Table 2.6). Higher germination rates of embryos (up to 88%) were obtained in this experiment though there were germination rates as low as 22% (Figures 2.4 and 2.5). Embryos of W02 gave significantly better germination rates than that of W01 on MS medium without PGRs and with and without AC (MC1). Thirty four percent of embryos of W01 germinated on MS medium without both PGRs and AC but this germination rate increased to 63% when the medium was supplemented with 0.5 mg l^{-1} BA + 0.2 mg l^{-1} IAA and 5 g l^{-1} AC. The germination rate (70%) of embryos of W02 on MS medium without PGRs and AC (MC1) increased to 80% when the medium was supplemented with 0.5 mg l^{-1} BA + 0.2 mg l^{-1} IAA and 5 g l^{-1} AC but the difference was not statistically significant. This shows the differences in the response of genotypes (W01 and W02). The lower germination rates of embryos of W02 on medium supplemented with (mg l^{-1}) 1.5 BA + 2 2,4-D (MC5) or 1.5 BA + 2 2,4-D + 0.2 IAA (MC6) were dramatically increased by the presence of AC in the media. The lower embryo germination rates of W02 on M5 and M6 can partly be attributed to the effects of increased levels of BA and 2,4-D in the absence AC because some of the embryos gave rise to callus without producing radicles and epicotyls, which was used as the criterion for germination.

Blackening rate was higher when media without AC was used while on the medium with AC 75% of explants did not blacken (Table 2.7). Formation of callus was influenced by medium composition, AC and genotype (Table 2.8). Callus was usually formed at the base of the shoots but occasionally formed from embryos with the absence of growth of radicle-epicotyl parts on the medium without AC.

Table 2.6: Effect of activated charcoal (AC) and medium composition (MC) on *in vitro* germination and seedling growth of two wild genotypes (Gt) (W01 and W02) of *E. ventricosum*. MC1= MS without PGRs; MC2= MS supplemented with (mg l^{-1}) 0.5 BA + 0.2 IAA; MC3= 0.5 BA + 0.2 2,4-D; MC4= 0.5 BA + 0.2 IAA + 0.2 2,4-D; M5= 1.5 BA + 2 2,4-D or M6= 1.5 BA + 2 2,4-D + 0.2 IAA; + AC= 5 g l^{-1} AC; and -AC= without AC. EG= embryo germination; SL= shoot length; NL/S= number of leaves per shoot; RL= root length; and NR/S= number of roots per shoot

Treatment	Parameter and F-probability level				
	EG	SL	NL/S	RL	NR/S
AC	0.037 *	<0.001 *	0.742 ns	<0.001 *	<0.001
Gt	0.005 *	0.013 *	0.742 ns	<0.001 *	0.002 *
MC	0.194 ns	0.046 *	0.005 *	0.140 ns	0.687 ns
AC x Gt	<0.001 *	0.015 *	0.028 *	<0.001 *	<0.001 *
AC x MC	<0.001 *	0.036 *	0.002 *	0.170 ns	0.143 ns
Gt x MC	<0.001 *	0.300 ns	0.119 ns	0.313 ns	0.554 ns
AC x Gt x MC	<0.001 *	0.406 ns	0.245 ns	0.224 ns	0.730 ns

*- indicates significant treatment effect

ns- indicates non-significant treatment effect

F-probability level ≤ 0.05

Shoot length and number of leaves per shoot were influenced by the interacting effects between AC and genotype and between AC and medium composition while root length and number of roots per shoot were influenced by interaction of AC and genotype. Better shoot length was obtained on MS medium without AC when the medium was supplemented with 0.5 mg l^{-1} BA + 0.2 mg l^{-1} IAA. The shortest shoot (3 mm) was recorded when MS medium without AC was supplemented with (mg l^{-1}) 1.5 BA + 2 2,4-D (M5) or 1.5 BA + 2 2,4-D + 0.2 (MC6), which was significantly improved by inclusion of AC. There was no root formation in the absence of AC and presence of BA + 2,4-D a month after embryo culture. Roots when formed on MS medium without PGRs and AC were short and black (Figure 2.5). During the same time an average of 6-8 roots per shoot with a length of 5-13 cm were

obtained by adding AC to the media (Tables 2.8 and 2.9). Roots produced in the presence of AC were white in color and healthy. Increased levels of BA and 2,4-D coupled with an absence of AC reduced growth of shoots and roots while promoting the formation of callus and blackening. Usually one leaf had unfolded per shoot within a month, but leaf growth was better when AC was included in the media.

Table 2.7: Effect of medium composition (MC) with and without activated charcoal (AC) on blackening of cultured zygotic embryos of two wild types (W01 and W02) of *E. ventricosum*. MC1= MS without PGRs; MC2= MS supplemented with (mg l^{-1}) 0.5 BA + 0.2 IAA; MC3= 0.5 BA + 0.2 2,4-D; MC4= 0.5 BA + 0.2 IAA + 0.2 2,4-D; MC5= 1.5 BA + 2 2,4-D or MC6= 1.5 BA + 2 2,4-D + 0.2 IAA; +AC= 5 g l^{-1} AC; and -AC= without AC

Activated charcoal (AC)	Medium composition (MC)	Percent of embryos growing without blackening	
		W01	W02
Without AC	MC1	25	33
	MC2	47	0
	MC3	56	67
	MC4	0	50
	MC5	32	100
	MC6	0	0
With AC	MC1	100	92
	MC2	75	100
	MC3	100	62
	MC4	76	100
	MC5	77	80
	MC6	88	100
SE		10.7	
LSD (5%)		31.3	

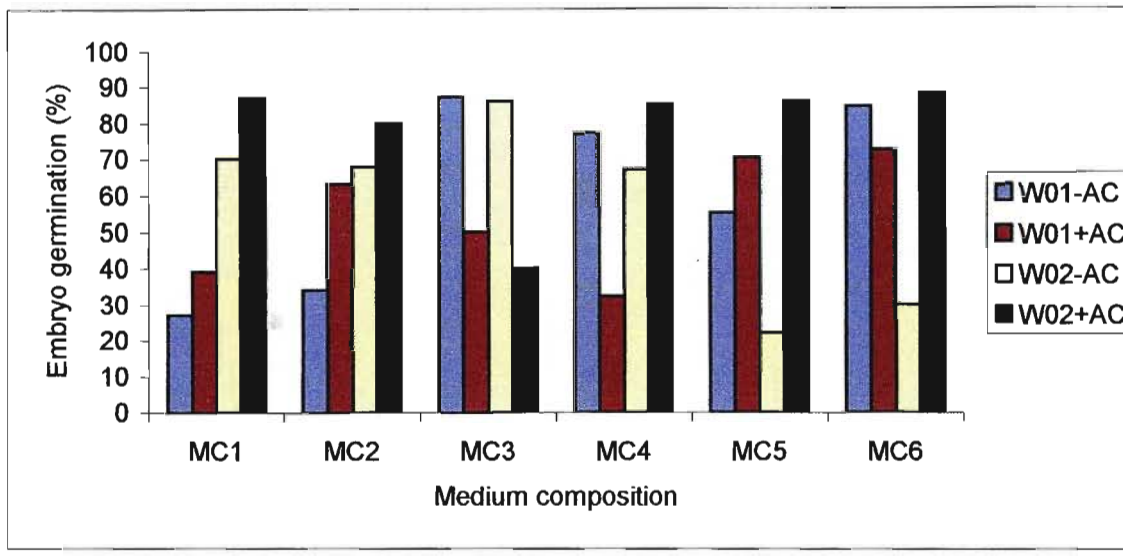

Figure 2.4: Effect of medium composition on germination of *in vitro* cultured embryos of two wild types (W01 and W02) of *E. ventricosum* with and without activated charcoal (AC), one month after embryo culture. MC1= MS without PGRs, MC2= MS supplemented with (mg l^{-1}) 0.5 BA + 0.2 IAA; MC3= 0.5 BA + 0.2 2,4-D; MC4= 0.5 BA + 0.2 IAA + 0.2 2,4-D; MC5= 1.5 BA + 2 2,4-D or MC6= 1.5 BA + 2 2,4-D + 0.2 IAA; + AC= 5 g l^{-1} AC; and -AC= without AC. SE= 7.7; and LSD (5%)= 22.5

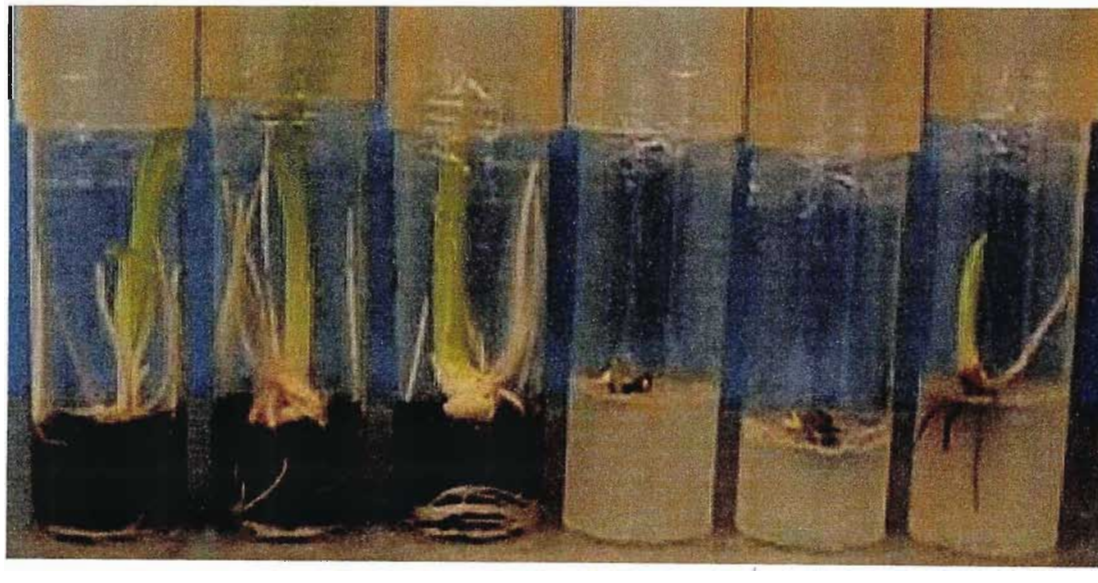

Figure 2.5: Effect of activated charcoal on growth of shoots and roots of *in vitro* seedlings from zygotic embryos of enset genotype W01 cultured on MS medium without plant growth regulators

Table 2.8: Effect of medium composition and activated charcoal (AC) on callus formation (%) and *in vitro* growth of seedlings, data averaged over two wild types of *E. ventricosum*, one month after embryo culture. MC1= MS without PGRs; MC2= MS supplemented with (mg l^{-1}) 0.5 BA + 0.2 IAA; MC3= 0.5 BA + 0.2 2,4-D; MC4= 0.5 BA + 0.2 IAA + 0.2 2,4-D; MC5= 1.5 BA + 2 2,4-D or MC6= 1.5 BA + 2 2,4-D + 0.2 IAA; +AC= 5 g l^{-1} AC; and -AC= without AC

Activated charcoal (AC)	Medium composition (MC)	Callus formation (%)	Shoot length (cm)	Number of roots $shoot^{-1}$	Number of leaves $shoot^{-1}$
Without AC	MC1	4	3.4	5	1
	MC2	49	5.0	2	0
	MC3	100	2.0	0	0
	MC4	94	2.2	0	2
	MC5	88	0	0	0
	MC6	100	0	0	0
With AC	MC1	0	5.5	6	1
	MC2	19	3.7	6	0
	MC3	6	4.6	7	1
	MC4	12	2.9	7	0
	MC5	37	4.7	8	1
	MC6	19	3.9	6	1
SE		8.5	0.9	1.4	0.2
LSD (5%)		24.7	2.7	4.2	0.6

Some of the seedlings from cultured embryos were used for micropropagation studies while others were acclimatized in a mist house when they attained lengths of 7-12 cm and produced three to five leaves and same roots per shoot *in vitro*. Vermiculite in pots was used as a medium for their growth. Hydroponic nutrient powder was dissolved in water (1 g powder per 1 l water) and applied once a week. Three weeks later, the seedlings were taken to the greenhouse and fertilized as before. After another three weeks, the seedlings were transferred to a mixture of soil compost: sand: peat: vermiculite (13:3:1:1 v/v), respectively.

Table 2.9: Effect of activated charcoal on growth of *in vitro* seedlings from zygotic embryos of two wild types (W01 and W02) of *E. ventricosum*, one month after embryo culture

Activated charcoal (AC)	Genotype	Shoot length (cm)	Root length (cm)	Number of leaves shoot^{-1}
Without AC	W01	2.1	1.4	0
	W02	2.1	0.5	1
With AC	W01	2.8	5.0	1
	W02	5.6	12.6	1
SE		0.5	0.6	0.1
LSD (5%)		1.5	1.8	0.4

The seedlings were watered every day for the first week and three to four times per week later. Some seedlings died after being transferred to the greenhouse. Nevertheless 75% final establishment was achieved.

2.4 Discussion

The elongating radicle-epicotyl parts of the enset embryos in the intact seeds, which were observed by rupturing some of the seeds, could not push through the micropylar collar to grow out through the micropylar opening and as a result the embryos died. Similarly, STOTZKY and COX (1962) reported a condition where embryos could not emerge through the micropylar canal. As the chalazal mass was removed in their study the embryos emerged in an abnormal way from the bottom of the seed rather than through the micropylar canal. This suggests the imposition of mechanical dormancy by the hard seed coat in enset and bananas (Musaceae). Mechanical dormancy is when the seed coat is too strong to allow embryo expansion during germination though water may be absorbed (HARTMANN and KESTER 1990). Water reached the embryos of enset in these studies when the seeds were soaked in water even for 30 min. Uptake of water by intact seeds of enset was also reported (BEZUNEH 1971). Musaceae seeds

permitted water to reach the embryo but did not result in a higher percentage of germination (GRAVEN *et al.* 1996). From these results, it became clear that the hard seed coat of enset does not totally block imbibition of water as water enters into the seed through the micropylar opening.

During excision of embryos from seeds of wild types of enset growth of microorganisms was observed on some of the embryos inside the seeds though the seed coats were intact, such embryos were discarded. However, 17% of the embryos excised from the seeds decontaminated for 30 min, looking visually clean at inoculation, were contaminated. The contamination became visible from the surface of the embryos a day after inoculation. Seeds of wild enset were collected after the mother plants fell on the ground due to age and fruits began decomposing. This may have contributed to higher rates of contamination. Decontamination of embryos in general was suggested when the seed coats are cracked or pathogens are known to exist within the seed coats (HU and WANG 1986). Decontaminating embryos of W02 for 5 min in 3.5% sodium hypochlorite caused total failure of embryo germination while it avoided contaminants, showing that at this concentration and time length, sodium hypochlorite was lethal to the embryos. Therefore further work is needed to reduce/avoid contamination without losing viability of embryos, beginning from maturity of fruits, harvesting and storage of the seeds.

Mature embryos generally require only inorganic salts supplemented with sucrose, whereas immature embryos have an additional requirement for vitamins, amino acids, growth regulators and sometimes an endosperm extract (GEORGE and SHERRINGTON 1984, HU and WANG 1986). However, supplementing MS medium with BA + IAA improved germination of embryos and shoot growth in stored seeds of Oniya and that of wild type W01. MS without PGRs resulted in higher germination of embryos of genotype W02. Similarly, use of BA did not significantly improve germination of *Musa acuminata* embryos (ASIF *et al.* 2001). Based on the genotype and length of time after harvesting, inclusion of lower concentrations of BA and IAA could be beneficial to embryo germination and shoot growth. BA (1.5 mg l^{-1}) and 2,4-D (2 mg l^{-1}) in the absence of AC promoted callusing. Inclusion of AC into the media avoided callus formation and reduced

blackening while it promoted germination of embryos in the presence of PGRs and growth of the shoots. Therefore, AC appeared to be a remedy for failure of root formation and growth, blackening and unwanted callus formation in embryo culture of enset, which were reported as constraints (BEZUNEH 1980). When used in tissue culture charcoal can assist in adsorbing toxic substances, which may be present in media as a result of autoclaving or are produced by cultured tissues, preventing unwanted callus growth and promoting root formation where some of the beneficial effect seems to be due to its ability to provide a dark environment (GEORGE and SHERRINGTON 1984). However, it was also mentioned that AC might adsorb PGRs. When the objective is to regenerate healthy shoots from zygotic embryos of enset, the use of AC was very beneficial.

Horizontal placement of the embryos that increased *in vitro* germination of enset embryos also improved that of *Musa balbisiana* embryos (AFELE and De LANGHE 1991). Exposing part of the haustorium appeared to favour embryo germination. Multiple shoot formation was observed in these studies. It was reported that MS medium with 0.45 mg l^{-1} BA + 0.17 mg l^{-1} IAA resulted in clumping of shoots (NEGASH *et al.* 2000) in germination of enset zygotic embryos. BEZUNEH (1980) described the basic morphology of the enset embryo, which has similarity with the embryo of *Musa* (McGAHAN 1961a). The embryo has a mushroom shape, which is characteristic of the Musaceae. The enlarged cap like portion, the haustorium, is the principal part of the cotyledon while the stalk-like portion, as well as a part of the cotyledon (McGAHAN 1961a) represents the epicotyl-hypocotyl-radicle axis. The stalk-like portion of the embryo is located within the micropylar collar.

CHAPTER THREE

MICROPROPAGATION OF *ENSETE VENTRICOSUM* FROM SHOOT TIPS OF *IN VITRO* GERMINATED ZYGOTIC EMBRYO SEEDLINGS

3.1 Introduction

Enset (*Ensete ventricosum* (Welw.) Cheesman) constitutes a sustainable agricultural system in south and southwestern Ethiopia. There, it is a source of staple food, feed and fiber. Products from enset are used in different forms in a traditional medicine. Nevertheless, cultivation of enset is constrained by various diseases such as enset wilt caused by *Xanthomonas campestris* pv *musacearum*, pests and abiotic factors. Enset germplasm is currently maintained in a field genebank and at risk of diseases, pests and adverse environmental conditions. Tissue culture techniques may enhance propagation, germplasm conservation and genetic improvement of this crop. Micropropagation through shoot tip culture offers many advantages over conventional propagation methods such as higher rates of multiplication, production of clean or disease free plant material. Plantlets of micropropagated banana and plantain establish more quickly, grow more vigorously and taller, have a shorter and more uniform production cycle and produce higher yields than conventional propagules (DREW and SMITH 1990, ROBINSON *et al.* 1993, VUYLSTEKE 1998).

In enset micropropagation, it was found difficult to initiate shoot tips *in vitro* from greenhouse grown suckers because of extensive blackening and unwanted callus formation (AFZA *et al.* 1996, MORPURGO *et al.* 1996, ZEWELDU 1997), which was addressed by the use of *in vitro* grown seedlings in the present work. The involvement of the callus phase in the micropropagation process may result in more occurrence of somaclonal variation (GEORGE and SHERRINGTON 1984). *In vitro* germinated zygotic embryo seedlings of enset were used as a source of explants along with greenhouse-grown material (NEGASH *et al.* 2000). However, advantages of using the *in vitro* grown seedlings of enset to avoid or reduce the difficulties encountered with the greenhouse material was not elaborated. In addition to the source of explants, composition of medium plays a major role in

increasing the number of propagules. The most critical component of the culture medium at the multiplication stage is a high level of cytokinin to promote axillary shoot proliferation (NEHRA and KARTHA 1994). Among various cytokinins used, BA is the most commonly employed at this Stage of culture for different species. The use of 2.25 or 4.5 mg l^{-1} BA *in vitro* was reported as a better treatment to reduce apical dominance of enset corms and induce growth of multiple shoots (NEGASH *et al.* 2000). However, the monopodial nature of the enset corm needs to be considered. Generally detailed information is lacking on enset shoot tip culture. In the present study, a differential response of shoot tip explants from *in vitro* grown seedlings and greenhouse grown suckers was addressed; a protocol for more rapid *in vitro* multiplication of *E. ventricosum* from shoot tips was developed; and effects of plant growth regulators (PGRs) to release lateral buds from apical dominance was documented in greater detail.

3.2 Materials and Methods

3.2.1 Plant material

Shoot tips of *in vitro* grown seedlings of enset genotype Oniya were used to conduct different experiments on *in vitro* multiplication. In one experiment *in vitro* response of shoot tips from *in vitro* grown seedlings was compared with shoot tips from greenhouse grown suckers. The *in vitro* grown seedlings were aseptically germinated from zygotic embryos and used after they attained a length of three to six cm. When the seedlings were used after further multiplication and maintenance *in vitro*, they were subcultured for three to four weeks onto MS medium devoid of PGRs prior to use for experimentation to reduce carrying over effects of PGRs. The suckers were vegetatively propagated in the greenhouse and used when they were six-months-old attaining heights of 50 to 90 cm. About 10-12 mm shoot tips were used intact, without injuring the apical domes, or mainly after splitting longitudinally into two through the apex.

3.2.2 Preparation and maintenance of gelled culture medium

MURASHIGE and SKOOG (MS) (1962) basal medium was used in all the experiments with 30 g l^{-1} sucrose and 7 g l^{-1} activated charcoal (AC). The pH of the medium was adjusted with KOH/HCl to 5.8 prior to autoclaving. The gelling property of agar decreased in the presence of AC and as a result 11 g l^{-1} agar was used. The medium was autoclaved at 121 °C for 20 min. Test tubes (25 x 100 mm) with 10 ml medium and jars with 30 ml medium were used based on size of the explants and regenerating plantlets. Usually the test tubes were used for inoculation and first subculture and the jars subsequently to further grow the plantlets. The explants were inoculated onto the medium under aseptic conditions, in the laminar flow hoods. Completely Randomized Design (CRD) was employed in all the experiments. Cultures were maintained in a growth room at 26 ± 1 °C under a 16 h light/8 h dark unless specified.

3.2.3 Multiplication of shoots in vitro

3.2.3.1 Effect of source of shoot tips on regeneration of plantlets in vitro for enset genotype Oniya

Shoot tips from two sources, *in vitro* grown seedlings and greenhouse grown suckers, were used. The shoot tips from suckers were first initiated *in vitro* for 5 weeks and used in the experiment at a multiplication stage. For the initiation, six-month-old suckers were uprooted from the greenhouse and trimmed to 2-3 cm long shoot tips. These explants were decontaminated for 15 min in 3.5% sodium hypochlorite with 2 drops of Tween 20 and rinsed three times with sterile distilled water. They were then reduced to 8-10 mm in length and inoculated onto MS medium supplemented with 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA + 7 g l^{-1} AC and gelled with 11 g l^{-1} agar. The shoot tips used for the initiation were intact, without injuring the apical domes. Two weeks later, shoots were subcultured onto MS medium containing AC without PGRs. After 3 weeks of subculture (5 weeks after inoculation), shoot tips from the initiated shoots were used to carry out the experiment along with the shoot tips from *in vitro* grown seedlings. The shoots from both sources were shortened to 10-12 mm and were split longitudinally into

two through the apex. The halved shoot tips were inoculated separately onto MS medium supplemented with 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA + 7 g l^{-1}AC and gelled with 11 g l^{-1} agar. The two sources of explants formed two treatments to be compared. Sixteen test tubes per treatment, one explant per test tube, were arranged in CRD.

3.2.3.2 Effect of type of shoot tips and plant growth regulators on in vitro *regeneration of multiple shoots for enset genotype Oniya*

Shoot tips from *in vitro* regenerated seedlings of zygotic embryos were used either intact or after splitting into two. The shoot tips were trimmed to length of about 10 mm and randomly divided into two groups. The shoot tips in the first group were used intact, without injuring the apical domes. The ones in the second group were split longitudinally through the apex to have two explants, which were cultured separately. The explants were inoculated onto MS medium supplemented with two compositions of PGRs (mg l^{-1}): 2.5 BA + 1 IAA or 2.5 BA + 1 NAA. Two types of shoot tips and two types of PGRs formed 4 treatments. Eight test tubes per treatment, one explant per test tube, were arranged in CRD. The cultures were subcultured on the same medium compositions one month after inoculation. After another month, the regenerating shoots and buds were subcultured on MS medium devoid of PGRs.

3.2.3.3 Effect of medium composition on in vitro *multiplication of shoot tips of enset genotype Oniya*

Shoot tips of *in vitro* germinated seedlings were cut to about 10 mm in length and were then split longitudinally into two through the apex. The halved shoot tips were inoculated onto 12 compositions of medium. The compositions of medium were: MS medium without PGRs; MS medium supplemented with (mg l^{-1}): 2.5 BA; 5 BA; 10 BA; 1 IAA; 2.5 BA + 1 IAA; 5 BA + 1 IAA; 10 BA + 1 IAA; 1 NAA; 2.5 BA + 1 NAA; 5 BA + 1 NAA; or 10 BA + 1 NAA. Ten test tubes per treatment, one explant per test tube, were used in CRD. The cultures were subcultured on the same media composition one month after inoculation and on MS medium without PGRs after one more month.

3.2.3.4 Effect of high concentration of benzyladenine on in vitro *induction of multiple shoots from shoot tips of enset genotype Oniya*

Shoot tips of *in vitro* regenerated seedlings of enset genotype Oniya were prepared in such a way that the apical domes were not mechanically injured during explant preparation. About 10 mm long shoot tips were excised and inoculated onto MS medium supplemented with seven concentrations of benzyladenine (BA). The BA concentrations were: 0, 10, 20, 40, 60, 80 or 100 mg l^{-1}. The zero concentration of BA represented medium without PGRs. With the rest of the BA concentrations, 1 mg l^{-1} IAA was used. Ten test tubes per treatment, one explant per test tube, were arranged in CRD.

3.2.3.5 Use of liquid medium for in vitro *multiplication of shoots from shoot tips of enset genotype Oniya*

Shoot tips were prepared from *in vitro* grown shoots of enset that were obtained from zygotic embryos. About 10 mm long shoot tips were split longitudinally into two through the apex to obtain two explants. Compositions of medium were: MS medium + no AC + no shaking ($MSAC_0Sh_0$); MS medium + AC + no shaking ($MSAC_1Sh_0$); MS medium + no AC + shaking ($MSAC_0Sh_1$); MS medium + AC + shaking ($MSAC_1Sh_1$); MS medium + AC + agar ($MSAC_1$ agar); MS media that were supplemented with 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA were: BA + IAA + no AC + no shaking ($BIAC_0Sh_0$); BA + IAA + AC + no shaking ($BIAC_1Sh_0$); BA + IAA + no AC + shaking ($BIAC_0Sh_1$); BA + IAA + AC + shaking ($BIAC_1Sh_1$); or BA + IAA + AC + agar (BIAC1 agar). For the liquid media 5 g l^{-1} AC was used and cultures were shaken, based on the treatment, at about 10 rpm while for the gelled media 7 g l^{-1} AC and 11 g l^{-1} agar were used. The explants were inoculated into the eight compositions of liquid media and onto two types of gelled media. Ten Erlenmeyer flasks per treatment, 15 ml liquid medium in each flask with one explant per flask, were used. Ten jars per treatment, with 25 ml medium, were used for gelled medium. Shoots and buds from both liquid and gelled media were subcultured onto gelled medium a month after inoculation. They were allowed to grow for two months subculturing onto fresh media after a month. Thereafter they were acclimatized.

3.2.4 Acclimatization of in vitro *plantlets*

The plantlets from gelled medium were acclimatized in a mist house after they became 5-10 cm long with 3-4 leaves. Those obtained from the liquid medium were taller up to 15 cm with thicker pseudostems. Number of leaves was similar but wider for plantlets obtained from the liquid medium. During acclimatization, three media were used to compare establishment of the plantlets. These media were: sand, vermiculite and a mixture of compost: sand: peat: vermiculite at a ratio of 13:3:1:1 (v/v), respectively. Ten plantlets were planted onto each of the three media. Hydroponic nutrient powder was dissolved in water (1 g powder per 1 l water) and applied once a week. The macronutrients in the hydroponic nutrient powder were 65% nitrogen, 2.7% phosphorus, 13% potassium, 1% calcium, 2.2% magnesium and 7.5% sulphur while the micronutrients were 0.15% iron, 0.02% manganese, 0.02% boron, 0.005% zinc, 0.002% copper and 0.001% molybdenum. Moisture was provided by an automatic misting system. Two weeks later, the plants were taken to the greenhouse and fertilized as before. They were watered every day for the first week, three to four times per week for the second and third weeks. After three weeks in the same pot in the greenhouse, the plantlets were transferred to a mixture of compost: sand: peat: vermiculite at a ratio of 13:3:1:1 (v/v), respectively, into larger pots. In the larger pots, the plants were watered every day for the first week, three to four times per week in the second week and once per week thereafter.

3.2.5 Data collection and statistical analysis

Data on number of shoots produced per explant were collected. When shoot tips that were split longitudinally through the apex were used, the number of shoots from the two halves was used to indicate rate of multiplication per shoot tip. A regenerated organ from the explant was considered a shoot when it produced a rolled leaf, otherwise it was considered as a bud. Shoot length was measured from the longest shoot. Buds per explant, leaves per shoot and roots per explant were counted. Length of bud from high concentrations of BA and diameter of shoot from liquid medium were measured. Blackening was recorded based on a score: 0= no blackening; 1= slightly black; 2= moderately black; and 3= extensively black.

Usually both the explant and medium become black. However, because of the presence of AC blackening was recorded only for the explants. Data collected two months after inoculation of shoot tips onto gelled medium and one month into a liquid medium was used for statistical analysis. When the number of treatments was two, the data was subjected to two-sample t-test to generate statistical evidence of the treatment effect. When the number of the treatments was more than two, the data was subjected to analysis of variance using an F-test. Least significant difference (LSD) at a 5% probability was used to separate significant treatment means after the analysis of variance. Standard error of means (SE) was given. All the statistical analyses were performed using GenStat 5 Release 4.2.

3.3 Results

3.3.1 Effect of source of shoot tips on in vitro *regeneration of plantlets of enset genotype Oniya*

In this experiment, shoot tips were prepared from two sources: seedlings of *in vitro* germinated zygotic embryos referred to as *in vitro* grown seedlings and greenhouse grown suckers. Shoot tips from greenhouse grown suckers were first initiated *in vitro* for five weeks and compared with shoot tips from *in vitro* grown seedlings at a multiplication stage. The shoot tips from the two sources responded significantly differently in terms of blackening of the explants, number of shoots and buds per shoot tip, number of leaves per shoot and number of roots per explant (Table 3.1). Shoot tips from *in vitro* grown seedlings did not exhibit a blackening problem while those from suckers of the greenhouse exhibited an average blackening score of about two, showing explants were moderately black. However, when the proportion of explants under different scores of blackening was considered, 38% of the explants were not black while 62% were highly black, which usually resulted in necrosis and death of the explants. In terms of multiplication rates, mean shoots of 3.7 and buds of 3.5 per shoot tip were regenerated from the two halves of the shoot tip of *in vitro* generated seedlings while less than one shoot per shoot tip was regenerated from shoot tips of greenhouse grown suckers because 44% of the shoot tips died, mainly due to

blackening and thus necrosis. *In vitro* growth of the shoots from *in vitro* grown seedlings was also better.

Table 3.1: Effect of source of shoot tips of enset genotype Oniya on blackening of explants and growth of shoots *in vitro*, two months after shoot tips were cultured for multiplication. MS medium + 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA was used. Blackening score: 0= no blackening; 1= slightly black; 2= moderately black; and 3= extensively black

Parameter	Means of treatment from		t-probability level
	In vitro shoot tip	Greenhouse shoot tip	
Blackening	0	1.9	<0.001 *
Number of shoots shoot tip^{-1}	3.7	0.6	<0.001 *
Number of buds shoot tip^{-1}	3.5	0.5	0.017 *
Shoot length (cm)	4.6	0.7	<0.001 *
Number of leaves $shoot^{-1}$	1.7	0.6	0.008 *

*- indicates significant difference between the two treatment means

3.3.2 Effect of type of shoot tips and plant growth regulators on in vitro *multiplication of shoots of enset genotype Oniya*

Two types of shoot tips were prepared from seedlings of enset genotype Oniya that were regenerated *in vitro* from zygotic embryos. In the first type, shoot tips were prepared without injuring the apical dome. In the second, shoot tips were split longitudinally into two through the apex with the aim to get two explants from a shoot tip and to injure the apical dome. Both the intact and longitudinally cut shoot tips were explanted onto two types of medium compositions. These were: MS medium supplemented with (mg l^{-1}): 2.5 BA + 1 IAA (BI) or 2.5 BA + 1 NAA (BN). The results showed that interactions between PGRs and the type of shoot tip did not significantly influence the number of shoots from the shoot tips and length of the shoots (Tables 3.2 and 3.3). However, shoot tips when split

longitudinally through the apex and treated with BA + IAA produced 4.2 shoots per shoot tip (per two halves). The effect of BA + IAA was not significantly different from that of BA + NAA on the number of shoots and length of the shoot (Tables 3.2 and 3.4). The shoot tips that were explanted intact gave only one shoot per shoot tip while when split longitudinally into two, the two halves together produced 3.6 shoots, averaged over the two compositions of medium. BA at concentration of 2.5 mg l^{-1} did not cause any multiple shooting when apical buds were not mechanically injured. The intact shoot tips resulted in longer shoots than the split shoot tips as they quickly grew from the apical buds. A simple correlation coefficient between number of shoots and length of shoot (r= -0.432, n= 32) shows a significant negative relationship between the two parameters. There was *in vitro* hyperhydricity of some of the regenerated shoots.

Table 3.2: Statistical significance of the effect of plant growth regulators and type of shoot tips on number and length of shoots, two months after shoot tips were cultured for multiplication. Plant growth regulators (PGRs): BI= 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA; and BN= 2.5 mg l^{-1} BA + 1 mg l^{-1} NAA; and type of shoot tips (ST): intact or split shoot tips

Treatment	F-probability level	
	Number of shoots shoot tip^{-1}	Shoot length
PGRs	0.203 ns	0.302 ns
Shoot tip type (ST)	0.001 *	<0.001 *
PGRs x ST	0.203 ns	0.471 ns

*- indicates significant treatment effect

ns- indicates non-significant treatment effect

F-probability level $\leq$ 0.05

Table 3.3: Interaction effect of plant growth regulators and type of shoot tips on multiplication of shoots and length of shoots *in vitro*, two months after shoot tips were cultured for multiplication. Plant growth regulators (PGRs): BI= 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA; BN= 2.5 mg l^{-1} BA + 1 mg l^{-1} NAA and type of shoot tips: ST1= intact shoot tip; and ST2= two halved shoot tips

Treatment		Number of shoots	Shoot length
PGRs	Shoot tip (ST)	Shoot tip^{-1}	(cm)
BI	ST1	1.0	10.8
	ST2	4.2	3.3
BN	ST1	1.0	10.5
	ST2	3.0	1.9
SE		0.2	0.8
LSD (5%)		ns	ns

ns- indicates non-significant difference between the treatment means

Table 3.4: Effect of plant growth regulators or type of shoot tips on number of shoots per shoot tip and length of shoots. Plant growth regulators (PGRs): BI= 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA; BN= 2.5 mg l^{-1} BA + 1 mg l^{-1} NAA; and type of shoot tips: ST1= intact shoot tip; and ST2= two halved shoot tips

Treatment		Number of shoots shoot tip^{-1}	Shoot length (cm)
PGRs	BI	2.6	7.1
	BN	2.0	6.3
SE		0.2	0.5
LSD (5%)		ns	ns
Type of shoot tips			
	ST1	1.0	10.7
	ST2	3.6	2.6
SE		0.2	0.5
LSD (5%)		0.5	1.6

3.3.3 Effect of plant growth regulators on regeneration of multiple shoots and buds in vitro for enset genotype Oniya

Shoot tips of *in vitro* regenerated plantlets were split longitudinally into two through the apex. The halved shoot tips were inoculated onto 12 medium compositions. The medium compositions were: MS medium without plant growth regulators (PGRs) (MC1); MS medium supplemented with (mg l^{-1}): 2.5 BA (MC2); 5 BA (MC3); 10 BA (MC4); 1 IAA (MC5); 2.5 BA + 1 IAA (MC6); 5 BA + 1 IAA (MC7); 10 BA + 1 IAA (MC8); 1 NAA (MC9); 2.5 BA + 1 NAA (MC10), 5 BA + 1 NAA (MC11); or 10 BA + 1 NAA (MC12). Growing shoot tips *in vitro* in the presence of PGRs significantly influenced growth and development of normal shoots and total number of shoots and buds per shoot tip. All treatments used resulted in a statistically similar number of small and hyperhydric buds, length of shoot, number of leaves per shoot and number of roots per explant (Table 3.5).

Table 3.5: Statistical significance of the effect of medium composition on regeneration of shoots *in vitro* from shoot tips of enset genotype Oniya. Medium compositions were: MS medium without PGRs; MS medium supplemented with (mg l^{-1}): 2.5 BA; 5 BA; 10 BA; 1 IAA; 2.5 BA + 1 IAA; 5 BA + 1 IAA; 10 BA + 1 IAA; 1 NAA; 2.5 BA + 1 NAA; 5 BA + 1 NAA; or 10 BA + 1 NAA

Growth parameter	F-probability level
Number of normal shoots shoot tip^{-1}	0.010 *
Number of small and hyperhydric buds shoot tip^{-1}	0.292 ns
Number of total shoots shoot tip^{-1}	0.050 *
Shoot length	0.247 ns
Number of leaves $shoot^{-1}$	0.495 ns
Number of roots $explant^{-1}$	0.119 ns

*- indicates significant treatment effect

ns- indicates non-significant treatment effect

F-probability level ≤ 0.05

A mean of 5.2 shoots and buds per shoot tip was produced on MS medium without PGRs (MC1) when the shoot tips were cut in half and cultured (Figure 3.1). Halved shoot tips which were inoculated onto MS medium + 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA (MC6) produced 6.8 shoots and buds, which was not significantly different from the number obtained from the medium without PGRs. However, 4.6 normal shoots were obtained from MC6, which was significantly better than 2.2 normal shoots obtained from MC1.

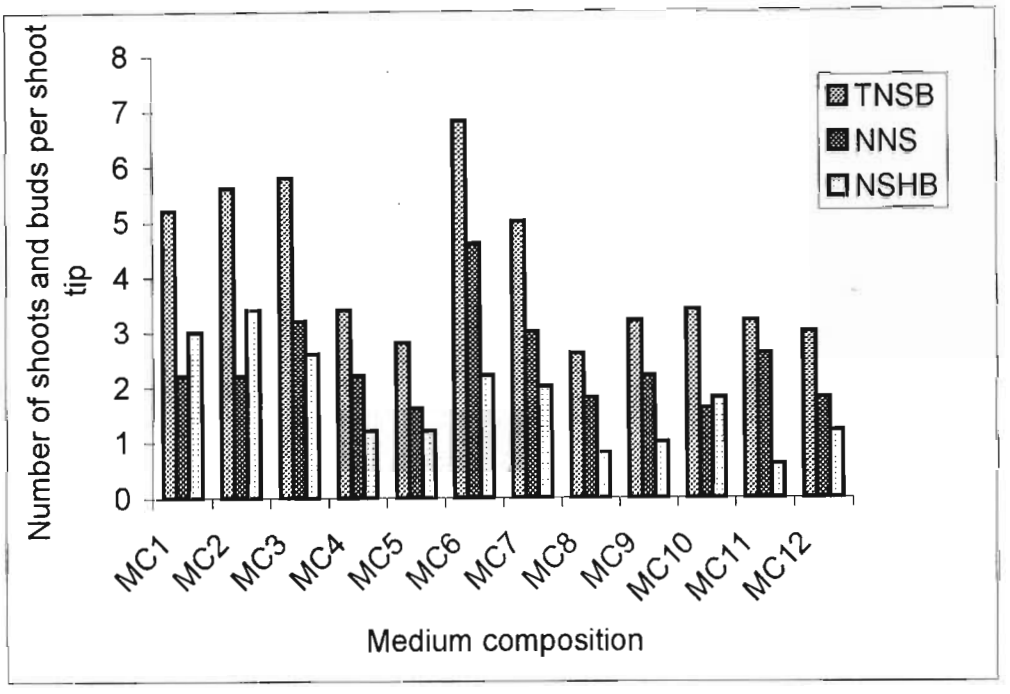

Figure 3.1: Total number of shoots and buds (TNSB), number of normal shoots (NNS) and number of small and hyperhydric buds (NSHB) per shoot tip produced on different medium compositions, two months after shoot tips were cultured for multiplication. The medium compositions were: MC1= MS medium without PGRs; MS medium supplemented with (mg l^{-1}): MC2= 2.5 BA; MC3= 5 BA; MC4= 10 BA; MC5= 1 IAA; MC6= 2.5 BA + 1 IAA; MC7= 5 BA + 1 IAA; MC8= 10 BA + 1 IAA; MC9= 1 NAA; MC10= 2.5 BA + 1 NAA; MC11= 5 BA + 1 NAA; MC12= 10 BA + 1 NAA. TNSB= total number of shoots and buds per shoot tip: SE= 1.0; and LSD (5%)= 2.9. NNS= number of normal shoots per shoot tip: SE= 0.5; and LSD (5%)= 1.5. NSVB= number of small and vitrified buds per shoot tip: SE= 0.8; and LSD (5%)= non-significant

Presence of 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA in the medium improved growth of shoots *in vitro*. Increasing concentrations of the BA from 2.5 to 5 or 10 mg l^{-1} with 1 mg l^{-1} IAA or NAA did not increase the number of shoots and buds. Wounding of the apical dome when shoot tips were cut longitudinally triggered production of adventitious buds without formation of callus (Figure 3.2a). For all shoot tips that were cut in half and cultured onto 12 compositions of medium, 17, 59, 15 and 9% produced 0, 1, 2 and 3 or more normal shoots per halved shoot tip, respectively. The majority of the split shoot tips (59%) gave rise to one complete normal shoot. Hyperhydricity, in which the regenerating buds were became translucent and turned a greenish callus (Figure 3.2b right), partly contributed to the lower number of normal shoots per shoot tip. A single shoot and multiple shoots were produced from halved shoot tips (Figure 3.2b). Lengths of shoots ranged from 2.3 to 5.1 cm, number of leaves per shoot from 1.2 to 2.3 and number of roots per explant from 1.4 to 3.4 (Table 3.6). However, the differences between their means were non-significant.

Table 3.6: Effect of medium composition on *in vitro* growth of shoots regenerated from shoot tips of *in vitro* grown seedlings of enset genotype Oniya. The medium compositions were: MC1= MS medium without PGRs; MS medium supplemented with (mg l^{-1}) BA and IAA or NAA: MC2= 2.5 BA; MC3= 5 BA; MC4= 10 BA; MC5= 1 IAA; MC6= 2.5 BA + 1 IAA; MC7= 5 BA + 1 IAA; MC8= 10 BA + 1 IAA; MC9= 1 NAA; MC10= 2.5 BA + 1 NAA; MC11= 5 BA + 1 NAA; or MC12= 10 BA + 1 NAA

Medium composition (MC)	Shoot length (cm)	Number of leaves $shoot^{-1}$	Number of roots $explant^{-1}$
MC1	3.5	2.2	1.0
MC2	2.9	2.3	1.5
MC3	5.1	3.4	1.2
MC4	3.6	3.0	0.3
MC5	2.8	1.4	0.2
MC6	4.5	2.7	0.9
MC7	4.2	2.8	0.9
MC8	4.4	2.8	0.4
MC9	3.3	1.9	0.3
MC10	3.8	2.4	0.7
MC11	3.9	2.3	0
MC12	2.3	1.5	0.5
SE	0.7	0.5	0.4
LSD (5%)	ns	ns	ns

ns- indicates non-significant difference between the treatment means

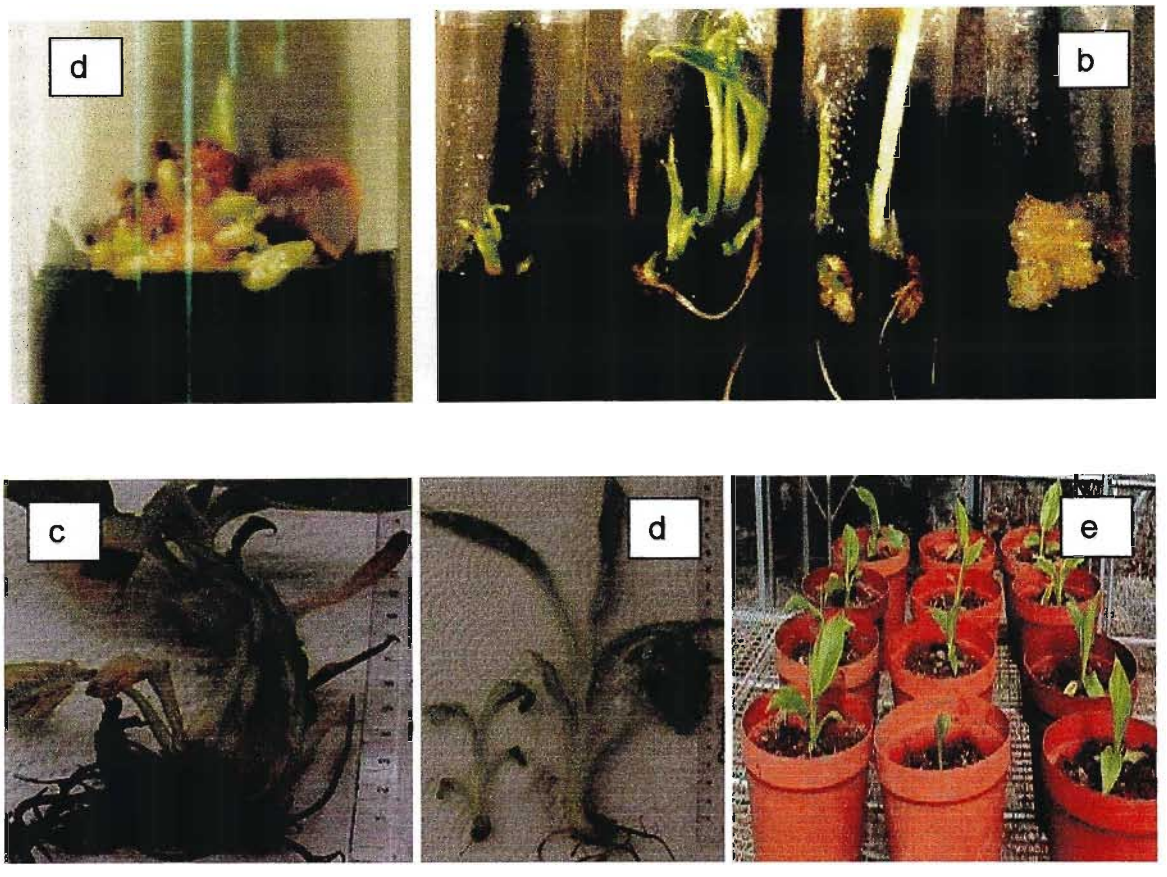

Figure 3.2: Micropropagation from shoot tips of enset genotype Oniya: a) Formation of buds from a halved shoot tip, one month after inoculation; b) Shoots of the same age from halved shoot tips showing differences in response: multiple shoots and hyperhydric buds converted to greenish callus, two months after inoculation, subcultured after a month; c) Multiple shoots per halved shoot tip from a liquid medium showing thicker pseudostems, three months after inoculation, subcultured every month; d) Comparison between shoots from gelled medium (small) and from a liquid medium (bigger), three months after inoculation; e) Plantlets that were acclimatized and established in the greenhouse, after about two months *ex vitro*. Explants in Figures a and b were cultured on MS medium + 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA; c and d were cultured on MS medium without PGRs. Seven g l^{-1} AC and 11 g l^{-1} agar were used

3.3.4 Effect of concentration of benzyladenine on multiplication of shoots in vitro *from shoot tips of enset genotype Oniya*

In this experiment, intact shoot tips, without mechanical injury to the apical domes, of genotype Oniya were excised and cultured on MS medium supplemented with 0, 10, 20, 40, 60, 80 or 100 mg l^{-1} BA. A high BA concentration was used in order to avoid or reduce apical dominance of enset shoot tips. All growth parameters such as number of shoots and buds, length of shoots and buds, number of leaves and roots from regenerated shoots were not significantly influenced by the BA concentrations (Table 3.7). The effect of BA on avoiding or reducing the inhibitory effect of apical dominance on lateral buds was non-significant.

Table 3.7: Statistical significance of the effect of BA concentrations on regeneration of shoots and buds *in vitro* from intact shoot tips of enset genotype Oniya, two months after shoot tips were cultured for multiplication. BA concentrations: 0, 10, 20, 40, 60, 80 and 100 mg l^{-1}

Growth parameter	F-probability level
Total number of shoots and buds shoot tip^{-1}	0.137 ns
Number of normal shoots shoot tip^{-1}	0.709 ns
Number of small and hyperhydric buds shoot tip^{-1}	0.253 ns
Shoot length	0.798 ns
Bud length	0.378 ns
Number of leaves $shoot^{-1}$	0.754 ns
Number of roots $explant^{-1}$	0.434 ns

ns- indicates non-significant treatment effect at F-probability level ≤ 0.05

The maximum multiplication rate was 1.7 shoots and buds per shoot tip, which was obtained at 40 mg l^{-1} BA (Figure 3.3). Of this, only 24% was normal shoots while the rest (76%) were small and hyperhydric buds that never grew into normal shoots. Most of the treatments resulted in 1.0 to 1.3 shoots and buds per shoot tip with poorly growing buds (Table 3.8).

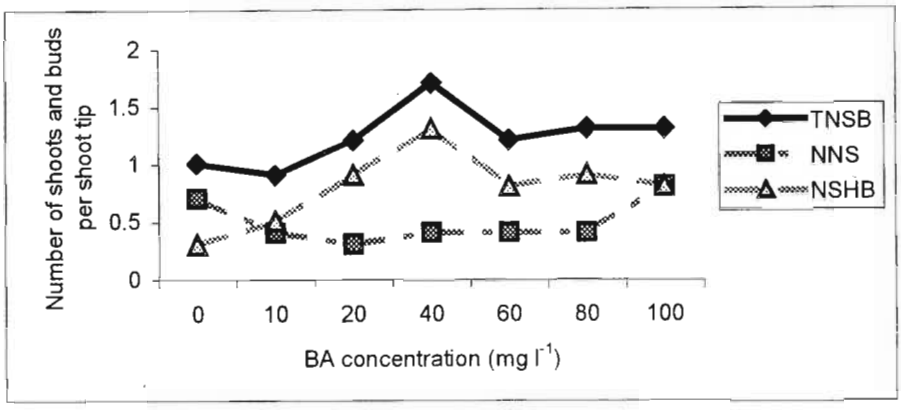

Figure 3.3: Trends of regeneration of multiple shoots/buds *in vitro* from intact shoot tips of enset genotype Oniya as effects of different concentrations of BA, two months after culturing shoot tips on BA-containing medium. BA 0 represents MS medium without PGRs; with other BA concentrations, 1 mg l^{-1} IAA was used. TNSB= total number of shoots and buds per shoot tip; NNS= number of normal shoots per shoot tip; and NSHB= number of small and hyperhydric buds

Table 3.8: Effect of BA concentrations on growth of shoots and buds *in vitro* from shoot tips of enset genotype Oniya, two months after culturing shoot tips on BA-containing medium. BA concentrations: 0, 10, 20, 40, 60, 80 and 100 mg l^{-1}. BA 0 represents MS medium without PGRs; with other BA concentrations, 1 mg l^{-1} IAA was used

Treatment (BA mgl^{-1})	Shoot length (mm)	Bud length (mm)	Number of leaves shoot^{-1}	Number of roots explant^{-1}
0	45.5	0.8	2.1	4.0
10	29.5	1.4	1.3	3.9
20	24.0	1.2	0.8	2.7
40	33.5	2.8	1.1	2.6
60	32.5	1.5	1.5	2.8
80	24.5	1.1	1.4	3.1
100	48.5	0.7	1.5	3.6
SE	1.3	0.1	0.5	0.6
LSD (5%)	ns	ns	ns	ns

ns- indicates non-significant difference between means of treatments

At BA 0, MS without PGRs, a greater number of normal shoots than small and hyperhydric buds was obtained. However, with all BA concentrations, a larger number of small and hyperhydric buds than normal shoots was obtained. All shoot tips grown with BA (10, 20, 40, 60, 80 and 100 mg l^{-1}) *in vitro*, 3% died, 72% gave rise to one shoot or bud from the apical bud while only 25% produced one or more lateral buds in addition to the apical bud. The lateral buds (Table 3.8) when produced were dominated by the shoots or buds that grew from the apical buds and were small in size.

3.3.5 Effect of liquid medium on blackening of explants and growth of shoots and buds in vitro

The use of liquid medium was one of the efforts to identify a suitable medium type and composition for multiplication of enset *in vitro*. The treatments were: MS medium + no AC + no shaking ($MSAC_0Sh_0$); MS medium + AC + no shaking ($MSAC_1Sh_0$); MS medium + no AC + shaking ($MSAC_0Sh_1$); MS medium + AC + shaking ($MSAC_1Sh_1$); MS medium + AC + agar ($MSAC_1$+ agar); MS medium supplemented with 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA were: BA + IAA + no AC + no shaking ($BIAC_0Sh_0$); BA + IAA + AC + no shaking ($BIAC_1Sh_0$); BA + IAA + no AC + shaking ($BIAC_0Sh_1$); BA + IAA + AC + shaking ($BIAC_1Sh_1$); and MS medium + BA + IAA + AC + agar ($BIAC_1$ + agar). There was a significant effect of the media combinations on blackening of explants and growth of shoots and buds (Table 3.9).

In the presence of AC, there was no blackening of explants on both gelled media ($MSAC_1$ + agar and $BIAC_1$ + agar). In the liquid medium, on MS medium without PGRs and with BA + IAA, the presence of AC without shaking reduced blackening of explants more than shaking without AC (Table 3.10). More shoots and buds were obtained when liquid medium was shaken in the presence of AC with or without PGRs or when the medium was gelled in the presence of BA + IAA + AC. Longer shoots were obtained from shaken liquid medium in the presence of AC. This difference was not significant. However, significantly thicker pseudostems of shoots were obtained when liquid medium was shaken in the presence of AC. A

mean pseudostem diameter of 1.2 to 2.2 mm was obtained when shoots were grown in liquid medium for a month as compared to 0.5 mm from gelled media.

Table 3.9: Statistical significance of effect of liquid medium on blackening and growth of shoots and buds *in vitro* from shoot tips of enset genotype Oniya, one month after inoculation of shoot tips

Parameter	F-probability level
Blackening of explant	<0.001 *
Total number of shoots and buds shoot tip^{-1}	<0.001 *
Number of shoots shoot tip^{-1}	0.008 *
Number of buds shoot tip^{-1}	<0.001 *
Length of shoot	0.057 ns
Diameter of shoot	0.007 *
Number of leaves $shoot^{-1}$	0.198 ns
Number of roots $explant^{-1}$	0.001 *

*- indicates significant treatment effect
ns- indicates non-significant treatment effect
F-probability level ≤ 0.05

Growth of the shoots from gelled and liquid media was evaluated after growing for two more months on gelled MS medium without PGRs (Table 3.11 and Figures 3.2c, d). Two-sample t-test showed that there were significant differences between the two groups in the means for shoot growth parameters. The results clearly showed that shoots from the liquid media had thicker pseudostems and more roots.

Table 3.10: Blackening of explant and growth of shoots and buds *in vitro* from shoot tips of enset genotype Oniya cultured in a liquid medium. Medium compositions: $MSAC_0Sh_0$= MS medium + no AC + no shaking; $MSAC_1Sh_0$= MS medium + AC + no shaking; $MSAC_0Sh_1$= MS medium + no AC + shaking; $MSAC_1Sh_1$= MS medium + AC + shaking; MSAC1 + agar= MS medium + AC + agar; MS medium supplemented with 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA: $BIAC_0Sh_0$= BA + IAA + no AC + no shaking; $BIAC_1Sh_0$= BA + IAA + AC + no shaking; $BIAC_0Sh_1$= BA + IAA + no AC + shaking; $BIAC_1Sh_1$= BA + IAA + AC + shaking; and BIAC1 + agar= MS gelled medium + BA + IAA + AC. AC= activated charcoal; BI= blackening of explants; NS= number of shoots per shoot tip; NB= number of buds per shoot tip; TSB= total number of shoots and buds per shoot tip; SL= shoot length; PD= pseudostem diameter; NL= number of leaves per shoot; and NR/E= number of roots per explant. Blackening was scored: 0= no blackening; 1= slightly black; 2= moderately black; and 3= extensively black. Seven g l^{-1} AC and 11 g l^{-1} agar were used

Treatment	BI	NS	NB	TSB	SL (mm)	PD (mm)	NL/S	NR/E
$MSAC_0Sh_0$	0.7	1.3	1.5	2.8	6	0.5	0.3	0.3
$MSAC_1Sh_0$	0.2	0.7	1.0	1.0	6	0.5	0.3	0.3
$MSAC_0Sh_1$	2.4	0	0	0	0	0	0	0
$MSAC_1Sh_1$	0	3.3	4.0	7.3	15	1.6	0.8	0.8
$MSAC_1$+Agar	0	2.3	2.3	4.6	6	0.5	0.4	0.4
$BIAC_0Sh_0$	0.7	0.8	0.2	1.0	3	0.6	0.3	0.3
$BIAC_1Sh_0$	0.1	1.5	0	1.5	4	0.5	0.3	0.6
$BIAC_0Sh_1$	3.0	0	0.2	0.2	0	0	0	0.3
$BIAC_1Sh_1$	0.4	3.0	3.2	6.2	21	2.2	0.8	1.9
$BIAC_1$+Agar	0	2.5	3.5	6.3	6	0.5	0.5	0.8
SE	0.2	0.3	0.2	1.5	0.5	0.4	0.2	0.3
LSD (5%)	0.5	2.0	1.8	3.7	ns	1.2	ns	0.8

ns- indicates non-significant difference between means of treatments

Table 3.11: Effect of gelled and liquid medium on growth of shoots *in vitro* for enset genotype Oniya. Data from samples of two sets of shoots three months after inoculation of shoot tips. Shoots from gelled media were obtained from shoot tips that were halved longitudinally through the apex and grown for three months *in vitro* on gelled medium with monthly subculturing; shoots from liquid medium were obtained from the same type of shoot tips that were cultured for one month in a liquid medium and then transferred to a gelled medium where they stayed for two more months with monthly subculturing to a fresh medium

Growth parameter	Means of treatment		t-probability level
	Shoots from gelled medium	Shoots from liquid medium	
Shoot length (cm)	13.3	17.8	0.106 ns
Shoot diameter (cm)	0.4	0.7	0.003 *
Number of roots shoot^{-1}	2.3	5.8	0.037 *
Number of leaves shoot^{-1}	3.3	4.2	0.049 *
Leaf width (cm)	1.7	2.9	0.033 *
Leaf length (cm)	5.3	8.3	0.020 *

*- indicates significant difference between the two means of treatments
ns- indicates non-significant difference between the two means of treatments
t-probability level ≤ 0.05

3.3.6 Regeneration and acclimatization of plantlets

Shoots produced roots at the multiplication stage possibly because of the presence of AC. However to get vigorous shoots they were transferred to jars on MS medium without PGRs but with AC. Plantlets from a liquid medium grew better than those from a gelled medium. Vermiculite and a mixture of compost: sand: peat: vermiculite at a ratio of 13:3:1:1 (v/v), respectively, gave similar establishment (75-80%). Most of the plantlets from the gelled medium formed concentric wilting of their pseudostems at the point where the pseudostem was in contact with sand. More plantlets (64%) from the liquid medium established better

than the ones from the gelled medium (50%) on the sand. Liquid medium produced vigorous plantlets that resulted in better acclimatization and establishment. The plantlets were acclimatized and established (Figure3.2e) in the greenhouse.

3.4 Discussion

Shoot tips from *in vitro* grown seedlings did not become black in the presence of AC. Intact shoot tips from greenhouse grown suckers, without wounding the apical dome, were initiated for five weeks in the presence of AC where blackening was slight. As the experiment was conducted at a multiplication stage, shoot tips from initiated shoots were split longitudinally through the apex. This wounding to the shoot tips seemed to aggravate blackening and caused death of 44% of the explants from the greenhouse suckers even when AC was used. Blackening due to phenolic oxidation is a serious problem in tissue culture of enset where a relatively high amount of flavonoides were found when callus tissue was examined (ZEWELDU 1997). In terms of regeneration capacity, shoot tips of *in vitro* grown seedlings gave an average of 3.7 shoots and 3.5 buds per shoot tip (from two halves) while only 31% of shoot tips from suckers of the greenhouse regenerated, accounting for less than one shoot and bud per shoot tip. Adventitious buds and shoots were formed directly from the explant without an involvement of callus phase. Micropropagation can be achieved through the production of adventitious buds directly from the explant (MURASHIGE 1974). The advantage of *in vitro* grown seedlings of zygotic origin in multiplication of enset *in vitro* could be due to the absence of blackening and juvenile nature of the explants. Better *in vitro* response of *in vitro* germinated seedlings for clonal multiplication in general and for the Gramineae and Coniferae is known (HU and WANG 1986, HALL 1999). The problem associated with *in vitro* grown seedlings is that seeds from out breeders can be genetically heterogeneous, which can cause unwanted variation in *in vitro* responses.

Multiplication of shoots of *Ensete ventricosum in vitro* from shoot tips of *in vitro* grown seedlings was further investigated in different ways. Shoot tips were used intact or after splitting longitudinally through the apex; split shoot tips were

exposed to various types and levels of PGRs *in vitro*. Intact shoot tips, without wounding the apical domes, were grown with high concentrations of BA and the response of shoot tip explants to liquid medium was also studied. NEGASH *et al.* (2000) reported the *in vitro* regeneration of one to two shoots per shoot tip when 2.25 mg l^{-1} BA + 0.2 mg l^{-1} IAA was used. In the present study, only one shoot per shoot tip was regenerated when intact shoot tips were cultured on a medium supplemented with (mg l^{-1}) 2.5 BA + 1 IAA. This showed that BA at this concentration did not induce growth of any lateral buds. After splitting the shoot tip longitudinally into two and culturing them separately, there was formation of multiple shoots on MS medium without PGRs. Halved shoot tips were grown *in vitro* with 10 mg l^{-1} BA alone or with 1 mg l^{-1} IAA or NAA did not induce formation of shoots and buds better than MS medium without PGRs. The number of shoots and buds per shoot tip from shaken liquid medium without PGRs in the presence of 5 g l^{-1} AC was comparable with that obtained from the medium in the presence of 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA. Therefore, wounding the apical dome by splitting appears necessary to release lateral buds of enset from apical dominance. An average of 2.6 shoots per shoot tip per 4 week interval were reported as a maximum rate of *in vitro* multiplication of enset on MS medium supplemented with 4.5 mg l^{-1} BA + 0.2 mg l^{-1} IAA (NEGASH *et al.* 2000). In the present study, only buds that cannot be used for the next cycle of multiplication were regenerated at the end of the fourth week and elongation of buds to shoots took place after subculturing for another four weeks. Therefore, we found an average of 6.8 shoots and buds per shoot tip in eight weeks when the tips were split longitudinally through the apex and cultured on MS medium supplemented with 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA. Of these 68% were normal shoots and 32% were small and/or hyperhydric. The use of split shoot tips in multiplication of enset *in vitro* and sorting out the dominated and/or hyperhydric buds were not reported before.

When split shoots of *Musa* Saba and 'Pelipita' were placed on MS medium supplemented with 5 mg l^{-1} BA for continued propagation, an average of 16 shoots were obtained per culture after four to six weeks (JARRET *et al.* 1985). In the present work, when a split shoot tip of enset was placed on MS medium with 5 mg l^{-1} BA + 1 mg l^{-1} IAA an average of 5.8 shoots and buds per shoot tip were obtained within eight weeks. This is less than what was obtained from banana

shoot tips. NANDWANI *et al.* (2000) reported regeneration of up to 28 shoots from shoot tip explants of banana cv Basrai on modified MS medium supplemented with 100 mg l^{-1} BA + 3 mg l^{-1} kinetin (Kn) + 0.5 mg l^{-1} IAA. In enset we obtained only 1.3 shoots and buds per intact shoot tip on MS medium supplemented with 100 mg l^{-1} BA + 1 mg l^{-1} IAA. Of all shoot tips grown with BA (10 to 100 mg l^{-1}) only 23% produced one lateral bud and 2% produced two or more lateral buds in addition to the apical bud. The results, thus, showed that there was some effect of BA to induce growth of lateral buds but it was not to any appreciable degree. The multiplication rate of enset was much lower than that of bananas. A larger number of shoots from shoot tips of banana than from that of enset can be explained by the sympodial morphology of banana corms where suckering occurs in the field as opposed to the monopodial morphology of enset corms where no side shoot formation occurs in the field.

The use of a liquid medium increased vegetative growth of shoots and resulted in longer and thicker shoots and buds. Such growth might be due to the uniform distribution of nutrients and the dilution of toxic explant exudates. The thickening of the pseudostem was different from hyperhydricity that led to callus formation from shoot tips on gelled media. Because shoots obtained from the liquid medium maintained their relative thickness and length after they were transferred to gelled medium without PGRs. Addition of AC to the liquid medium was highly beneficial for growth of the shoots *in vitro*. Five g l^{-1} AC that was used in the liquid medium deposited on some of the shoot tips and created unfavourable condition. From the liquid medium, we found up to seven shoots and buds per shoot tip (two halves) of enset, from which three to five shoots grew uniformly. The use of liquid medium for *in vitro* multiplication of enset was not reported. However, the rate of multiplication obtained in the liquid medium was comparable with 6.6 shoots per shoot tip of banana (CRONAUER and KRIKORIAN 1984b). Shoot tips of enset obtained from *in vitro* grown shoots were small in size and some of the explants submerged to the base of the liquid media and died, perhaps from lack of aeration even though the medium was shaken. Therefore, interaction between size of explant, volume of the medium, amount of AC and time of transfer to gelled medium need further investigation. Because of better growth of shoots and conversion rate of buds to shoots, the use of a liquid medium seems promising. Shoots produced roots at

the multiplication stage even in the presence of BA. The plantlets were transferred to MS medium without PGRs and grown for one month prior to acclimatization. They were acclimatized for two weeks in the mist house and 75-80% were established in the greenhouse. At the greenhouse stage all plants seem normal and similar. It is difficult to identify off type at this stage.

The rate of multiplication of shoots of enset *in vitro* varied from 3.7 to 6.8 shoots and buds per shoot tip per two months on gelled MS medium + 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA while it went up to 7.3 on a liquid medium. If we consider only 3 shoots per shoot tip to be normal and complete from gelled medium, it is possible to multiply 6 cycles in a year with two months for a multiplication cycle, that gives 3^6 = 729 normal shoots per year. As it was possible to safely obtain 4 normal shoots per shoot tip from a liquid medium the number of shoots that can be produced from a single shoot per year can be estimated to be over 4000. Using conventional propagation methods, 70 suckers per corm were produced from three-year-old Halla enset genotype (DIRO *et al.* 2002). If 200 suckers per corm under conventional propagation is considered (BEZUNEH and FELEKE 1966), which is rarely obtained, the rate of *in vitro* propagation is much faster. Enset growers use two to six-year-old plants for propagation. This long cycle is not important with the clones in the hands of the growers because they usually maintain enset plants of different ages and stages in their homesteads and thus they can find plant material of their choice to propagate every year. So, it is possible to consider 200 suckers per year under conventional propagation for comparison purpose. However, *in vitro* multiplication can be beneficial when new cultivars are introduced. Plant material from disease/pest resistant clones is needed in large amounts. In addition, the protocol of *in vitro* multiplication can be used for *in vitro* enset germplasm conservation. The rate of *in vitro* multiplication may be improved with further investigation by reducing hyperhydricity and callusing of differentiated buds, improving growth of buds into shoots, increasing the rate of multiplication per shoot tip and shortening the length of multiplication cycle.

CHAPTER FOUR

IN VITRO REGENERATION FROM SHOOT TIPS OF GREENHOUSE-GROWN *ENSETE VENTRICOSUM*

4.1 Introduction

Shoot tips are used as explants in micropropagation of different plant species by encouraging lateral buds to grow and multiply. One of the advantages of using shoot tips is that better genetic fidelity can be ensured because of the organized nature of the explants. *In vitro* germinated seedlings are sometimes chosen as a source of shoot tips because they are juvenile and respond best. Likewise, shoot tips from seedlings of *in vitro* germinated zygotic embryos of enset (*Ensete ventricosum*) also responded better than those from greenhouse-grown suckers (Chapter three). However, when *in vitro* germinated seedlings are used out breeder seeds may cause difficulties in maintaining trueness to type. This variation can be avoided by using shoot tips from suckers that are multiplied in the greenhouse from a single mother plant. Moreover, enset is a perennial plant that produces seeds only after a long juvenile period, after more than four years even in the warm climate of Areka area where enset research is situated in Ethiopia (UNDP/ECA 1996). Therefore, the use of shoot tips from vegetatively multiplied mother plants that are grown in the greenhouse or in the field in enset micropropagation is of importance.

Different decontamination approaches were developed based on the source of the plant material for bananas (HAMILL *et al.* 1993). In enset, endogenous contaminants were reported after shoot tips were decontaminated once with sodium hypochlorite (ZEWELDU 1997). Therefore, it would be useful to generate information on decontamination procedures for both greenhouse and field-grown enset material to initiate aseptic culture. Blackening of explants, which was a problem in shoot tip culture of enset, was associated more with shoot tips from greenhouse-grown plants than with ones from *in vitro* germinated zygotic embryos (Chapter three). Because of injury to plant tissue during isolation of explants from stock plants, the metabolism of phenolic compounds is stimulated (DEBERGH and

READ 1991). In general, phenolics are labile products very easily oxidized. Many of these compounds are phytotoxic and will lead to death of plant tissue if released into cells (COLLIN and EDWARDS 1998). Blackening of shoot tips of enset, therefore, needs attention when regenerating microplants.

After initiation of aseptic shoot tip culture, multiplication of the propagules and regeneration of complete plants are important steps in micropropagation. The use of cytokinin such as BA and/or injury to the apical domes helps to reduce apical dominance and induce growth of lateral buds. Very limited information is available on the initiation and multiplication of shoot tips of *Ensete ventricosum in vitro* without having extensive blackening and callus formation (NEGASH *et al.* 2000). In the present work, different experiments were executed using mainly greenhouse-grown mother plants of two enset genotypes, Keberia and Mazia, to reduce/avoid blackening and formation of callus at the initiation and multiplication stages of shoot tip culture and to regenerate multiple shoots.

4.2 Materials and Methods

4.2.1 Plant material

Vegetative plant material of two enset genotypes, Keberia and Mazia, was obtained from Ethiopia and propagated in the greenhouses of the Research Centre for Plant Growth and Development, School of Botany and Zoology, University of Natal. Keberia is a popular enset clone due to its sweet corm after cooking and a better quality of processed product. Mazia is preferred for its tolerance to diseases and its higher yield. A mixture of compost: sand: peat: vermiculite at a ratio of 13:3:1:1 (v/v) was used to produce suckers in pots from the vegetative plant material. Three level teaspoons (about 10 g) hydroponic nutrient powder was dissolved in 5 l water and applied once a week to the suckers and soil under the suckers. The macronutrients in the hydroponic nutrient powder were 65% nitrogen, 2.7% phosphorus, 13% potassium, 1% calcium, 2.2% magnesium and 7.5% sulphur while the micronutrients were 0.15% iron, 0.02% manganese, 0.02% boron, 0.005% zinc, 0.002% copper and 0.001% molybdenum. The suckers were watered every week. There was aphid infestation in the greenhouses but it

was controlled by the application of Malathion (50% EC) at a rate of 6.25 ml per 5 l water sprayed until leaves were covered by the spray solution. The spraying was repeated when necessary. Greenhouse-grown 6-12 months-old suckers were used as a source of shoot tips for the experiments on *in vitro* propagation. One-year-old field-grown suckers were used within 8 days after uprooting from the experimental field of Areka Agricultural Research Centre, Ethiopia, for comparison of decontamination procedures.

4.2.2 Decontamination methods for shoot tip explants

This experiment was conducted to study the effect of decontamination method and explant size on contamination of cultures during the initiation of the shoot tips *in vitro*. Vegetatively multiplied suckers of enset genotype Mazia were uprooted from the greenhouse. Successive leaf bases were removed by hand. A block of tissue two to three cm in length was excised and decontaminated for 15 min in 3.5% sodium hypochlorite with two drops of Tween 20. The blocks were grouped into two. Those in the first group were rinsed three times with sterile distilled water, as a first decontamination method (D1). From blocks in the second group, bleached tissues were removed leaving about 1.5 to 2 cm long shoot tips. These blocks were again decontaminated in the same way as before for 5 min and rinsed three times with sterile distilled water, as the second decontamination method (D2). Explants of 5 mm (S1) and 10 mm long shoot tips (S2) were excised from the blocks that were decontaminated with the two methods outlined above. The explants were inoculated onto MS medium supplemented with (mg l^{-1}) 2.5 BA + 1 IAA. Sucrose of 30 g l^{-1} was added to the medium. The medium was gelled with 2 gl^{-1} gelrite. The pH was adjusted to 5.8 prior to autoclaving. The medium was autoclaved at 121°C for 20 min. Two decontamination methods (D1 and D2) and two sizes of explants (S1 and S2) formed 4 treatments. Fifteen test tubes, one explant per test tube, were arranged in a completely randomised design (CRD). The cultures were incubated in a growth room under a 16 h light/8 h dark at 26 ± 1 °C. Light irradiance of 40-43 μmol m^{-2} s^{-1} was provided by cool white fluorescent tubes.

The second experiment on the decontamination method and initiation of shoot tips was carried out using shoot tips of enset genotype Mazia from greenhouse and field-grown mother plants as explants. The shoot tips were decontaminated, following the steps in the first decontamination method (D1), for 15 min in 3.5% sodium hypochlorite with two drops of Tween 20 and rinsed three times with sterile distilled water. About 10 mm long shoot tips were excised and inoculated onto three compositions of media: MS medium without both plant growth regulators (PGRs) and activated charcoal (AC); MS medium without PGRs but with 7 g l^{-1} AC; and MS medium supplemented with 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA + 7 g l^{-1} AC. Eight g l^{-1} agar for AC free medium and 11 g l^{-1} agar for AC containing medium was used as a gelling agent. Two sources of explants and three compositions of medium formed 6 treatments. Fifteen test tubes, one explant per test tube, were arranged in CRD. The cultures were incubated in a growth room under a 16 h light/8 h dark at 26 ± 1 °C and an irradiance of 43 μmol m^{-2} s^{-1} as outlined above.

4.2.3 Effect of light regime, activated charcoal and ascorbic acid on blacking and growth of shoot tips of enset genotypes Keberia and Mazia in vitro *at the initiation stage*

Two to three cm long blocks of shoot tips were excised from greenhouse-grown suckers of enset genotypes Keberia and Mazia, without injuring the apical domes. The blocks of tissues were decontaminated for 15 min in 3.5% sodium hypochlorite with two drops of Tween 20 and rinsed three times with sterile distilled water. About 10 mm long shoot tips were excised aseptically from the decontaminated blocks of tissues and used as explants. A medium for explant inoculation was prepared without AC, with 7 g l^{-1} AC, without ascorbic acid and with 25 mg l^{-1} ascorbic acid. The shoot tips from both enset genotypes were inoculated onto the media. MS medium supplemented with 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA and gelled with 8 g l^{-1} agar in the absence of AC and with 11 g l^{-1} agar in the presence of 7 g l^{-1} AC. The factorial combination of the light regime, activated charcoal, ascorbic acid and enset genotype made a total of 16 treatments. Nine shoot tips per treatment, one shoot tip per test tube, were arranged in CRD and incubated under 16 h light/8 h dark conditions.

4.2.4 Multiplication of shoots in vitro *from initiated shoot tips of enset genotypes Keberia and Mazia*

4.2.4.1 Effect of medium composition and activated charcoal on in vitro *multiplication of shoots*

Shoot tips of enset genotypes Keberia and Mazia were initiated on MS medium supplemented with 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA in the presence of 7 g l^{-1} AC and gelled with 11 g l^{-1} agar. These initiated shoots, two months after first inoculation, were used in the multiplication experiment. Shoot tips 10-12 mm in length were prepared and split longitudinally through the apex to obtain two halves. The halved shoot tips of both genotypes were inoculated onto 4 medium compositions with and without AC. The medium compositions were: MS medium without PGRs; MS medium supplemented with (mg l^{-1}): 2.5 BA + 1 IAA; 5 BA + 1 IAA; or 10 BA + 1 IAA. The pH of the medium was adjusted to 5.8 prior to autoclaving and gelled with 11 g l^{-1} agar and autoclaved at 121 °C for 20 min. Two enset genotypes and 4 compositions of medium with and without AC constituted 16 treatments. Eight test tubes per treatment, each containing a halved shoot tip, were arranged in CRD. The cultures were incubated in a growth room under a 16 h light/8 h dark at 26 ± 1 °C.

4.2.4.2 Effect of decapitation of initiated shoot tips on multiplication of shoots in vitro

Shoot tips of enset genotypes Keberia and Mazia were initiated on MS medium supplemented with 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA + 7 g l^{-1} AC and gelled with 11 g l^{-1} agar. The shoots were subcultured after one month on MS medium without PGRs + 7 g l^{-1} AC and grew for another one month. Ten to 12 mm long shoot tips were excised from the shoots and split longitudinally into two through the apex. The apical meristems were carefully removed from the halved shoot tips for decapitation. The decapitated shoot tips were inoculated onto three medium compositions for multiplication. The medium compositions were MS medium without PGRs; MS medium supplemented with (mg l^{-1}) 2.5 BA + 1 IAA; or 1.5 BA + 3 IBA. To all the medium compositions 7 g l^{-1} AC was added and the medium was

gelled with 11 g l^{-1} agar. Two genotypes and three medium compositions constituted six treatments. Ten test tubes, each containing one decapitated shoot tip, were used per treatment in CRD.

4.2.4.3 Effect of liquid medium on formation of multiple shoots/buds in vitro

Shoot tips of *in vitro* grown shoots of two enset genotypes Keberia and Mazia were split longitudinally through the apex and inoculated into a liquid MS medium supplemented with 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA + 1 g l^{-1} AC. Six ml medium was used per jar and subcultured after two weeks to a fresh medium. One g l^{-1} AC was used. Jars instead of Erlenmeyer flasks were used because it was difficult to remove the shoot tips through the narrow neck of the flasks after they formed clumps of buds.

4.2.5 Data collection and statistical analysis

In the study of decontamination methods of explants, data on contamination was recorded as a binary response that took y= 0 when there was no contamination and y= 1 when culture was contaminated. Blackening of explants was recorded based on a score: 0= no blackening; 1= slightly black; 2= moderately black; and 3= extensively black. Because of the presence of AC in the treatments, blackening of the medium was not recorded. Callusing was also scored: 0= no callus formation, 1= swelling of the explant; and 2= callus formed. The number of shoots and buds per shoot tip was recorded to address regeneration of plantlets. When the shoot tips were split longitudinally through the apex, the two halves together were considered as a shoot tip. The regenerated organ was considered as a shoot when it developed a rolled leaf; otherwise it was recorded as a bud. Shoot length was measured from top of the corm to the tip of the longest leaf. The number of leaves per shoot and number of roots per explant were counted.

Since the binary response (y) from the experiments on decontamination methods had a Bernoulli's distribution the data was subjected to a linear logistic regression model following procedures of statistical modeling of binary response (McCONWAY *et al.* 1999). Fitted regression lines were obtained from estimates of

regression coefficients of the explanatory variables, the treatments. Based on the equation from the fitted regression lines, probability of having contamination was computed for the size of explant, method of decontamination and source of explant and interpreted. Other data were subjected to two-sample t-test for experiments with two treatments or to an F-test for analysis of variance for experiments with more than two treatments. When the treatment effects were statistically significant in the analysis of variance, means were separated with least significant difference (LSD) at a 5% probability level. Standard error of means (SE) was given along with means of the treatments. Simple correlation coefficients were computed to indicate the relationships between parameters. GenStat 5 Release 4.2 was used for the statistical analysis.

4.3 Results

4.3.1 Decontamination methods for shoot tip explants

Two sizes of shoot tip explants, 5 mm long and 10 mm long, of enset genotype Mazia from the greenhouse were decontaminated using two methods. First, explants were decontaminated for 15 min only once in 3.5% sodium hypochlorite. Second, the explants were decontaminated first for 15 min and again for 5 min in the sodium hypochlorite. The fitted linear logistic regression line for the effect of explant size and decontamination method on contamination of the culture, from Table 4.1, was: log (Ψ)= -2.315 + 0.777 S - 0.777 D, where Ψ= the odds ratio, S= shoot tip explant size and D= decontamination method. The logistic linear regression line for the effect of source of shoot tip explant on the contamination of culture was: log (Ψ)= -3.14 + 2.80 ES, where Ψ= the odds ratio and ES= explant source. Equations of these regression lines were used to calculate probabilities of having contamination (Table 4.2).

There was no effect of size of explant and decontamination method on probability of having contamination. This was indicated by the approximate x^2 probability of regression deviance of 0.468 and t-probability of 0.395 (Table 4.1), which are non-significant. When 10 mm long shoot tips were decontaminated only once for 15 min, the probability of having contamination was 0.177. This probability was

slightly higher than that of the other treatments but the difference is not significant. Repeating the decontamination procedure for 5 min (D2) caused slightly more death of shoot tips (Figure 4.1).

Table 4.1: Estimates of parameters from a linear logistic regression model on the contamination of cultures *in vitro* for shoot tips of enset genotype Mazia: a) Effect of shoot tip explant size and decontamination method; and b) Effect of shoot tip explant source on the contamination of culture. SE= standard error of means; and t (*)= t-value with corresponding levels of t-probability. S= explant size; D= decontamination method; and ES= explant source

Parameter	Estimate	SE	t (*)	t-prob	Antilog estimate
a) Constant	-2.315	0.796	-2.91	0.004 *	0.099
S	0.777	0.913	0.85	0.395 ns	2.174
D	-0.777	0.913	-0.85	0.395 ns	0.459
b) Constant	-3.14	1.02	-3.07	0.002 *	0.043
ES	2.80	1.10	2.54	0.011 *	16.430

*- indicates significant effect of the estimate
ns- indicates non-significant effect of the estimate
t-probability level ≤ 0.05

The number of shoots less than one shows death of some of the explants. The explants from greenhouse and field-grown mother plants were decontaminated once for 15 min in 3.5% sodium hypochlorite, rinsed three times with sterile distilled water and inoculated onto the medium. There was strong evidence of a regression effect with approximate x^2 probability of 0.001 that there was an effect of source of explant on probability of having contamination. The t-value of 2.54 shows significant contribution of source of explant to the probability of contamination of the culture.

Table 4.2: Effect of size of shoot tip explant and method of decontamination or source of shoot tip explant on the probability of having culture contamination for enset genotype Mazia, computed using the estimates in Table 4.1 in fitted linear logistic regression lines. Response variable was: y= 0, for no contamination; and y= 1, for contaminated culture. Explanatory variables were: shoot tip explant size (S): S1= 0, for 5 mm; and S2= 1, for 10 mm. Decontamination method (D): D1= 0, for explant decontaminated once (for 15 min); and D2= 1, for explant decontaminated twice (first for 15 min and repeated for 5 min). Shoot tip explant source (ES): ES1= 0, for shoot tip explants from greenhouse-grown suckers; and ES2= 1, for shoot tip explants from field-grown suckers

Treatment	Probability of having culture contamination, p (y= 1)
a) Explant size and decontamination method	
Five mm long explant, decontaminated once	0.090
Ten mm long explant, decontaminated once	0.177
Five mm long explant, decontaminated twice	0.043
Ten mm long explant, decontaminated twice	0.090
b) Explant source	
Greenhouse grown suckers	0.041
Field grown suckers	0.416

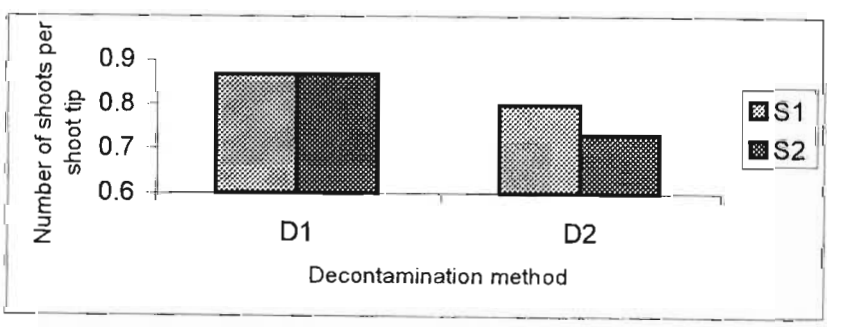

Figure 4.1: Number of shoots per shoot tip *in vitro* produced from explants of two sizes (S1 and S2) that were decontaminated in two ways (D1 and D2) for enset genotype Mazia. Length of shoot tip explant (S): S1= 5 mm; and S2= 10 mm. Decontamination method (D): D1= decontaminating once (for 15 min); and D2= decontaminating twice (first for 15 min and repeated for 5 min). SE= 0.1; and LSD (5%)= non-significant

When shoot tip explants from field-grown mother plants were used, the probability of culture contamination was 0.416. This high probability level could also be explained by the antilog of estimate that showed that there were 16 times more chance to obtain contamination with the explants from field-grown mother plants than with the ones from the greenhouse.

4.3.2 Effect of light regime, activated charcoal and ascorbic acid on blacking and growth of shoot tips in vitro *of enset genotypes Keberia and Mazia at the initiation stage*

There was an interaction effect of source of explant and composition of medium on blackening of shoot tip explants at the initiation stage (Table 4.3). When MS medium was used without plant growth regulators (PGRs) and activated charcoal (AC) (MC1) the explants both from greenhouse and field-grown suckers were extensively black (Figure 4.2). Addition of 7 g l^{-1} AC (MC2) significantly decreased blackening of the shoot tips obtained from greenhouse-grown suckers while it was less effective in reducing the blackening of shoot tips from field-grown suckers in the absence of PGRs.

Table 4.3: Statistical significance for the effect of source of explant and medium composition on blackening and growth of the shoot tips at the initiation stage, data recorded one month after inoculation of the shoot tips of genotype Mazia

Treatment	F-probability level		
	Blackening	Shoot number	Shoot length
Explant source (ES)	0.467 *	0.108 ns	0.090 ns
Medium composition (MC)	0.002 *	0.653 ns	0.061 ns
ES x MC	0.031 *	0.191 ns	0.041 *

*- indicates significant treatment effect

ns- indicates non-significant treatment effect

F-probability level ≤ 0.05

The number of shoots per shoot tip that survived was not influenced by the treatments. However, in most of the treatments some shoot tips died in the culture process and number of shoots was less than one per shoot tip from both sources of the shoot tips (Figure 4.3). The highest average shoot length was obtained on MC2 (MS medium with AC but without PGRs) for shoot tips culture from greenhouse-grown suckers.

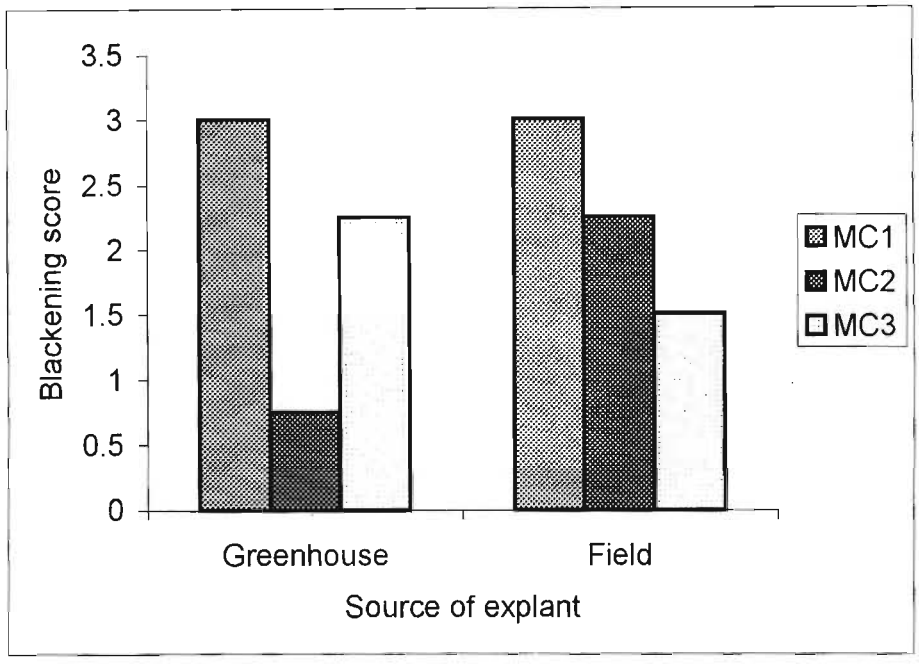

Figure 4.2: Effect of source of explant and medium composition on blackening of shoot tip explants of enset genotype Mazia at *in vitro* initiation stage. Medium compositions were: MC1= MS medium without AC and without PGRs; MC2= MS medium without PGRs + AC; and MC3= MS medium + AC + 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA. Blacking score: 0= no blackening; 1= slightly black; 2= moderately black; and 3= extensively black. Data recorded one month after inoculation of shoot tips: SE= 0.14; and LSD (5%)= 0.4

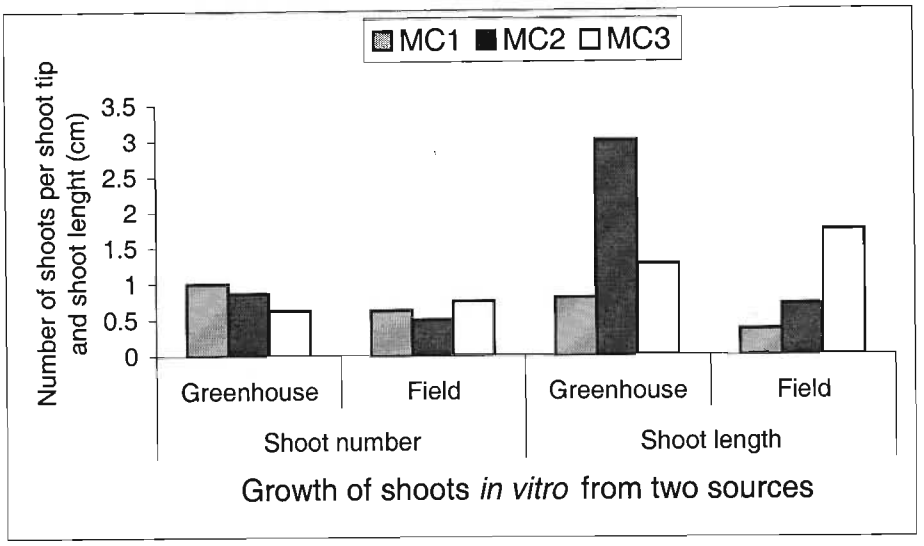

Figure 4.3: Growth of shoots in number and length *in vitro* from shoot tip explants from greenhouse and field-grown mother plants of enset genotype Mazia on different medium compositions. Medium compositions were: MC1= MS medium without PGRs and AC; MC2= MS medium without PGRs + AC; and MC3= MS medium + AC + 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA. For the shoot number: SE= 0.15; and LSD (5%)= non-significant. For the shoot length: SE= 0.54; and LSD (5%)= 1.53

From all the treatments tested against blackening only AC showed a statistically significant effect (Table 4.4). Addition of 7 g l^{-1} AC significantly reduced blackening of explants (Figures 4.4 and 4.10a, b). Inoculation of explants onto medium without or with 25 mg l^{-1} ascorbic acid or incubation of the culture in a growth room under a 16 h light/8 h dark or in the dark did not result in significantly different blackening scores of the explants of both genotypes. However, when the shoot tips were incubated in the light without AC, the number of shoots per shoot tip that survived was better when ascorbic acid was added to the medium than when it was excluded (Figure 4.5). AC irrespective of the light regime and ascorbic acid improved the number of shoots per shoot tip that survived for further subculture. In the absence of AC, shoot length was reduced when the culture was incubated in the light without ascorbic acid and in the dark with ascorbic acid. Differential responses of enset genotypes in terms of shoot length were observed (Figure 4.6). Genotype Mazia exhibited poor shoot growth, which was improved by inclusion of AC in the medium.

Table 4.4: Statistical significance for the effect of light regime, activated charcoal and ascorbic acid on blackening and initiation of shoot tips of enset genotypes Keberia and Mazia, one month after inoculation of the shoot tips. LR= light regime; Gt= genotype; AC= activated charcoal; and AA= ascorbic acid

Parameter	F-probability level			
	Blackening of explant	Callusing of explant	Number of shoots shoot tip^{-1}	Shoot length
LR	0.428 ns	0.746 ns	0.172 ns	0.831 ns
Gt	0.322 ns	<0.001 *	0.411 ns	0.027 *
AC	<0.001 *	<0.001	0.003 *	<0.001 *
AA	0.552 ns	0.746 ns	0.784 ns	0.494 ns
LR x Gt	0.114 ns	0.746 ns	0.784 ns	0.253 ns
LR x AC	0.843 ns	0.746 ns	0.784 ns	0.943 ns
Gt x AC	0.235 ns	<0.001 *	0.411 ns	0.018 *
LR x AA	0.235 ns	0.332 ns	0.003 *	0.848 ns
Gt x AA	0.843 ns	0.746 ns	0.411 ns	0.603 ns
AC x AA	0.235 ns	0.746 ns	0.411 ns	0.865 ns
LR x Gt x AC	0.076 ns	0.746 ns	0.057 ns	0.333 ns
LR x Gt x AA	0.114 ns	0.332 ns	0.784 ns	0.219 ns
LR x AC x AA	0.322 ns	0.332 ns	0.015 *	<0.001 *
Gt x AC x AA	1.000 ns	0.746 ns	0.172 ns	0.996 ns
LR x Gt x AC x AA	0.322 ns	0.332 ns	0.784 ns	0.780 ns

*- indicates significant treatment effect

ns- indicates non-significant treatment effect

F-probability level ≤ 0.05

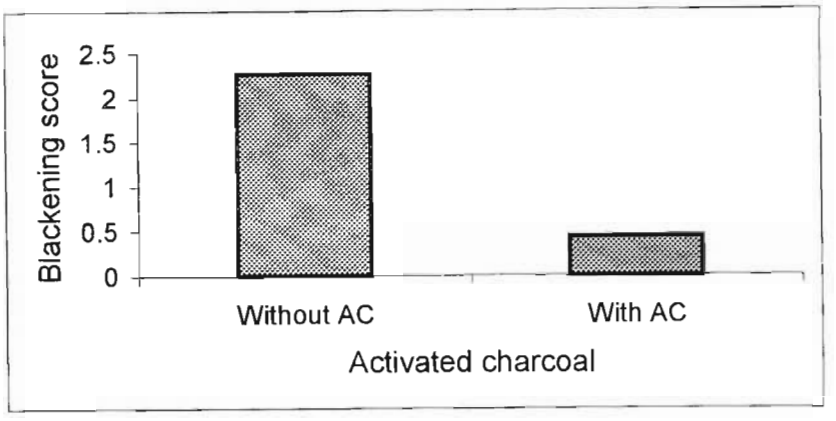

Figure 4.4: Effect of activated charcoal (AC) on blackening of shoot tip explants *in vitro* at the initiation stage, one month after inoculation of the shoot tips. Blackening score: 0= no blackening; 1= slightly black; 2= moderately black; and 3= extensively black. Data was averaged over two enset genotypes Keberia and Mazia. SE= 0.1; and LSD (5%)= 0.3

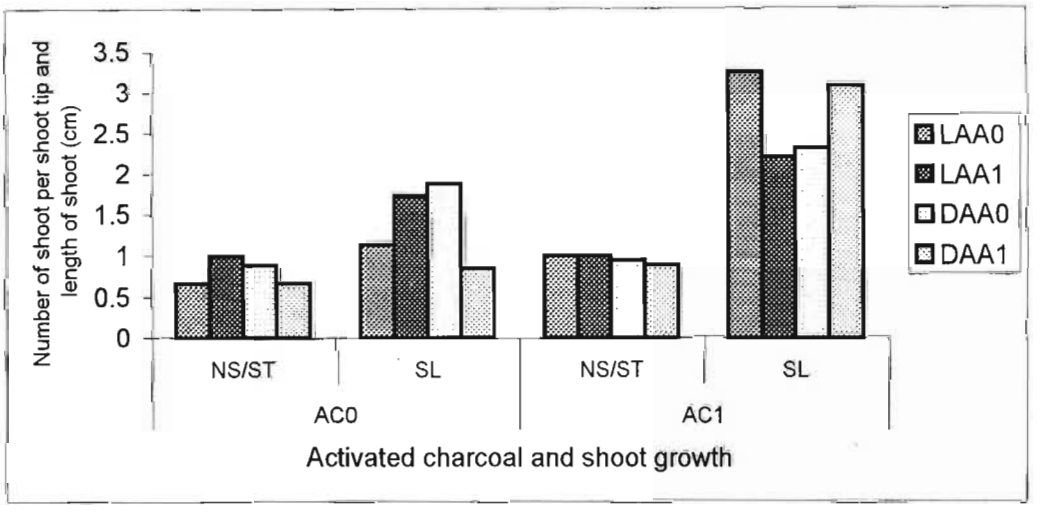

Figure 4.5: Effect of activated charcoal (AC), light regime and ascorbic acid on number of shoots per shoot tip and length of shoot at *in vitro* initiation stage. AC0= without activated charcoal (AC); AC1= with 7 g l^{-1} AC; L= light; D= dark; AA0= without ascorbic acid (AA); and AA1= with 25 mg l^{-1} AA. NS/ST= number of shoots per shoot tip; SL= Shoot length. Data was averaged over two enset genotypes Keberia and Mazia, one month after inoculation of the shoot tip. For NS/ST: SE= 0.07; and LSD (5%)= 0.2. For SL: SE= 0.36; and LSD (5%)= 1.0

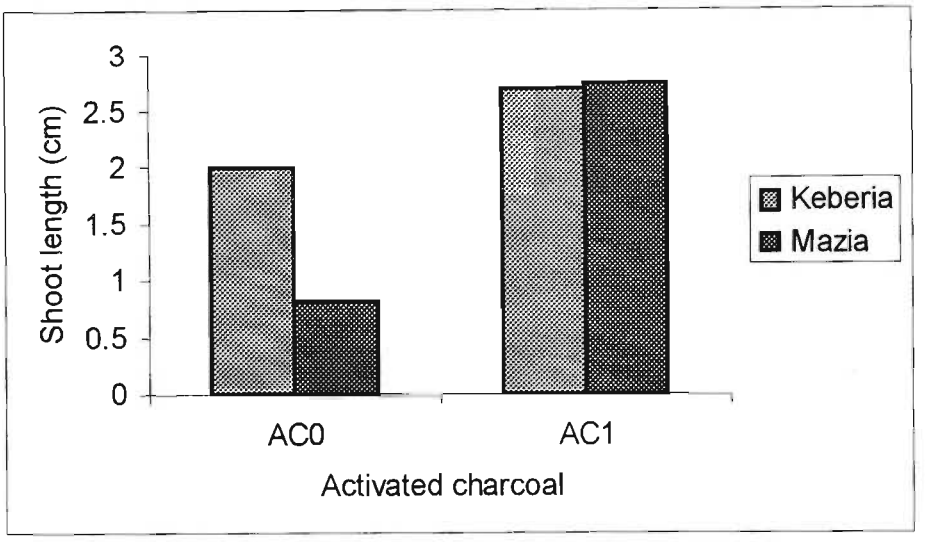

Figure 4.6: Effect of activated charcoal (AC) on shoot length of enset genotypes Keberia and Mazia *in vitro* at the initiation stage, one month after inoculation of the shoot tips. AC0= without activated charcoal (AC); and AC1= with 7 g l^{-1} AC. SE= 0.25; and LSD (5%)= 0.71

In the absence of AC, enset genotype Keberia grew faster than Mazia and produced a shoot length of about 2 cm on average while genotype Mazia produced less than 1 cm long shoots over two months. At the initiation stage, shoot tips of enset genotype Mazia became swollen *in vitro* when cultured on medium without AC, which was not observed at the initiation stage on explants of the same genotype in the presence of AC and on explants of genotype Keberia in both the absence and presence of AC (Table 4.5). Association between blackening, callusing and shoot growth parameters at the initiation stage is indicated by simple correlation coefficients (Table 4.6). Blackening of the explants positively associated to callus formation. This could partly be due to the occurrence of both blackening and callusing simultaneously in the absence of AC particularly with genotype Mazia. Increased blackening related to decreased number and length of shoot, which was evidenced by significant negative correlation coefficients, as blackening caused death of some of the shoot tips and poor growth of the others usually in the absence of AC.

Table 4.5: Effect of activated charcoal on callusing of shoot tip explants of two enset genotypes Keberia and Mazia at the initiation stage, one month after inoculation of the shoot tips. Callusing score: 0= no callusing; 1= swelling; and 2= callus formed

Genotype	Activated charcoal (AC)	Score for callus formation
Keberia	Without AC	0
	With AC	0
Mazia	Without AC	0.6
	With AC	0
SE		0.04
LSD (5%)		0.12

Table 4.6: Simple correlation coefficients showing association of blackening and callusing of shoot tip explants and growth parameters of shoots *in vitro* at the initiation stage. Bl= Blackening; Cl= Callusing; NS= Number of shoots per shoot tip; and SL= Shoot length

Bl	1.000			
Cl	0.169 *	1.000		
NS	-0.227 *	0.151 ns	1.000	
SL	-0.333 *	-0.335 *	0.441 *	1.000 ns
	Bl	Cl	NS	SL

*- indicates significant correlation

ns- indicates non-significant correlation

n= 144; t-probability level ≤ 0.05

4.3.3 Multiplication of shoots in vitro *from initiated shoot tips of enset genotypes Keberia and Mazia*

At the multiplication stage, when shoot tips were split longitudinally through the apex, blackening and callus formation of the explants were influenced by the interaction effect of activated charcoal (AC), enset genotype and medium

composition (Table 4.7). The number of shoots per shoot tip was influenced by the interaction effect of genotype with either AC or medium composition while shoot length was influenced by medium composition with either AC or genotype (Table 4.8). AC, genotype, medium composition or their interactions did not affect production of buds per shoot tip. The number of leaves and roots was affected by a combined effect of AC and medium composition. In the absence of AC, enset genotype Keberia was more black than genotype Mazia on MS medium with 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA (MC2).

Table 4.7: Statistical significance of the effect of composition of medium and activated charcoal on *in vitro* blackening and callusing of shoot tip explants of enset genotypes Keberia and Mazia at the multiplication stage, two months after culturing the shoot tips. AC= activated charcoal; Gt= genotype; and MC= Medium compositions, which were: MS medium without PGRs; MS medium supplemented with (mg l^{-1}): 2.5 BA + 1 IAA; 5 BA + 1 IAA; or 10 BA + 1 IAA

Treatment	F-probability level	
	Blackening	Callusing
AC	0.040 *	1.000 ns
Gt	0.062 ns	0.768 ns
MC	<0.001 *	0.001 *
AC x Gt	0.707 ns	0.041 *
AC x MC	<0.001 *	0.007 *
Gt x MC	0.221 ns	<0.001 *
AC x Gt x MC	<0.001 *	<0.001 *

*- indicates significant treatment effect

ns- indicates non-significant treatment effect

F-probability level ≤ 0.05

Table 4.8: Statistical significance for the effect of composition of medium and activated charcoal on shoot growth of enset genotypes Keberia and Mazia *in vitro* at the multiplication stage, two months after culturing the shoot tips. AC= activated charcoal; Gt= genotype; and MC= Medium compositions, which were: MS medium without PGRs; MS medium supplemented with (mg l^{-1}): 2.5 BA + 1 IAA; 5 BA + 1 IAA; or 10 BA + 1 IAA. NS= number of shoots per shoot tip; SL= shoot length; NB= number of buds per shoot tip; NL= number of leaves per shoot; and NR= number of roots per explant

Treatment	F-probability level				
	NS	LS	NB	NL	NR
AC	<0.001 *	<0.001 *	0.467 ns	<0.001 *	<0.001 *
Gt	0.110 ns	0.539 ns	0.254 ns	0.659 ns	0.176 ns
MC	0.694 ns	0.010 *	0.097 ns	0.006 *	<0.001 *
AC x Gt	0.026 *	0.112 ns	0.917 ns	0.095 ns	0.095 ns
AC x MC	0.379 ns	0.011 *	0.762 ns	0.040 *	0.002 *
Gt x MC	0.043 *	0.048 *	0.371 ns	0.129 ns	0.119 ns
AC x Gt x MC	0.336 ns	0.413 ns	0.641 ns	0.705 ns	0.070 ns

*- indicates significant treatment effect

ns- indicates non-significant treatment effect

F-probability level ≤ 0.05

The average score of blackening steadily increased from almost zero to about three as the BA concentration increased from 0 to 10 mg l^{-1} in MC1 to MC4. The blackening was significantly reduced by the inclusion of AC (Figure 4.10c and Table 4.9). On MC2 medium, enset genotype Mazia exhibited greater formation of callus than genotype Keberia. Enset genotype Keberia produced about one shoot and one bud per shoot tip both in the absence and presence of AC while genotype Mazia usually regenerated shoots in a few cases in the absence of AC (Figure 4.7).

Table 4.9: Effect of medium composition and activated charcoal on blackening and callusing of shoot tip explants of enset genotypes Keberia and Mazia, two months after culturing the shoot tips for multiplication. AC0= without activated charcoal (AC); AC1= with 7 g l^{-1} AC; Gt= genotype; MC1= MS medium without PGRs; MS medium supplemented with PGRs (mg l^{-1}): MC2= MS medium + 2.5 BA + 1 IAA; MC3= MS medium + 5 BA + 1 IAA; and MC4= 10 BA + 1 IAA. Bl= blackening; and Cl= callusing

Activated charcoal (AC)	Genotype (Gt)	Medium composition (MC)							
		MC1		MC2		MC3		MC4	
		Bl	Cl	Bl	Cl	Bl	Cl	Bl	Cl
AC0	Keberia	0.4	0	1.5	0.1	1.8	0.1	2.8	0.8
	Mazia	0.3	0	0.4	1.5	1.6	0	2.6	0.3
AC1	Keberia	1.5	0	1.1	0.5	1.0	0.3	1.1	0.1
	Mazia	0.2	0	2.5	0	0.9	0.9	0.4	0
Blackening (Bl)									
SE						0.3			
LSD (5%)						0.9			
Callusing (Cl)									
SE						0.2			
LSD (5%)						0.6			

The number of shoots per shoot tip, after culturing for two months on the initiation media and for another two months on the multiplication media, was not more than one on most of the medium compositions for both genotypes (Figure 4.8). Supplementing MS medium with 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA slightly improved regeneration of shoots of genotype Keberia. The regeneration of plantlets of genotype Keberia was better on MS medium devoid of PGRs (MC1) than on MS medium with 10 mg l^{-1} BA + 1 mg l^{-1} IAA (MC4). Both MC1 in the absence of BA and MC4 in the presence of 10 mg l^{-1} BA resulted in regeneration of about one shoot per shoot tip of genotype Mazia. Regardless of AC, MS medium without PGRs produced a better shoot length for both enset genotypes.

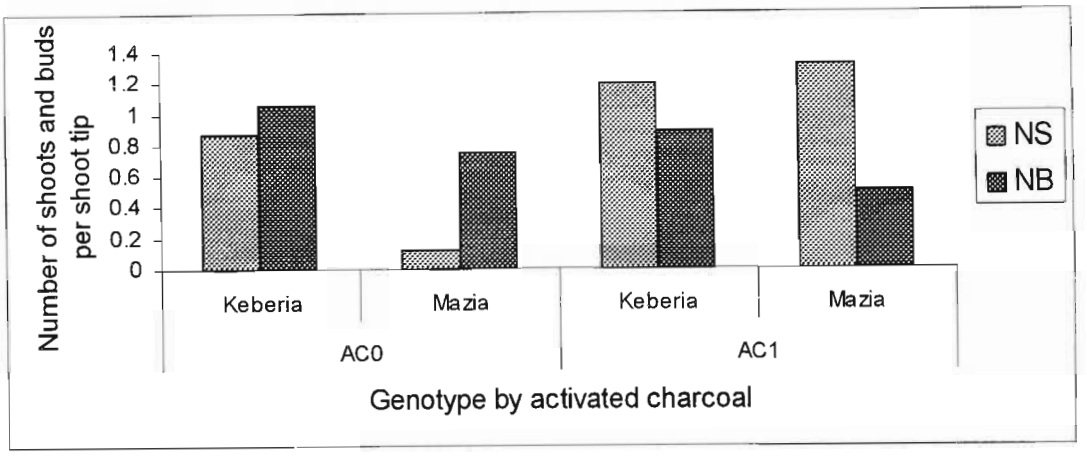

Figure 4.7: *In vitro* regeneration of shoots and buds from shoot tip explants of enset genotypes Keberia and Mazia in the absence and presence of activated charcoal (AC), two months after culturing the shoot tips for multiplication. AC0= without activated charcoal (AC); AC1= with 7 g l^{-1} AC; NS= number of shoots per shoot tip; NB= number of buds per shoot tip. For NS: SE= 0.2; and LSD (5%)= 0.5. For NB: SE= 0.3; and LSD (5%)= non-significant

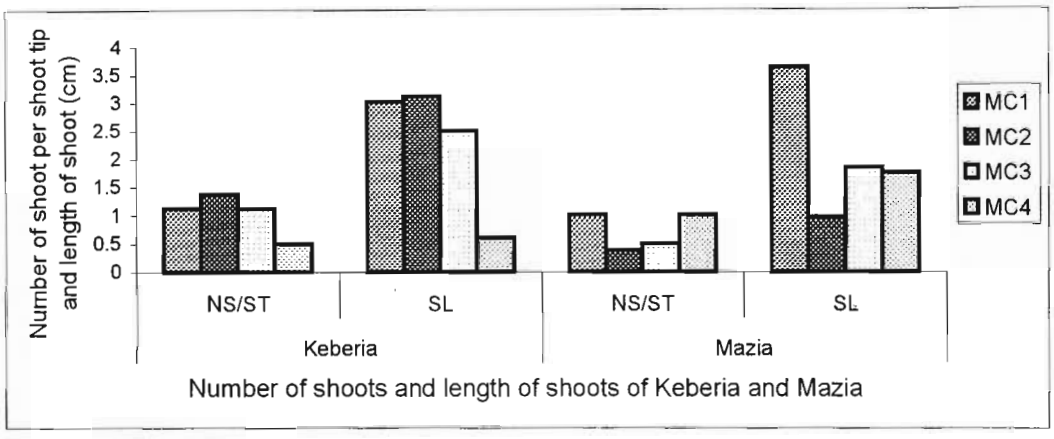

Figure 4.8: Effect of medium composition on the number of shoots per shoot tip and length of shoot of enset genotypes Keberia and Mazia, two months after culturing the shoot tips for multiplication. Medium compositions were: MC1= MS medium without PGRs; MS medium supplemented with PGRs (mg l^{-1}): MC2= 2.5 BA + 1 IAA; MC3= 5 BA + 1 IAA; MC4= 10 BA + 1 IAA. NS/ST= number of shoot per shoot tip; and SL= shoot length. For NS/ST: SE= 0.3; and LSD (5%)= 0.8. For SL: SE= 0.6; and LSD (5%)= 1.8

The presence of AC considerably increased shoot length when it was evaluated as an average value of the two enset genotypes (Figure 4.9). Increasing the BA concentration from 5 mg l^{-1} to 10 mgl^{-1} decreased shoot length of genotype Keberia. In the absence of AC and presence of BA, shoots produced few leaves per shoot while growth of roots was inhibited (Table 4.10). Significantly more leaves and roots were formed when AC was added to the medium in the absence of BA.

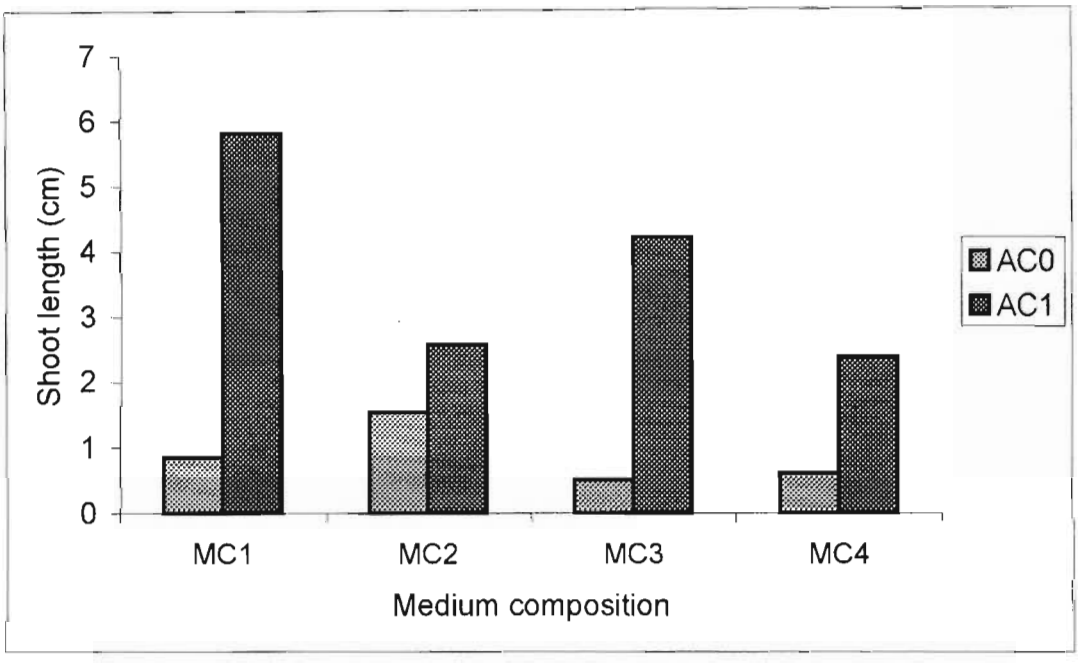

Figure 4.9: Effect of medium composition and activated charcoal on shoot length *in vitro*, two months after shoot tips were cultured onto the multiplication medium. AC0= without activated charcoal (AC); AC1= with 7 g l^{-1} AC. Medium compositions: MC1= MS medium without PGRs; MS medium supplemented with (mg l^{-1}): MC2= 2.5 BA + 1 IAA; MC3= 5 BA + 1 IAA; or MC4= 10 BA + 1 IAA. Data was averaged over two enset genotypes Keberia and Mazia. SE= 0.6; and LSD (5%)= 1.8

Table 4.10: Effect of activated charcoal and medium composition on growth of leaves and roots of shoot *in vitro*, two months after inoculation of shoot tips onto the multiplication medium. Data was averaged over two enset genotypes Keberia and Mazia. AC0= without activated charcoal (AC); and AC1= with 7 g l^{-1} AC. Medium compositions: MC1= MS medium without PGRs; MS medium supplemented with (mg l^{-1}): MC2= 2.5 BA + 1 IAA; MC3= 5 BA + 1 IAA; or MC4= 10 BA + 1 IAA

Activated charcoal (AC)	Medium composition (MC)	Number of leaves Shoot^{-1}	Number of roots Explant^{-1}
AC0	MC1	0.5	0.1
	MC2	0.6	0
	MC3	0.1	0
	MC4	0	0
AC1	MC1	2.3	3.2
	MC2	1.0	1.5
	MC3	1.4	1.6
	MC4	1.1	1.0
SE		0.2	0.3
LSD (5%)		0.7	0.8

Simple correlation coefficients in Table 4.11 show the relationship between blackening, callusing and growth parameters of the shoots at the multiplication stage. Blackening of explants significantly and inversely correlated to the number of shoots per shoot tip, length of shoot, number of leaves and roots. As the formation of callus increased, the number of shoots per shoot tip, length of shoots and number of leaves per shoot decreased. The increased number of shoots per shoot tip associated with increased length of shoot and increased number of leaves and roots.

Shoot tips of enset genotypes Keberia and Mazia were halved, decapitated and inoculated onto three types of medium compositions: MS medium without PGRs;

MS medium with 2.5 mg l^{-1} BA + 1 mg l^{-1}; and MS medium with 1.5 mg l^{-1} BA + 3 mg l^{-1} IBA. The number of shoots and buds per shoot tip, length of shoots and number of leaves per shoot was influenced by the genotype (Table 4.12). A larger number of shoots per shoot tip of genotype Keberia and larger number of buds for genotype Mazia were produced (Table 4.13) when shoot tips were decapitated. Shoot length and emergence of leaves were better for Keberia. From the study made on the use of liquid medium for *in vitro* multiplication, halved shoot tips gave fast growing shoots from the apical buds and smaller buds from lateral buds (Figure 4.10d). Removing the bigger shoots and subculturing the buds on a fresh medium improved growth of the buds. On average, three shoots and buds per shoot tip of genotype Keberia and 11 shoots and buds per shoot tip of genotype Mazia were produced in a month (Table 4.14). Of the shoots and buds produced from shoot tips of genotype Mazia in the liquid medium, three to five shoots per shoot tip grew normally.

Table 4.11: Simple correlation coefficients showing blackening and callusing of explants and growth of shoots *in vitro* at the multiplication stage. Bl= blackening; Cl= callusing; NS= number of shoots per shoot tip; SL= shoot length; NB= number of buds per shoot tip; NL= number of leaves per shoot; and NR= number of roots per explant

Bl	1.000						
Cl	-0.021 ns	1.000					
NS	-0.303 *	-0.276 *	1.000				
SL	-0.397 *	-0.308 *	0.754 *	1.000			
NB	0.016 ns	-0.110 ns	-0.114 ns	-0.092 ns	1.000		
NL	-0.393 *	-0.295 *	0.758 *	0.935 *	-0.116 ns	1.000	
NR	-0.346 *	-0.118 ns	0.441 *	0.655 *	-0.063 ns	0.610 *	1.000
	Bl	Cl	NS	SL	NB	NL	NR

*- indicates significant correlation

ns- indicates non-significant correlation

n= 128; and t-probability level ≤ 0.05

Table 4.12: Statistical significance for the effect of medium composition on the regeneration of plantlets from decapitated shoot tips of enset genotypes Keberia and Mazia, two months after culturing shoot tips for multiplication. Medium compositions were: MS medium without PGRs; MS medium supplemented with (mg l^{-1}): 2.5 BA + 1 IAA; or 1.5 BA + 3 IBA. Gt= genotype; and MC= medium composition. AC was used with all medium compositions

Treatment	F-probability level			
	No. of shoots shoot tip^{-1}	No. of buds shoot tip^{-1}	Shoot length	No. of leaves $shoot^{-1}$
Gt	0.015 *	0.003 *	<0.001 *	<0.001 *
MC	0.163 ns	0.505 ns	0.076 ns	0.420 ns
Gt x MC	0.241 ns	0.404 ns	0.047 *	0.243 ns

*- indicates significant treatment effect

ns- indicates non-significant treatment effect

F-probability level ≤ 0.05

Table 4.13: *In vitro* response of decapitated shoot tips of enset genotypes Keberia and Mazia, two months after shoot tip culturing for multiplication. Medium compositions were: MS medium without PGRs; MS medium supplemented with (mg l^{-1}): 2.5 BA + 1 IAA; or 1.5 BA + 3 IBA. AC was used with all medium compositions

Genotype	Number of shoots shoot tip^{-1}	Number of buds shoot tip^{-1}	Shoot length (cm)	Number of leaves $shoot^{-1}$
Keberia	1.6	0.3	4.0	1.8
Mazia	0.8	1.4	0.8	0.4
SE	0.3	0.2	0.5	0.2
LSD (5%)	0.7	0.7	1.4	0.6

Table 4.14: Multiplication of shoot of enset genotypes Keberia and Mazia *in vitro* in a liquid medium (MS medium + 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA + 1 g l^{-1} AC), one month after culturing in the liquid medium

Growth parameter	Mean measurement		t-probability level
	Keberia	Mazia	
Number of shoots shoot tip^{-1}	0.3	0.1	0.552 ns
Number of buds shoot tip^{-1}	1.4	5.4	<0.001 *
Number of shoots and buds shoot tip^{-1}	3.1	11.4	<0.001 *

*- indicates significant difference between the two means

ns- indicates non-significant difference between the two means

t-probability level ≤ 0.05

4.3.4 Regeneration of plantlets and acclimatization ex vitro

The plantlets with shoots and roots from the multiplication stage were transferred to MS medium without PGRs and grown for a month. The plantlets from gelled medium were acclimatized in a mist house after they attained lengths of 5-10 cm producing three to four leaves. Those obtained from the liquid medium were taller, up to 15 cm with thicker pseudostems at acclimatization. The number of leaves was similar but wider for plantlets obtained from the liquid medium. All plantlets were planted on a mixture of compost: sand: peat: vermiculite at a ratio of 13:3:1:1 (v/v), respectively. Hydroponic nutrient powder was dissolved in water (1 g powder per 1 l water) and applied once a week. Moisture was provided by an automatic misting system. Two weeks later, the plants were transferred to the greenhouse and fertilized as before. After three weeks in the greenhouse, the seedlings were transferred to the same mixture of soil but into larger pots. They were watered every day for the first week and three to four times per week later. Establishment of about 70% for plantlets from the gelled medium and 95-100% for plantlets from the liquid medium was achieved.

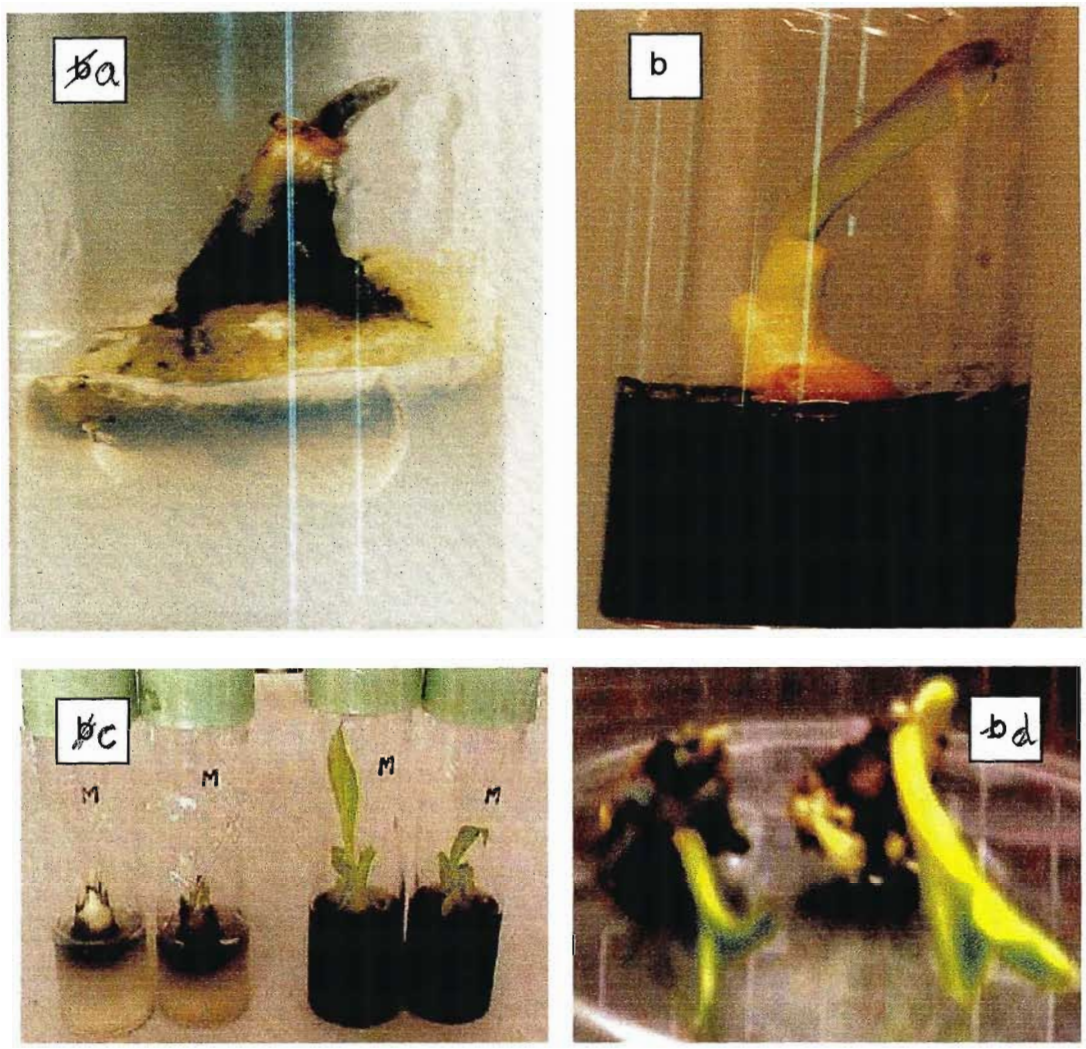

Figure 4.10: Regeneration of shoots and buds from shoot tips of greenhouse-grown suckers of enset genotype Mazia on MS medium with 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA: a) Blackening and callusing of intact shoot tip in the absence of AC, three months after inoculation; b) Initiation of intact shoot tip without blackening and callus formation in the presence of AC, one month after inoculation; c) Effect of AC on blackening of halved shoot tips, one month after splitting for multiplication; and d) Growth of shoots and buds from lateral and apical buds of halved shoot tips, one month after culturing in the liquid medium with 1 g l^{-1} AC

4.4 Discussion

In the decontamination methods of shoot tips, size of the explant and decontamination method used did not have a significant effect on the probability of having a contaminated culture. This suggested that either 5 or 10 mm long shoot tips could be used with either of the decontamination methods, decontaminating the explants once or twice, without significant difference in encountering culture contamination. However, for simplicity and less damage to the explants, 8-10 mm shoot tips from the greenhouse suckers could be used decontaminating only once for 15 min in 3.5% sodium hypochlorite. This method was not effective to decontaminate shoot tips obtained from field-grown mother plants where about 42% of the shoot tips were contaminated. The results using a linear logistic regression model showed that there were 16 times more chance of encountering contamination with explants from field grown suckers than with the explants from greenhouse grown suckers. Many holes with necrotic wounds inside the cortex of the corm were observed during excision of the shoot tips from field grown suckers, which was not observed in the shoot tips from greenhouse grown suckers. This indicated that by propagating enset in the greenhouse it would be possible to significantly reduce chances of culture contamination. The contamination was of both fungal and bacterial nature and observed two days after inoculation of the shoot tips and continued appearing for a week. There were a few cases with explants from field-grown suckers where bacterial contamination started from the explant after 15 days of inoculation, which could probably be endogenous contaminants. Such late contamination was not observed in the case of explants from greenhouse-grown suckers. The initial explant was found as the major source of contamination in tissues culture (LEIFERT and WOODWARD 1997). Stimulating vigorous growth of papaya in a greenhouse as a source of explant helped to improve growth and to control endogenous contamination *in vitro* (DREW 1988). Endogenous contamination was reported in enset tissue culture (ZEWELDU 1997). In banana, when mother plants that were obtained from the field were stored for 10 days and shoot tips were cultured, 51% contamination was encountered using the same decontamination procedure (HAMILL *et al.* 1993).

When greenhouse-grown suckers of enset genotype Keberia and Mazia were used, 70 to 90% of the shoot tips were initiated without blackening by adding 7 g l^{-1} activated charcoal (AC) to MS medium with or without 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA. In banana, addition of 25 mg l^{-1} ascorbic acid alone to the medium prevented blackening more effectively than AC (GUPTA 1986). Addition of activated charcoal, nevertheless, reduced browning of palm explants and culture media and increased survival of the explants and organogenesis better than an antioxidant, ascorbic acid (TISSERAT 1979). It also controlled browning of explants and stimulated shoot growth of *Strelitzia reginae* (MENSULI-SODI *et al.* 1993). In the present work, addition of 25 mg l^{-1} ascorbic acid to the medium was not as effective as AC in reducing blackening of shoot tips of enset *in vitro*. However, the presence of ascorbic acid improved survival and elongation of the shoot in the light condition in the absence of AC showing that ascorbic acid reduced the negative impact of blackening even though the outer part of the corm of the shoot was black. The rate of survival and growth of shoots was also better when shoot tips were incubated in the dark than in the light without both ascorbic acid and AC but the shoots tended to etiolate.

Elongation of the pseudostem part of the shoot tip during the initiation stage occurred rapidly through extension of pre-existing leaf initials after inoculation but became slower in general after subculture and was pronounced in the absence of AC. At this stage the shoot tips were not split longitudinally as the purpose was to initiate aseptic culture for further multiplication and thus meristematic domes of the shoot tips were not injured. This also could contribute to the reduction of blackening of the explants. Swelling of some shoot tips of enset genotype Mazia was observed when they were cultured without AC. Otherwise callus formation was not a problem at the initiation stage in the presence of AC. Blackening of explant due to oxidation of polyphenol and callus formation (AFZA *et al.* 1996, MORPURGO *et al.* 1996, ZEWELDU 1997) were critical problems in enset shoot tip culture. The negative relationship between blackening and number and length of shoots at the initiation stage showed that reducing the blackening increased the survival rate and growth of shoots from existing apical buds. AC added to the medium adsorbs the phenolic compounds and so reduces tissue blackening; the maintenance of the newly initiated cultures in darkness for one week lowers

activity of polyphenol oxidase; and reducing agents such as ascorbic acid added to the medium prevent blackening by polymerization of phenolic quinones, thereby removing one of the substrates that leads to blackening of the tissues (COLLIN and EDWARDS 1998). Based on the results on blackening score, number of shoots that survived and length of shoot, it is possible to initiate intact shoot tips from the greenhouse in the presence of AC on MS medium without PGRs or with 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA.

At the multiplication stage, splitting of the shoot tips was found necessary (Chapter three). The splitting, however, causes meristematic dome wounding and results in more blackening of the shoot tip explants. For this reason, shoot tips without splitting were initiated for a longer time (eight weeks) in the presence of AC whereafter they were split longitudinally into two and cultured onto different media compositions in the presence or absence of AC. Regardless of the concentration of BA, most of the halved shoot tips from enset genotype Mazia failed to regenerate shoots in two months in the absence of AC while some halved shoot tips of this genotype gave buds. Shoot tips of genotype Keberia more often grew into shoots even in the absence of AC. This showed that the presence of AC was more important to enset genotype Mazia than to Keberia. The morphogenetic response of cultured shoot tips of *Musa* spp (BANERJEE and DE LANGHE 1985, WONG 1986) and papaya (LITZ and CONOVER 1981) was also influenced by genotypes. Blackening scores should be observed along with number and growth of regenerated shoots because sometimes blackening may occur on the surface of the corms without significantly affecting growth of the shoots. Callus formation at the multiplication stage was higher with genotype Mazia. However, for both genotypes it was generally low in the presence of AC.

When shoot tips were split longitudinally, it was difficult to exactly divide the apical dome into two equal parts. One part of the halved shoot tips usually had a larger part of the apical dome and regenerated shoots. The other part of the halved shoot tips received a smaller portion of the apical dome or may even have been cut without part of the apical dome and regenerated bud or grew into leafy structure without forming a bud. This, therefore, showed that the presence of the apical meristem with the split shoots was important for the organogenesis of explants

from greenhouse-grown suckers of enset genotypes Keberia and Mazia. As a result, two halves of the shoot tip more often gave rise to one bud and one shoot, without leading to multiple shooting regardless of the wound to the apical domes and the presence of BA in gelled MS medium. There is no quantitative report on the response of shoot tip explants from greenhouse-grown mother plants of enset (*Ensete ventricosum*). WONG (1986) also found more survival of split shoot tips with apical buds of banana. For the multiplication of bananas, 5 mg l^{-1} BA was considered as a standard concentration (VUYLSTEKE 1998) while 10 mg l^{-1} BA resulted in more than 35 shoots per shoot tip in banana cultivar, Robusta (DORE SWAMY *et al.* 1983) and also was the most effective concentration in promoting shoot initiation in abaca (*Musa textilis*) (MANTE and TEPPER 1983). In the present study, however, growing halved shoot tips with 5 or 10 mg l^{-1} BA did not cause significant formation of multiple shoots in enset genotypes Keberia and Mazia when the mother plants were grown in the greenhouse. Regeneration of one to two shoots/buds per shoot tip on gelled medium without interference of callus formation was demonstrated using halved shoot tips.

Decapitation of shoot tips resulted only in about two shoots and buds per shoot tip of genotypes Keberia and Mazia *in vitro*, which was few. MA and SHII (1972 in Israeli *et al.* 1995) obtained multiple shoots in banana through decapitation of the apical buds. Concentration of PGRs, 1.5 mg l^{-1} BA + 3 mg l^{-1} IBA, used for multiplication of shoots of *Ensete superbum* (MATHEW and PHILIP 1996) did not result in multiple shooting of the genotypes of *E. ventricosum* even after decapitation of the apical domes. Shoot tips from the greenhouse were big enough for decapitation but blackening and thus death of the explants was a problem. In this experiment, shoot tips derived from greenhouse that were grown *in vitro* were decapitated. However, the shoot tips were so small that they were not suitable for decapitation. Decapitation of the apical bud is used in conventional propagation of enset (BEZUNEH and FELEKE 1966). In a liquid medium, an average of about three shoots and buds per shoot tip of genotype Keberia and 11 shoots and buds per shoot tip of genotype Mazia were regenerated when shoot tips of *in vitro* grown plantlets were split longitudinally and cultured, which was not obtained on gelled media. Better proliferation of shoot tips of Musa (CRONUER and KRIKORIAN 1984b) and pineapple (MATHEWS and RANGAN 1979) was

obtained when initiated explants were subcultured in liquid medium of the same formulation. Genotype Keberia was poor in producing viable buds and as a result only few of the initiated buds grew into complete plantlets. About three to five normal shoots per shoot tip of genotype Mazia grew in two months from the multiple adventitious buds differentiated in the liquid medium. This showed that multiple shoots could be initiated from halved shoot tips but the regeneration capacity depended on types of genotype. Therefore, further work on *in vitro* multiplication of different enset genotypes is important with emphasis to a liquid medium.

Plant regeneration with shoots and roots was obtained on the multiplication media. The presence of AC had more impact than BA on promoting root formation in enset. In banana, absence of roots in the presence of relatively high BA concentrations associated with better multiplication (CRONAUER and KRIKORIAN 1984b). The absence of roots did not associate with multiple shoot formation in enset; it rather associated with generally poor regeneration. Shoots regenerated in the presence of BA both from gelled and liquid medium were transferred to jars on MS medium devoid of PGRs for further growth. Plantlets were planted *ex vitro* onto a mixture of compost: sand: peat: vermiculite at a ratio of 13:3:1:1 (v/v), respectively. They acclimatized for 15 days in a mist house providing moisture by an automatic misting system. The plantlets were established in the greenhouse on the same soil mixture in bigger pots. Plantlets from the liquid medium showed better establishment (90 to 100%) than those from the gelled medium. Plantlets from the liquid medium had developed thicker pseudostems that possibly contributed to better establishment.

CHAPTER FIVE

CALLUS CULTURE AND SOMATIC EMBRYOGENESIS IN *ENSETE VENTRICOSUM*

5.1 Introduction

Enset (*Ensete ventricosum* (Welw.) Cheesman, family Musaceae) is a perennial herbaceous plant that is cultivated in Ethiopia having great local importance as a food crop. However, its cultivation is constrained by various diseases such as enset wilt caused by *Xanthomonas campestris* pv *musacearum*, pests and abiotic factors. Therefore as in other crops, enset improvement for desirable traits is of paramount importance. This can be done using both conventional and non-conventional methods. Somatic embryogenesis used in the non-conventional methods, has various practical application in plant production and improvement (RAZDAN 1993, ISRAELI *et al.* 1995, GANAPATHI *et al.* 1999) such as clonal propagation that can be automated using a bioreactor, synthesis of artificial seeds and source of regenerable protoplasts that can be used for somatic hybridization and other genetic manipulation. Adventitious shoots from callus can be used to produce plantlets for planting when there is genetic uniformity or somaclones can be used as breeding material. Callus is a coherent and amorphous tissue, formed when plant cells multiply in a disorganized way (GEORGE and SHERRINGTON 1984, GEORGE 1993) that can give rise to organs such as shoots and roots through organogenesis and/or to embryos through somatic embryogenesis. Formation of adventitious shoots from a callus is favored by low auxin and high cytokinin concentrations while root formation generally takes place in a medium with a relatively high auxin and low cytokinin concentration (PIERIK 1987). Somatic embryogenesis can be induced indirectly from callus, cell suspension and protoplast cultures or directly from cells of organized structures such as stem segments or zygotic embryos. The indirect induction of somatic embryogenesis is widely used (GEORGE 1993).

Callus was induced from corm explants of *E. ventricosum* and plants were regenerated through formation of adventitious shoots and somatic embryos at

different levels of cytokinins (AFZA *et al.* 1996, MORPURGO *et al.* 1996) and through formation of adventitious shoots using combinations of auxin and cytokinin (ZEWELDU 1997). In *E. superbum*, the shoot tips from *in vivo* plants produced a proliferating callus from which adventitious shoots were regenerated but somatic embryos were not formed (MATHEW *et al.* 2000). Embryogenic callus was induced from the corm tissue at the base of *in vitro* generated plants in the presence of BA and 2,4-D and somatic embryos were differentiated and matured on hormone-free medium (MATHEW *et al.* 2000, MATHEW and PHILIP 2003).

There are only few reports available on callus culture and somatic embryogenesis in *E. ventricosum* (AFZA *et al.* 1996, MORPURGO *et al.* 1996, ZEWELDU 1997). These are limited to unspecified genotypes of the species. In this study, regeneration of adventitious shoots and somatic embryos from calli of different genotypes of *E. ventricosum* were investigated.

5.2 Materials and Methods

5.2.1 Plant material

Seeds of enset genotype Oniya stored for six years and vegetative plant material of genotypes Keberia and Mazia were obtained in August 2001 from Areka Agricultural Research Centre, Ethiopia. All the genotypes are under cultivation. Genotype Mazia is resistant to enset wilt disease caused by *Xanthomonas campestris* pv *musacearum* (WOLDEMICHAEL 2000) and preferred for its higher final kocho yield. Keberia is an early maturing type and preferred for its sweet cooked corms. The vegetative plant material of genotypes Keberia and Mazia was propagated in the greenhouses of the Research Centre for Plant Growth and Development, School of Botany and Zoology, University of Natal, where the studies were undertaken. Three level teaspoons (about 10 g) hydroponic nutrient powder was dissolved in 5 l water and applied once a week to the suckers until the leaves were covered with spray solution and to the soil under the suckers. Zygotic embryos and *in vitro* seedlings from the stored seeds of Oniya and suckers of Keberia and Mazia, 6-8 months after propagation, were used for the studies. The studies were undertaken between September 2001 and October 2002.

5.2.2 Callus culture and somatic embryogenesis

5.2.2.1 Callus culture and regeneration of adventitious shoots from Oniya genotype

Callus was induced from zygotic embryos of stored seeds of clone Oniya that was cultured on MS medium supplemented with 0.5 mg l^{-1} BA + 0.2 mg l^{-1} IAA. The callus was multiplied on the same medium composition, adding 1 g l^{-1} casein hydrolysate as a source of organic nitrogen. Further proliferations of the callus from the embryos and plant regeneration were studied on MS medium with five types/concentrations of PGRs. These were (mg l^{-1}): 0.5 BA + 0.2 IAA; 0.5 BA + 0.2 IAA + 0.1 2,4-D; 0.5 BA + 0.2 IAA + 0.2 2,4-D; 1 BA + 0.4 IAA + 0.4 2,4-D; and 1.25 2,4-D. Callus was maintained and multiplied by subculturing every 8 weeks onto MC2 + 1 g l^{-1} casein hydrolysate for eight months after callus initiation. Thereafter, the callus was treated on MS medium with different PGRs and concentrations (mg l^{-1}): 1.13 BA; 4.51 BA; 1.13 BA + 0.28 2,4-D; and 0.28 2,4-D + 4.51 BA. For the regeneration of shoots, organogenic callus from the zygotic embryos was cultured on MS medium without PGRs in the light, using a 16 h light/8 h dark and an irradiance of 43 μmol m^{-2} s^{-1} at 25 ± 1 °C.

5.2.2.2 Callus culture and somatic embryogenesis in Oniya and Mazia genotypes

Shoot tips of greenhouse-grown suckers of enset genotype Mazia were trimmed to obtain 2-3 cm long explants. The shoot tips were decontaminated for 15 min in 3.5% sodium hypochlorite with two drops of Tween 20, and then rinsed three times with sterile distilled water. Five to eight mm long shoot tips were excised and cultured onto MS medium supplemented with 2.5 mg l^{-1} BA + 1 mg l^{-1} NAA + 30 g l^{-1} sucrose and gelled with 8 g l^{-1} agar. pH of the medium was adjusted to 5.8 before autoclaving. The shoot tip explants were subcultured twice at a three week interval on the same medium composition, during which time callus was formed at the base of some of the shoot tip explants. The callus with some portion of corm explant was then cultured on MS medium supplemented with (mgl^{-1}) 0.5 BA + 0.2 IAA + 0.1 2,4-D + 1 g l^{-1} casein hydrolysate and subcultured twice at monthly intervals on the same medium composition. The calli induced from shoot tips of the greenhouse- grown Mazia and the one from zygotic embryos of stored seeds of

Oniya and maintained on MS medium containing 0.5 mg l^{-1} BA + 0.2 mg l^{-1} IAA + 0.1 mg l^{-1} 2,4-D were cultured on half strength MS medium (full strength of vitamins) plus 0.5 g l^{-1} casein hydrolysate supplemented with (mg l^{-1}) 0.5 BA + 0.2 IAA + 0.2 2,4-D or 1.15 BA + 0.25 2,4-D. Callus was also induced on MS medium supplemented with 10 mg l^{-1} BA + 1 mg l^{-1} NAA from shoot tips of vegetatively propagated greenhouse- grown enset genotypes Keberia and Mazia. Test tubes (10 x 2.5 cm) and disposable petri dishes (6.5 cm in diameter) both with 10-12 ml medium were used to culture and subculture the shoot tips and calli, respectively.

5.2.2.3 Histological observation

Specimens of embryogenic callus were fixed for 48 h in 0.05 M cacodylate buffer containing 3% glutaraldehyde and washed with 0.05 M cacodylate buffer in two changes after which the specimens were further fixed for 1 h in 0.05 M cacodylate buffer containing 2% osmium tetraoxide. Thereafter, the specimens were washed, one after the other, with 0.05 M cacodylate buffer in two changes, 2% uranyl acetaldehyde, distilled water and graded ethanol series (10, 30, 50 and 70% for the first day and 80, 90 and 100% for the second day) and finally drained with propylene oxide. The specimens were embedded in Epon-Araldite mixture and cured in an oven at 70 °C for 48 h. Ultra thin sections of about 80 nm thick were cut and picked up on 200 hexagonal mesh copper grids. The grids were stained for 10 min with uranyl acetate and rinsed with double distilled water, and stained again for 10 min with lead citrate and rinsed with double distilled water. Sodium hydroxide pellets were placed in the lead citrate staining petri dish to absorb CO_2 and prevent stain precipitate forming on the sections. The sections were viewed with Philips CM120 BioTwin transmission electron microscope.

5.2.2.4 Maturation of the somatic embryos

Eight different medium compositions (MC) were used to study their impact on maturation of the somatic embryos from shoot tips of genotype Mazia. Compositions of the medium were: MS medium without PGRs + 30 g l^{-1} sucrose with either 8 g l^{-1} agar (MC1) or 16 g l^{-1} agar (MC2); MS medium supplemented with: 0.5 mg l^{-1} BA + 0.2 mg l^{-1} IAA + 0.2 mg l^{-1} 2,4-D + 30 g l^{-1} sucrose with either

8 g l^{-1} agar (MC3) or 16 g l^{-1} agar (MC4); 0.5 mg l^{-1} BA + 0.2 mg l^{-1} IAA + 0.2 mg l^{-1} 2,4-D + 60 g l^{-1} sucrose with either 8 g l^{-1} agar (MC5) or 16 g l^{-1} agar (MC6); and 0.53 mg l^{-1} ABA + 60 g l^{-1} sucrose with either 8 g l^{-1} agar (MC7) or 16 g l^{-1} agar (MC8).

Data on number and growth of adventitious shoots, 5 and 12 months after culturing zygotic embryos of enset genotype Oniya for callus initiation, callus fresh weight and number of somatic embryos, about five months after inoculation of shoot tips of enset genotype Mazia, were recorded and subjected to analysis of variance using GenStat 5 Release 4.2. Significant treatment means were separated using least significant difference (LSD) at a 5% probability. Standard errors of means (SE) were also computed.

5.3 Results

5.3.1 Regeneration of adventitious shoots from callus culture

Growth of the callus was generally slow and callus developed into heterogeneous structures: watery, friable and organogenic callus, even within the same treatment. However, there were clear treatment effects (Table 5.1). A large amount of organogenic callus and earlier shoot growth was obtained when MS medium was supplemented with 0.5 mg l^{-1} BA + 0.2 mg l^{-1} IAA while MS medium supplemented with (mg l^{-1}) 0.5 BA + 0.2 IAA + 0.2 2,4-D followed by MS medium supplemented with (mg l^{-1}) 0.5 BA + 0.2 IAA + 0.1 2,4-D was good for production of watery and friable callus. Increasing BA, IAA and 2,4-D from 0.5, 0.2 and 0.1 or 0.2, respectively, to 1, 0.4 and 0.4 mg l^{-1} did not improve proliferation of the callus. MS medium with 1.25 mg l^{-1} 2,4-D resulted in browning and inhibited growth of callus. Vigorous adventitious shoots were regenerated from organogenic callus of zygotic embryos of stored seeds of genotype Oniya (Figure 5.1c). Regeneration of roots was followed by growth and development of shoots. Complete shoots were also regenerated from the callus, 8-10 months after callus initiation (Table 5.2) but their growth was slow and some shoots were hyperhydric.

Table 5.1: Effect of medium composition on growth of callus and regeneration of adventitious shoots from zygotic embryos of enset genotype Oniya, five months after callus initiation. Treatments (mg l^{-1}): MC1 = 0.5 BA + 0.2 IAA; MC2 = 0.5 BA + 0.2 IAA + 0.1 2,4-D; MC3 = 0.5 BA + 0.2 IAA + 0.2 2,4-D; MC4 = 1 BA + 0.4 IAA + 0.4 2,4-D; and MC5 = 1.25 2,4-D

Treatment	Type of callus (%)			Number of adventitious buds jar^{-1}
	Watery and friable	Organogenic	Brown	
MC1	24	76	0	5
MC2	47	52	0	5
MC3	58	42	0	5
MC4	32	24	43	4
MC5	0	0	93	0
SE	2	1	1	1
LSD (5%)	6	6	4	2

Table 5.2: Regeneration of adventitious shoots of enset genotype Oniya from 8-month-old callus, after four months on regeneration medium (MS). Concentrations of PGRs (mg 1^{-1}): MC1 = 1.13 BA; MC2 = 4.51 BA; MC3 = 0.28 2,4-D + 1.13 BA; and MC4 = 0.28 2,4-D + 4.51 BA

Type of medium		Number of shoots jar^{-1}		Longest shoot (cm)	Number of leaves $shoot^{-1}$
For callus culture	For shoot regeneration	Healthy	Hyperhydric		
MC1	MS	2	0	8	3
MC2	MS	2	4	5	3
MC3	MS	6	10	2	2
MC4	MS	4	4	3	2
SE		0.9	2.1	1.3	0.3

Callus was induced from shoot tips of greenhouse-grown enset genotype Mazia on MS medium containing BA + NAA. As callus produced at the base of the shoots, the shoot bud became black and died after nine weeks of inoculation on MS medium + 2.5 mg l^{-1} BA + 1 mg l^{-1} NAA (Figure 5.1a). The callus was further subcultured twice at monthly intervals on MS medium supplemented with (mg l^{-1}): 0.5 BA + 0.2 IAA + 0.1 2,4-D to multiply the callus and to reduce blackening of the tissues (Figure 5.1b). Tissue blackening reduced as intact corm tissues became more disorganized. Adventitious shoots were also regenerated from callus of shoot tips of greenhouse-grown enset genotypes Keberia and Mazia. This callus was used to study somatic embryogenesis.

5.3.2 Somatic embryogenesis and histological observation of the embryogenic callus

Embryogenic callus with many proembryos on the surface (Figure 5.2a) was obtained on MS medium with (mg l^{-1}): 0.5 BA + 0.2 IAA + 0.2 2,4-D or 1.15 BA + 0.25 2,4-D, after about 21 weeks after shoot tip inoculation. Somatic embryos showed variable growth and development (Figure 5.2b). The somatic embryos had no vascular connections with the underlying parental tissue and were enclosed by an epidermal layer of cells disrupted at the end of the root primordia. Sections taken from embryogenic callus and viewed on the transmission electron microscope showed cells with embryogenic characteristics such as small cytoplasm, prominent nucleus and large starch grains (Figures 5.3a, b). The number of somatic embryos was significantly influenced by explant source, which were zygotic embryos of stored seeds of Oniya and shoot tips of greenhouse grown Mazia (Table 5.3). The effects of source of explant on the number of somatic embryos produced were not significantly changed with different media compositions (Table 5.4). Callus from shoot tips enset genotype Mazia produced a larger number (average of 18) of somatic embryos per petri dish than the callus from zygotic embryos of stored seeds. The latter produced on average only three somatic embryos per petri dish (Table 5.5). Profuse embryo-like circular structures were observed on callus from the zygotic embryos of the stored seeds. When callus was induced from shoot tips of *in vitro* grown seedlings from zygotic embryos of the same stored seeds, somatic embryos were obtained without

difficulty on MS medium supplemented with 0.5 BA + 0.2 IAA + 0.2 2,4-D in mg l^{-1}. When callus was induced from embryos of the stored seeds, it seems that age of the embryos contributed to the poor response of callus in producing somatic embryos. Many adventitious shoots were regenerated from the callus. Somatic embryogenesis in the three genotypes of enset (Keberia, Mazia and Oniya) (Table 5.6) was summarized.

Table 5.3: Statistical significance of the effect of explant source and medium composition on growth of callus and formation of somatic embryos

Treatment	F-probability level	
	Callus fresh weight	Number of somatic embryos
Explant source (ES)	0.841 ns	<0.001 *
Medium composition (MC)	0.303 ns	0.146 ns
ES x MC	0.767 ns	0.505 ns

*- indicates significant treatment effect

ns- indicates non-significant treatment effect

F-probability level ≤ 0.05

Table 5.4: Effect of interaction between source of explants and medium composition on callus fresh weight and number of somatic embryos. Treatments (mg l^{-1}): MC1= 0.5 BA + 0.2 IAA + 0.2 2,4-D; or MC2= 1.15 BA + 0.25 2,4-D

Explant source	Medium composition	Callus fresh weight (mg petri $dish^{-1}$)	Number of Somatic embryos petri $dish^{-1}$
Mazia shoot tips	MC1	1003	22
	MC2	725	14
Oniya zygotic embryos	MC1	983	4
	MC2	823	2
SE		201	3
LSD (5%)		ns	ns

ns- indicates non-significant differences between the treatment means

Table 5.5: Effect of explant source on callus growth and formation of somatic embryos

Explant source	Callus fresh weight (mg pd^{-1})	Number of somatic embryos petri $dish^{-1}$
Mazia shoot tips	864	18
Oniya zygotic embryos	905	3
SE	142	2
LSD (5%)	ns	8

Table 5.6: Summary of somatic embryogenesis from different genotypes/ explants of *E. ventricosum* induced on half strength MS medium (with full amount of vitamins) plus 0.5 g l^{-1} casein hydrolysate supplemented with (mg l^{-1}) 0.5 BA + 0.2 IAA + 0.2 2,4-D (MC1)

Enset genotype	Explant used to induce callus	Remarks on formation of somatic embryos (SEs)
Oniya	Zygotic embryos of stored seed	Few SEs and many circular structures were obtained
Oniya	Shoot tips of *in vitro* generated seedlings from zygotic embryos of the stored seeds	Callus was induced on MS + 0.5 mg l^{-1} BA + 0.2 mg l^{-1} IAA and SEs were obtained on (MC1)
Mazia	Shoot tips from greenhouse-grown suckers	Fairly large number of SEs were produced
Keberia	Shoot tips from greenhouse-grown suckers	Callus was induced on MS + 10 mg l^{-1} BA + 1 mg l^{-1} NAA in 9 to 12 weeks and SEs were obtained on MC1

Figure 5.1: Callus culture and plant regeneration from *E. ventricosum* shoot: a) Callus induction from tip of enset genotype Mazia on MS medium supplemented with (mg l^{-1}) 2.5 BA + 1 NAA, 9 weeks after inoculation; and b) Callus culture on MS medium supplemented with (mg l^{-1}) 0.5 BA + 0.2 IAA + 0.1 2,4-D, 4 weeks after subculture; and c) Adventitious shoots regenerated *in vitro* from callus of zygotic embryo of stored seed of enset genotype Oniya

Figure 5.2: Somatic embryogenesis of *E. ventricosum* genotype Mazia produced on MS medium supplemented with (mg l^{-1}) 0.5 BA + 0.2 IAA + 0.2 2,4-D, 21 weeks after shoot tip inoculation: a) Embryogenic callus showing developing somatic embryos; and b) somatic embryos at different stages of development

Figure 5.3: Histological observation of sections from embryogenic callus: Dense cytoplasm with prominent nucleus and large starch grains (a) and the starch grains at higher magnification (b) for genotype Mazia

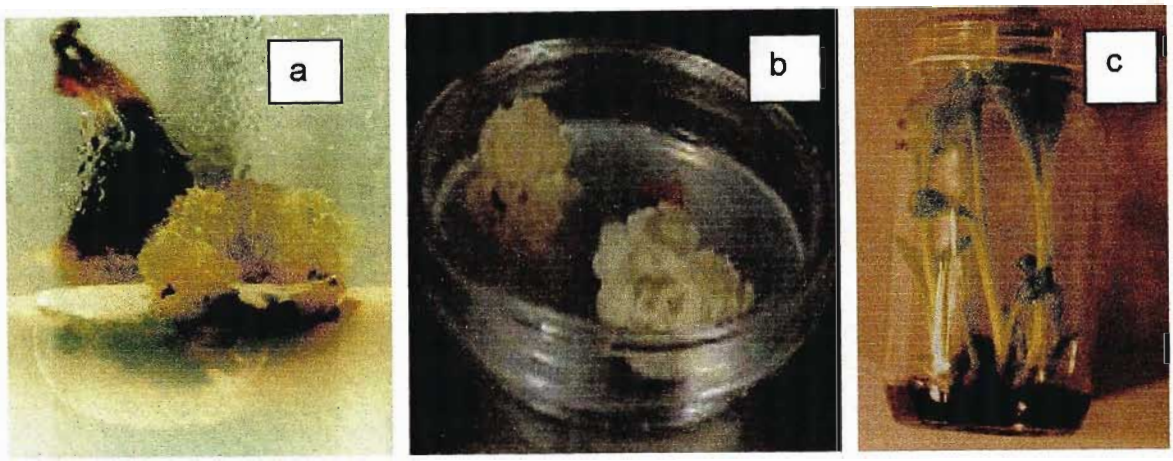

Figure 5.1

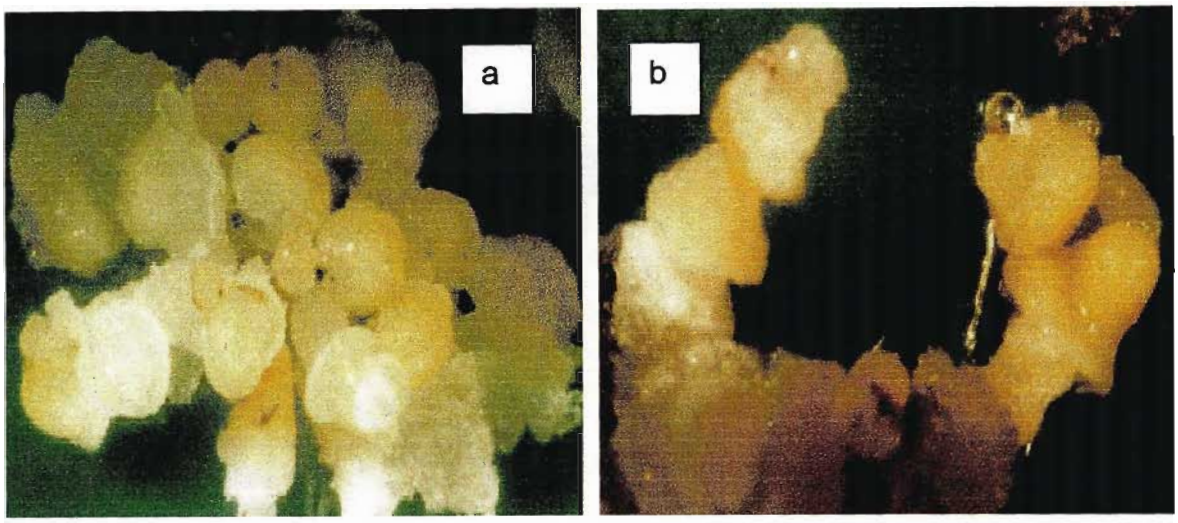

Figure 5.2

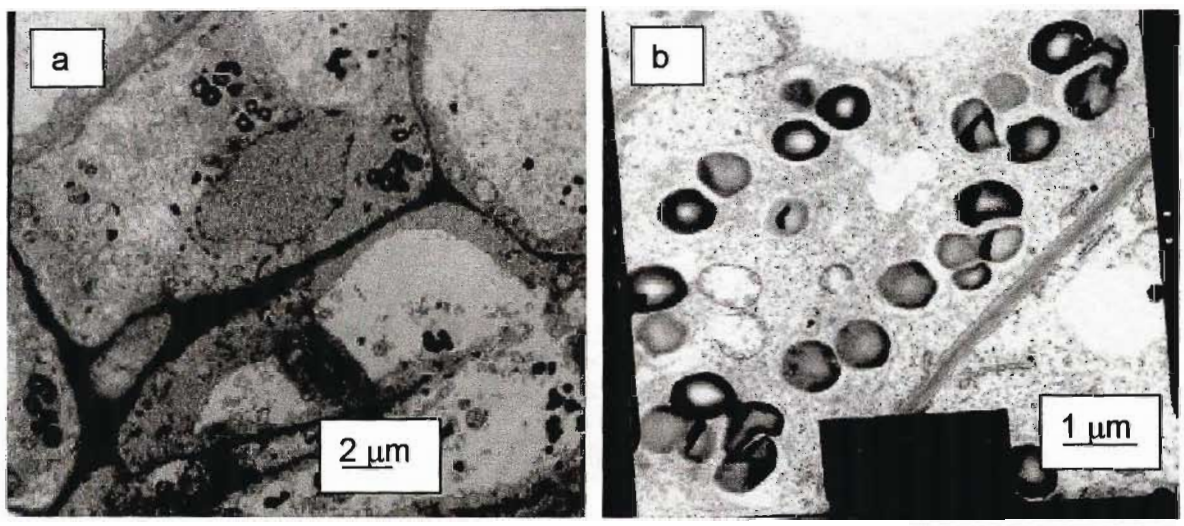

Figure 5.3

Maturation of somatic embryos was investigated using different compositions of media (Table 5.7). Even though regeneration of plantlets from the somatic embryos was not achieved in this study, there was a remarkable elongation of most of the somatic embryos (up to 12 mm from a length of 4-5 mm at time of transferral) on MS medium without PGRs + 30 g l^{-1} sucrose + 8 g l^{-1}agar after which they became black and died. When MS medium was used without PGRs with 60 g l^{-1} sucrose + 8 or 16 g l^{-1} agar, some embryos increased in length, became black and then died within a month. When PGRs (0.5 BA + 0.2 IAA + 0.2 2,4-D all in mg l^{-1}) that were used to induce somatic embryogenesis were added to the medium, the embryos formed callus or became swollen and most of them remained alive without blackening. In the presence of 0.53 mg l^{-1} ABA the embryos became black and died without any elongation or callus formation.

Table 5.7: Effect of different compositions of medium on growth of somatic embryos (SEs) from shoot tip callus of enset genotype Mazia. Medium compositions were (PGRs in mg l^{-1} and sucrose and agar g l^{-1}): MS + 30 sucrose + 8 agar (MC1); MS + 60 sucrose + 16 agar (MC2); MS medium supplemented with: 0.5 BA + 0.2 IAA + 0.2 2,4-D + 30 sucrose + 8 agar (MC3); 0.5 BA + 0.2 IAA + 0.2 2,4-D + 30 sucrose + 8 agar + 16 agar (MC4); 0.5 BA + 0.2 IAA + 0.2 2,4-D + 60 sucrose + 8 agar (MC5); 0.5 BA + 0.2 IAA + 0.2 2,4-D + 60 sucrose + 16 agar (MC6); and 0.53 ABA + 60 sucrose with either 8 agar (MC7) or 16 agar (MC8), data one month after subculture

Medium composition	Blackening of SEs and medium	Growth conditions of somatic embryos (SEs)
MC1	SEs and medium were black/brown	All SEs died after most of them elongated
MC2	SEs were black, medium not black	All SEs died
MC3	No blackening occurred	About 75% of the SEs showed proliferation of callus while about 25% died
MC4	No blackening occurred	70% of the SEs showed proliferation of callus while the rest died
MC5	No blackening occurred	70% of the SEs were swelling while the rest died
MC6	No blackening occurred	60% of the SEs were swelling and the rest died
MC7	SEs and the medium were black	All SEs died without swelling and callusing
MC8	The explants and medium were black	All SEs died without swelling and callus formation

5.4 Discussion

Callus was induced from zygotic embryos of enset genotype Oniya on MS medium with 0.5 mg l^{-1} BA + 0.2 mg l^{-1} IAA. Organogenic callus was obtained on the same medium composition and adventitious shoots were fully regenerated on MS medium devoid of PGRs. The most important characteristic of callus (DODDS and ROBERTS 1995), in general, is that it has a potential to develop normal roots, shoots and embryoids that can form plants. ZEWELDU (1997) regenerated adventitious shoots from callus of shoot tips of enset on MS medium with 4.5 mg l^{-1} BA + 0.017 mg l^{-1} IAA. In the present study, 4.5 mg l^{-1} BA was used with or without 0.28 mg l^{-1} 2,4-D and adventitious shoots were regenerated. Somatic embryos were obtained from callus induced from the shoot tips of greenhouse-grown enset genotype Mazia and from callus obtained from zygotic embryos of stored seeds of enset genotype Oniya on MS medium with (mg l^{-1}): 0.5 BA + 0.2 IAA + 0.2 2,4-D (MC1) or 1.15 BA + 0.25 2,4-D (MC2). Somatic embryos were also regenerated from callus obtained from shoot tips of enset genotype Keberia on MS medium with (mg l^{-1}): 0.5 BA + 0.2 IAA + 0.2 2,4-D. But the amount of callus that was obtained was small that it was not tested under different treatments. Even though the difference in number of somatic embryos between these two media compositions (MC1 and MC2) was not statistically significant, the inclusion of IAA in the media improved the quality of callus and embryos in terms of browning and growth. Browning of culture was also a problem in studies on somatic embryogenesis in banana (GANAPATHI *et al.* 1999). Gelled MS medium + 1.5 mg l^{-1} BA + 2 mg l^{-1} 2,4-D was used to induce embryogenic callus from *in vitro* plantlets of *E. superbum* (MATHEW *et al.* 2000, MATHEW and PHILIP 2003). We found that somatic embryogenesis in *E. ventricosum* can be induced at lower concentrations of BA (0.5 mg l^{-1}) and 2,4-D (0.2 mg l^{-1}). It is usually preferred to use lower concentration of 2,4-D to reduce the chance of regenerating genetically variable plantlets. BA (1.13, or 4.5 mg l^{-1}) without auxin was used to induce adventitious buds and somatic embryos from corm explants of *E. ventricosum* (AFZA *et al.* 1996). In the present study, 1.13 or 4.5 mg l^{-1} BA without auxin, when used to culture callus obtained from zygotic embryos of stored enset seeds, resulted in adventitious shoots but no somatic embryos were produced. Regeneration of plantlets from somatic embryos was reported for *E. superbum* on MS without PGRs (MATHEW

et al. 2000, MATHEW and PHILIP 2003). However, the use of MS medium without PGRs gave elongated somatic embryos but did not result in the regeneration of plantlets from the embryos of *E. ventricosum* in these studies. Thus, further optimization of the requirements for regeneration of plantlets from somatic embryos of *Ensete* species is needed.

The indirect production of adventitious shoots from explants via a callus stage may cause genetic instability of the progeny (GEORGE and SHERRINGTON 1984, RICE *et al.* 1992). According to KRIKORIAN (1989) callus is one of the less organized starting materials that may generate plantlets with more variations. However, callus cultures, through formation of adventitious shoots and roots, is used to micropropagate monopodial orchids such as *Phalaenopsis* and *Vanda*, which are characterized by a single unbranched axis that posses few available axillary shoots for use as explants (TISSERAT and JONES 1999). *E. ventricosum* is also a monopodial plant. In micropropagation of bananas, early detection and sorting of variants based on morphological characteristic are practically used (ISRAELI *et al.* 1991, ISRAELI *et al.* 1995). In the present study, adventitious shoots of *E. ventricosum* were established in the greenhouse and it was difficult to morphologically evaluate variations among them at an early stage of plant growth and development. The most common variants are dwarfs and mosaic types in bananas that can only be detected towards the end of the nursery stage (ISRAELI *et al.* 1991) while in plantains, early detection is more complicated since variations commonly appear in the inflorescence (ISRAELI *et al.* 1995). It is also possible to identify dwarf types at early stage of micropropagation in Cavendish banana through molecular methods. Therefore, further studies are needed to characterize the genetic stability of adventitious shoots obtained from callus culture of enset. Selection of noble variants for desirable traits would also contribute to genetic improvement of enset.

CHAPTER SIX

GENERAL CONCLUSIONS

Enset (*Ensete ventricosum* (Welw.) Cheesman) is a diploid (2n=18) herbaceous perennial belonging to the Musaceae. It is an important crop as a source of food, feed and fiber in Ethiopia. Enset is tolerant to moisture stress once established and is also environmentally friendly. As a result, it forms a sustainable agricultural system in the south and southwesten Ethiopia. In addition to its use as a source of staple food, products from enset are used in traditional medication some of which are to heal broken or fractured bones, to stop diarrhea and to ease the discharge of the placenta after childbirth. A starch that can be used for paper, textile and adhesive industries is produced from enset and the potential use of the starch in tablet formulation is being studied. However, production of enset is threatened by various factors such as diseases and pests, limitation in germplasm conservation and genetic improvement where tissue culture techniques can have a contributing role. Information is generally scanty on tissue culture of enset. Therefore, *in vitro* propagation techniques of enset such as zygotic embryo culture, micropropagation from shoot tips of *in vitro* grown seedlings, plant regeneration from shoot tips of greenhouse-grown mother plants and callus culture and somatic embryogenesis were investigated.

Germination of intact stored seeds of enset genotypes Mariya and Oniya was investigated under different sets of treatments using seeds that settled in water in a beaker, discarding the floating ones. All the seeds used failed to germinate. It was, however, observed that water reached the embryo inside the intact seed and some embryos of genotype Oniya began to germinate but failed to emerge out of the seed coat through the micropylar opening. This indicated the importance of mechanical dormancy in enset in which the seed coat is too strong to allow embryo expansion during germination though water may be absorbed. After the intact seeds failed to germinate, zygotic embryos were aseptically excised and cultured *in vitro*. The germination of the embryos from stored seeds of enset genotype Oniya was obtained on MS medium both with and without plant growth regulators (PGRs). But a better germination rate was achieved when the embryos

were placed horizontally, longitudinal axis of the embryo was placed flat halfway embedded into the medium and on MS medium supplemented with 0.5 mg l^{-1} BA + 0.2 mg l^{-1} IAA. Embryos from stored seeds of genotype Mariya failed to germinate *in vitro* too. Germination of excised zygotic embryos from seeds of two wild enset types was also studied shortly after seed harvesting. In this case, a germination rate over 85% was obtained on MS medium both with and without PGRs, depending on the genotypes. Poor embryo development might contribute to the germination variability. Poorly developed embryos were characterized by a shrunk surface and a pale yellowish colour that differed from creamy white normal embryos. Addition of 5 g l^{-1} activated charcoal (AC) to the culture medium significantly improved germination of zygotic embryos in the presence of PGRs and growth of the seedlings *in vitro*.

Shoot tips of *in vitro* grown seedlings from zygotic embryos of enset genotype Oniya were used to conduct experiments on micropropagation. Multiple shoots and buds were regenerated, without a callus phase, from halved shoot tips that were grown on MS medium that was gelled with agar or in a liquid medium. There was no blackening of explants in the presence of AC on gelled medium while slight blackening occurred in the liquid medium in the presence of AC. Using these media, commonly up to seven shoots and buds per shoot tip were produced within two months. Of these three to five shoots per shoot tip were normal. Supplementing the liquid and gelled medium with 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA improved growth of shoots although it did not cause a significant increase in the number of shoots and buds from halved shoot tips over MS medium devoid of PGRs. Addition of 1 and 7 g l^{-1} AC to a liquid and gelled medium, respectively, improved growth of shoots *in vitro*. Better growth of shoots and conversion of buds into shoots were achieved when liquid medium was used. Growing shoot tips *in vitro* with uninjured apical domes on MS medium with up to 100 mgl^{-1} BA resulted in only 1.3 to 1.7 shoots and buds per shoot tip. The buds were small in size and usually hyperhydric. Therefore, the BA did not break apical dominance in enset to any appreciable degree that can be of practical use. A wound during splitting shoot tips into two through the apex appears necessary to induce growth and development of lateral buds from enset shoot tips *in vitro*.

Shoot tips from greenhouse and field-grown suckers of enset genotype Mazia were used to develop procedures for decontamination. Shoot tips, 2-3 cm in length, from greenhouse-grown suckers could efficiently be decontaminated for 15 min in 3.5% sodium hypochlorite with two drops of Tween 20, rinsed three times with sterile distilled water. Shoot tip explants of 8-10 mm in length were found the optimum size for shoot tip culture. This method did not reduce contamination of shoot tips from field-grown mother plants to a tolerable level. It is, therefore, beneficial to propagate mother plants in the greenhouse as a source of explants. Further investigation is needed for alternative decontamination procedures for explants from field-grown mother plants. Shoot tips from greenhouse-grown suckers of genotypes Keberia and Mazia were used in the initiation and multiplication of shoots. The use of whole shoot tips, without wounding the apical dome, at the initiation stage together with 7 g l^{-1}AC significantly reduced blackening of the explants. As a result, 70 to 90% shoot tips from the greenhouse-grown suckers of enset genotypes Keberia and Mazia were initiated without blackening and callusing on gelled MS medium with or without 2.5 mg l^{-1} BA + 1 mg l^{-1} IAA. At the multiplication stage the splitting of the shoot tips, which was found necessary to induce growth of the lateral buds, aggravated blackening and death of the explants. Consequently, shoots were left to grow for 8 weeks *in vitro* at the initiation stage without splitting through the apex and thus without wounding the apical domes. So, increasing the length of the initiation time of intact shoot tips, in the presence of AC, reduced blackening and death of the split shoot tips at the multiplication stage.

The use of gelled MS medium with 2.5, 5 or 10 mg l^{-1} BA with or without 1 mg l^{-1} IAA did not cause formation of multiple shoots *in vitro* from shoot tips of greenhouse-grown suckers even though shoots were split. Commonly one shoot or bud regenerated from halved shoot tips. Shoot tips were also decapitated to release the lateral buds from apical dominance and cultured on MS medium gelled with agar. The decapitated shoot tips of Keberia gave more shoots while that of Mazia resulted in more buds. However, the regeneration was poor, one to two shoots per decapitated shoot tip regardless of PGRs used. Shoot tips that were split longitudinally into two through the apex and cultured into a liquid medium gave three to five normal shoots per shoot tip (the two halves) in two month from

explants of greenhouse suckers of genotype Mazia. Keberia responded poorly to the liquid medium. Callus was induced from shoot tips of enset genotypes Keberia, Mazia and Oniya and zygotic embryos of Oniya. Organogenic callus was induced from zygotic embryos of genotype Oniya on MS medium containing 0.5 mg l^{-1} BA + 0.2 mg l^{-1}IAA and adventitious shoots from the callus were regenerated on MS medium devoid of PGRs. Somatic embryos were produced from calli of the three enset genotypes. However, a better response was obtained from genotype Mazia in producing a greater number of somatic embryos from the embryogenic callus on half strength MS medium supplemented with (mg l^{-1}) 0.5 BA + 0.2 2, 4-D + 0.2 IAA or 1.15 BA + 0.25 2,4-D + 0.5 g l^{-1} casein hydrolysate. Enset genotype Mazia is resistant to enset wilt caused by *Xanthomonas campestris* pv *musacearum*. As information on *in vitro* propagation of *E. ventricosum* is generally very limited, it would be useful to standardize the use of the liquid medium for multiplication of shoots *in vitro* from many genotypes; hyperhydricity during multiplication of shoots from *in vitro* grown seedlings and germination of somatic embryos to regenerate plantlets through somatic embryogenesis require further studies.

Shoots produced roots at the multiplication stage due to the presence of AC. They were transferred to MS medium without PGRs for further growth and development prior to acclimatization. The plantlets were acclimatized in the mist house for two weeks on vermiculite or a mixture of compost: sand: peat: vermiculite at a ratio of 13:3:1:1 (v/v). Hydroponic nutrient powder was dissolved in water and applied once a week. Then the plantlets were transferred to the greenhouse. Better establishment of plantlets from a liquid medium was achieved because of vigorous growth of shoots *in vitro* and *ex vitro*. Tissue culture techniques are not an inexpensive venture. Therefore, performance of tissue cultured enset plants under field condition; occurrence of off types and cost benefit of the system need to be addressed in the future.

A significant contribution of the present work is that new information was generated on the germination of stored seeds that was obtained by zygotic embryo culture. Formation of callus and poor root development (BEZUNEH 1980) of the cultured zygotic embryos were controlled by addition of 5 g l^{-1} AC to the medium. The information can be used to test seed viability when seeds are stored

for a long time and also to germinate embryos in the breeding process. Blackening of shoot tip explants was addressed by the use of *in vitro* grown seedlings from zygotic embryos, the addition of AC to culture medium and increasing the length of the initiation time of intact shoot tips from the greenhouse-grown suckers before splitting the shoot tips for multiplication. Addition of AC also reduced swelling and callusing of the shoot tips of greenhouse-grown suckers *in vitro*. Blackening and formation of unwanted callus were critical problems in enset shoot tip culture (AFZA *et al.* 1996, MORPURGO *et al.* 1996, ZEWELDU 1997). The use of split shoot tips and a high concentration of BA to reduce the effect of apical dominance and the use of a liquid medium for *in vitro* multiplication of *E. ventricosum* was not reported before. Information on somatic embryogenesis of genotype Mazia, which is important in enset cultivation, was also generated.

The information generated in the present study should hopefully contribute to clonal multiplication, germplasm conservation and genetic improvement of enset. Enset is easy to propagate using conventional vegetative propagation methods and it is a subsistence crop, which is not yet cultivated commercially. The importance of micropropagation would, therefore, be to propagate new genotypes and/or specific pathogen-tolerant clones, like enset clone(s) tolerant to a pathogen, *Xanthomonas campestris* pv *musacearum* that cases enset wilt. Shoot tip culture can also supplement a field genebank in conserving enset genetic resources. Genetic variability can be created using callus culture and important variants can be selected as callus culture can readily be used. Somatic embryos could be produced from different enset genotypes including from genotype Mazia, which is resistant to the bacterial pathogen that causes enset wilt. Therefore, with further research emphasis, there is the potential for somatic hybridization and genetic manipulation. However, since enset is fertile and produces viable embryos the use of existing variability through conventional breeding and selection would be practically important. In the breeding process the poor germination of intact seeds of enset can be overcome by *in vitro* germination of excised embryos. Therefore, tissue culture techniques need to be used when developing a high intensity enset breeding program.

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Appendix 1: Preparation of MURASHIGE and SKOOG (MS) (1962) medium

Stock No.	Medium	Mass (g l^{-1})	Stock volume ml per a litre of final solution
1	NH_4NO_3	165	10
2	KNO_3	95	20
3	$CaCl_2.2H_2O$	44	10
4	$MgSO_4.7H_2O$	37	10
5	NaFeEDETA	4	10
6	KH_2PO_4	17	10
7	H_3BO_3	0.62	
	$MnSO_4.4H_2O$	2.23	10
	$ZnSO_4.7H_2O$	0.86	
	KI	0.083	
8	NA_2MoO_4	0.025	
	$CuSO_4.5H_2O$	0.0025	10
	$CoCl_2.6H_2O$	0.0025	
9	Thiamine HCl (Aneurine)	0.01	
	Niacin (Nicotinic acid)	0.05	10
	Pyridoxin	0.05	
	Glycine	0.2	

Pipettes were rinsed with distilled water after transferring each stock solution to a volumetric flask. All the stocks, 100 mg l^{-1} myo-inositol and sucrose were mixed in the volumetric flask, made up to just below volume using distilled water. It was stirred using a magnetic stirrer. The pH meter was calibrated using standard pH 7 and pH of the medium was adjusted to 5.8 and the volume was corrected with distilled water. Agar of 8 g l^{-1} without activated charcoal (AC) and 11 g l^{-1} with AC was used.