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SOMATIC EMBRYOGENESIS, EMBRYO CONVERSION, MICROPROPAGATION AND FACTORS AFFECTING GENETIC TRANSFORMATION OF *THEOBROMA CACAO* L.

A Thesis in

Food Science

by

Abdoulaye Traoré

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

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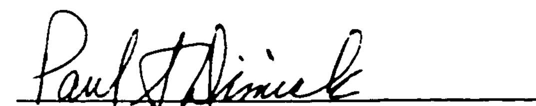

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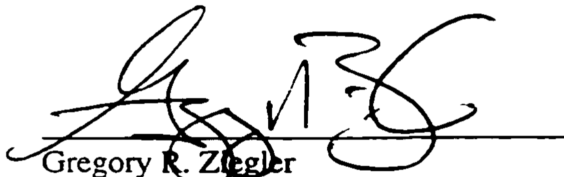

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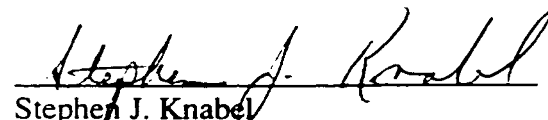

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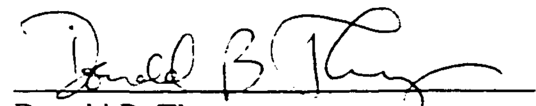

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ABSTRACT

The effects of 5 carbon sources (glucose, fructose, maltose, sorbitol, sucrose) and 2 explant types (staminode and petal) on cacao somatic embryogenesis were evaluated. Glucose, fructose and sucrose were found to support cacao somatic embryo production, while no embryos were produced with sorbitol or maltose in the media. Staminode explants were more responsive to somatic embryogenesis than petals, producing up to 99% embryogenic explants and over 20 embryos per responsive explant. In contrast, petal explants produced a maximum of 34% embryogenic explants and a maximum of 8 embryos per responsive explant. Four different media (MS, WP, DKW, and DKW supplemented with potassium nitrate and amino acids) were tested for their ability to support and promote shoot development during embryo conversion. DKW medium supplemented with potassium nitrate and amino acids yielded the highest percentage of normal and total shoot production (59% and 92% respectively). DKW and WPM media supported a higher percentage of conversion (77% each) than did MS medium (59%). The ivory colored type II somatic embryos more frequently produced taproots than did the translucent type I embryos (65% and 35% respectively). *In vitro* pruning of plantlets with abnormal shoots resulted in axillary meristem growth and conversion into normal plantlets with a rate of 79%. A micropropagation system, including shoot proliferation, was developed for cacao by pruning *in vitro* somatic embryo derived plantlets. The addition of TDZ to the medium did not affect shoot production from internodal and apical explants. Higher rooting efficiencies of shoot explants were achieved using IBA. Micropropagated and somatic embryo derived plants were acclimated to greenhouse conditions with a success rate averaging 90%. Genetic transformation of cacao staminode tissue was attempted using Agrobacterium or biolistic techniques. Transgenic callus and proembryonic tissue expressing GFP were recovered but no embryo development was obtained. Agrobacterium and the antibiotic cefotaxime at 400mg/L were found to negatively influence staminode explant growth and somatic embryo production.

TABLE OF CONTENTS

	Page
LIST OF FIGURES	x
LIST OF TABLES	xiii
ACKNOWLEDGMENTS	xiv
Chapter 1 INTRODUCTION AND STATEMENT OF THE PROBLEM	1
Chapter 2 LITERATURE REVIEW	6
ORIGIN AND DISTRIBUTION OF CACAO	6
GENOTYPIC VARIABILITY	7
Criollo	7
Forastero	7
Trinitario	8
ETHNOBOTANY OF CACAO	8
BOTANY OF CACAO	11
Plant Growth	11
The Leaves	12
The Trunk	13
The Root System	13
Flowers and Fruit Formation	14

PROPAGATION OF CACAO 15

Seeds 15

Vegetative Propagation Systems 16

Rooted Cuttings 16

Grafting 18

Budding 19

Tissue Culture 20

Somatic Embryogenesis 20

Somatic Embryogenesis From Zygotic Tissue 20

Somatic Embryogenesis From Maternal (Somatic) Tissue 22

Micropropagation 24

SOMACLONAL VARIATION 26

TELOMERE CHANGES DURING TISSUE CULTURE 28

Chapter 3 EFFECTS OF CARBON SOURCE AND EXPLANT TYPE ON SOMATIC EMBRYOGENESIS OF FOUR CACAO (*Theobroma cacao* L.) GENOTYPES 30

INTRODUCTION 30

MATERIALS AND METHODS 32

Plant Material 32

Media Formulations 32

Somatic Embryogenesis 33

Data Collection and Analysis 33

RESULTS 34

Effect of Carbon Source on Explant Growth 34

Effect of Carbon Source on Embryo Production 35

Influence of Genotype on Embryo Production 36

Influence of Explant Type 41

DISCUSSION 42

Chapter 4 CONVERSION AND ACCLIMATION OF SOMATIC EMBRYOS IN CACAO (*Theobroma cacao* L.) 46

INTRODUCTION 46

MATERIALS AND METHODS 48

Somatic Embryogenesis 48

Media Preparation 49

Culture Conditions 50

Data Analysis 50

In Vitro Pruning 51

Acclimation and Plant Development 51

RESULTS 52

Effect of Different Basal Salts 52

Relation Between Embryo Morphology and Conversion 54

Effect of *in Vitro* Pruning 58

Acclimation and Plant Development 59

DISCUSSION 64

Chapter 5 A MICROPROPAGATION SYSTEM FOR CACAO (*Theobroma cacao* L.) USING SOMATIC EMBRYO DERIVED PLANTLETS 69

INTRODUCTION 69

MATERIALS AND METHODS 71

Plant Material 71

In Vitro Pruning 71

Root Induction 72

Root Development and Plantlet Maintenance 72

Effect of TDZ on Internodal and Apical Explants 72

Plant Acclimation 73

Shoot Proliferation 73

RESULTS 74

Effect of TDZ on Axillary Bud Development of Internodal and Apical Explant 74

Shoot Proliferation 75

Effect of IBA on Rooting 79

Acclimation of Micropropagated Plantlets 83

DISCUSSION 86

Chapter 6 FACTORS LIMITING GENETIC TRANSFORMATION OF CACAO (*Theobroma cacao* L.) 91

INTRODUCTION 91

MATERIALS AND METHODS 92

RESULTS AND DISCUSSION 94

Chapter 7 CONCLUSION AND FUTURE PERPESPECTIVES 101

Appendix A MEDIA FORMULATIONS 107

Appendix B COCOA GREENHOUSE FERTILZER 120

REFERENCES 122

LIST OF FIGURES

Figures:		Page
Figure 3.1	Growth of cacao petal and staminode explants in response to five different carbon sources	37
Figure 3.2	Effect of carbon source on growth of Scavina 6 cacao staminode and petal explants during somatic embryogenesis	38
Figure 3.3	Effect of carbon source and explant type on somatic embryo production in four cacao genotypes using DKW based protocol	39
Figure 3.4	Effect of carbon source on somatic embryo induction from 4 cacao genotypes using PCG media	40
Figure 4.1	Effects of media type on the conversion of Scavina 6 cacao somatic embryos	53
Figure 4.2	Somatic embryo conversion and plant regeneration in cacao	56
Figure 4.3	Relation between embryo type and shoot production during conversion of Scavina 6 cacao somatic embryos	57

Figure 4.4	Relation between embryo type and root production during conversion of Scavina 6 somatic embryos	57
Figure 4.5	Growth of cacao seedlings and S.E. derived plantlets during acclimation	60
Figure 4.6	Development of somatic embryo derived plant during and after acclimation	63
Figure 5.1	Internodal and apical shoot culture of cacao somatic embryo explants	76
Figure 5.2	Effect of TDZ on axillary bud development of internodal and apical explant from cacao somatic embryo derived plantlets	77
Figure 5.3	Shoot proliferation after *in vitro* pruning of cacao somatic embryo derived plantlets	78
Figure 5.4	Rooting of somatic embryo derived cacao shoot tips	81
Figure 5.5	Effect of IBA induction time on rooting of cacao shoot explants	82
Figure 5. 6	Acclimation of micropropagated cacao plantlets	85
Figure 6.1	Simplified diagram of Agrobacterium mediated genetic transformation and somatic embryogenesis of cacao using staminode tissue	97

Figure 6.2 Somatic embryogenesis from cell culture and genetic transformation using the biolistic method 98

Figure 6.3 Factors affecting somatic embryogenesis during genetic transformation with Agrobacterium 99

Figure 6.4 GFP expression in cacao staminode tissue explant after genetic transformation with Agrobacterium 100

Figure 7.1 Integrated system for clonal propagation of cacao (*Theobroma cacao* L.) 106

LIST OF TABLES

Table		page
Table 4. 1	Morphological characteristics of type I and type II cacao somatic embryos	55
Table 4. 2	Effect of *in vitro* pruning on conversion of plantlet with abnormal shoots	59
Table 4. 3	Response of seven different cacao clones to acclimation	62
Table 5.1	Response of three different cacao clones to shoot proliferation after *in vitro* pruning	79
Table 5.2	Response of three different cacao clones to *in vitro* rooting	83
Table 5.3	Response of three different cacao clones to acclimation	84

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Chapter 1

INTRODUCTION AND STATEMENT OF THE PROBLEM

Theobroma cacao L. is a tropical tree crop originating from the Amazon basin. Because of special fat and flavor content of its seeds, cacao has evolved from an Aztec drink into a multimillion dollar chocolate industry, playing a very important role in the economy of many tropical countries where it is cultivated. Most of the cacao plantations around the world contain seed derived plants, and because of genetic segregation in seedlings, only a small percentage of trees in the plantations are elite plants. A large number of trees that occupy valuable space in the field are very susceptible to pests and diseases or do not have acceptable yield. This has negatively affected the profit margin of most farmers who sometime abandon cocoa farming. In order to have higher production, many farmers have to create larger farms, (for example in Africa) so they clear more rainforest causing an environmental concern.

Because of long generation time, a complex genetic incompatibility between clones and the narrow genetic background of cultivated cacao, genetic improvement trough classical breeding has been slow and difficult (Kennedy et al., 1987). Clonal propagation of elite plants has long been regarded as a good means to improve cacao

production, and vegetative propagation using various systems for grafting and rooting of cuttings have been developed (Wood and Lass 1985).

During development, a cacao tree exhibits a dimorphic growth pattern that starts with a vertical or orthotropic growth to form the main trunk. Approximately 12 to 18 months after germination a crown of 3 to 5 horizontal branches called a jorquette is formed after the disintegration of the apical meristem. These branches exhibit a horizontal or plagiotropic growth to form the tree canopy. Because of their abundance, plagiotropic shoots have been the main source of explants for vegetative propagation in cacao (Cheesman, 1936). However plants produced from such explants do not exhibit a typical cacao dimorphic growth, and require training to form an acceptable canopy (Cheesman, 1936; Bertrand and Agbodjan, 1989). In addition, plagiotropic rooted cuttings lack a taproot and can be susceptible to drought in regions with prolonged dry seasons like Africa (Bertrand and Agbodjan, 1989). Vegetative propagation of cacao using orthotropic rooted cuttings can produce plants with a taproot and a dimorphic growth, but compared to plagiotropic shoots, orthotropic ones are produced in limited numbers by the tree (Cheesman, 1936; Bertrand and Agbodjan, 1989).

The development of plant tissue culture offers an alternative method to clonally propagate a large number of true-to-type elite cacao plants. However, many attempts to develop *in vitro* shoot propagation systems for cacao have shown its recalcitrance to tissue culture (Orchard et al., 1979; Passey and Jones, 1983; Flynn et

al., 1990; Figueira and Janick, 1994; Lardet et al., 1998). Since first reported by Essan (1977), somatic embryogenesis has been regarded as an alternative method to clonally propagate cacao. Although early developments in cacao somatic embryogenesis have produced embryos from zygotic tissues (Essan, 1977; Passey et al., 1980), plant regeneration from these embryos has been problematic (Wang and Janick, 1984; Duhem et al., 1989; Wen et al., 1991). Additionally, because of genetic segregation in seeds, clonal propagation from immature zygotic tissue explants used in that system has a limited value (Alemanno et al., 1997). Somatic embryogenesis and plant regeneration systems using explants from maternal tissues would allow the propagation of true-to-type elite plants. In addition, it can be used to develop a genetic transformation system for cacao.

Cacao somatic embryogenesis (SE) using maternal tissue was first reported by Sondahl et al. (1989) using immature petals, however plant conversion was limited. Cacao somatic embryos have been produced from different flower parts (Lopez-Baez et al., 1993; Sondahl et al., 1993; Alemanno et al., 1996; Li et al., 1998) and nucellar tissue (Figueira and Janick, 1993). In spite of the recent success in somatic embryo production by some groups (Lopez-Baez et al., 1993; Sondahl et al., 1993; Alemanno et al., 1997), conversion and acclimation of cacao somatic embryos into plants has remained a limiting step in the practical application of the system to clonally propagate cacao. To date only four studies reported successful plant regeneration from cacao somatic embryos (Sondahl et al., 1993; Lopez-Baez et al., 1993;

Alemanno et al., 1997 and Li et al., 1998). Although a routine recovery of large numbers of viable plants from cacao somatic embryos was reported by Li et al. (1998), the efficiency of embryo production remains low in many genotypes (Lopez-Baez et al., 1993; Alemanno et al., 1996; Li et al., 1998).

While these reports on cacao somatic embryogenesis have focused on optimizing growth regulators as a means to improve somatic embryogenesis and plant recovery, comparative studies of basal salts and carbon sources have not received attention. Traditionally, MS medium (Murashige and Skoog, 1962) and sucrose have been used to produce cacao somatic embryos (Essan, 1977; Pence et al., 1980; Wang and Janick, 1984; Adu-Ampomah et al., 1987; Sondahl et al., 1993; Lopez-Baez et al., 1993; Alemanno et al., 1996). However in each case embryo conversion remained difficult. Elhag et al. (1987) conducted a comparative study of sucrose, glucose and fructose effects on cacao somatic embryogenesis using calli induced from zygotic tissues. These authors indicated that embryogenesis increased when sucrose was replaced by glucose or fructose.

Somatic embryogenesis using tissue of maternal origin has also been reported using sucrose (Sondahl et al., 1989; Figueira and Janick, 1993; Lopez-Baez et al., 1993; Alemanno et al., 1997). Recently, Li et al., (1998) reported a cacao somatic embryogenesis procedure using maternal tissue (staminode and petals), along with DKW medium (Driver and Kuniyuki, 1984) and glucose alone or in combination with sucrose. With this new procedure Li et al. (1998) also reported a routine plant

recovery. In spite of this progress, somatic embryogenesis remains expensive and needs to be optimized further in order to be a practical method of cacao clonal propagation. A comparative study of various carbon sources, tissues explants and basal salts will identify the most appropriate combination of these factors for cacao tissue culture. It takes at least 4 to 6 months to produce plantlets from staminodes using somatic embryogenesis. The development of a micropropagation system using the plantlets from somatic embryogenesis will allow a quick multiplication step that will also increase its efficiency.

The objectives of this project were:

1) To optimize embryo production by identifying better carbon sources, media and explant types for cacao somatic embryogenesis.
2) To improve embryo conversion by identifying the best type of embryos for conversion and improving culture conditions for the conversion of all types of cacao somatic embryos.
3) To monitor somatic embryo derived plants during growth and development for any abnormalities.
4) To increase the efficiency of plantlet production by developing a micropropagation protocol using somatic embryo derived plantlets.
5) To develop a genetic transformation and plant regeneration system for cacao using somatic embryogenesis

Chapter 2

LITERATURE REVIEW

Origin and distribution of cacao

Theobroma cacao L. is a member of the Sterculiaceae family, which also includes *Herrania, Guazuma* and *Cola*. Of the twenty-two species that are included in the genus, *Theobroma cacao* is the only one cultivated. Other well-known species are *Theobroma bicolor* and *Theobroma grandiflorum*. *Theobroma cacao* originated from the central Amazon and spread westward and northward to the south of Mexico forming two major populations separated by the Panama isthmus (Cuatrecasas, 1964). These two populations evolved to be the Criollo type found mostly in Central America and the Forastero, which is found in South America. A third group of cacao called Trinitario was derived from crosses between Criollo and Forasteros in Trinidad. In the 16th century cacao was well established in Central America where it was rediscovered by the Spanish who later disseminated it in the Caribbean and South America (Wood and Lass, 1985). Around 1600, cacao was spread across the Pacific Ocean to the Philippines and later to Sulawesi, Java, Sri Lanka and India. Introduction of cacao cultivation in Africa begins in Saõ Tomé in 1822 with Amelonado cacao (Forastero sub-type) from Bahia in Brazil. From Saõ Tomé, cacao was spread to Fernando Po in 1855 and later to Nigeria, Ghana, and Côte d'Ivoire

(Wood and Lass, 1985). However, in Cameroon, cacao was introduced in the Victoria region by the Germans using a collection of Trinitario pods from South America (Wood and Lass, 1985).

Genotypic Variability

Criollo

Criollos were the original cacaos domesticated by the Maya Indians. The group is characterized by its good aroma, but it has a weak chocolate flavor. Criollo beans do not require a long period of fermentation. The pods are green or red in color, soft in texture and contain less than 30 beans, whitish or light purple in color. Due to their susceptibility to major cacao diseases such as Phytophtora, Ceratocystis and Mirids, Criollo cultivation has been progressively abandoned (Wood and Lass, 1985). The group is now found in isolated populations in Central America, Caribbean Islands, Madagascar, Comoro Island, Sri Lanka, Indonesia (Java) and the Samoa islands (Mossu, 1992).

Forastero

This group includes most of the cacao cultivated today or found in the wild. It is characterized by its vigor, strong chocolate flavor and the high fat content of its beans. The pod of a Forastero cacao is hard and contains a large number of beans (more than 30) which are dark purple in color. Forasteros are a very heterogeneous

population that contains the West African Amelonado type, the Comun and Para type found in Brazil, and variable other types named according to the river in the regions where they were collected (Mossu, 1992).

Trinitario

This group is highly heterogeneous and exhibits characteristics intermediate between Forasteros and Criollos. It is believed to be derived from crosses between Forasteros and Criollos in Trinidad (Wood and Lass 1985). The pods, variable in shape and color, contain beans that produce chocolate of quality intermediate of Criollos and Forastero. Trinitarios are grown all over the world and have replaced most of the Criollos in Central and South America.

Ethnobotany of cacao

Cocoa was extensively used by the Maya and Aztec Indians, when it was rediscovered by the Spanish explorers Christopher Columbus and Hernando Cortés in the early 1500s (Lupien, 1999). Roasted cocoa beans were ground and mixed with maize, water and spices to make a traditional beverage called "chocolatl", which was consumed in large quantities by the Aztec emperor Montezuma (Cook, 1982). In addition to being used for making a traditional drink, cocoa beans were important in medicine and traditional rituals of the Mayas and Aztecs, who also used them as

currency. The tree was believed to have a divine origin by these populations and that prompted the Swedish botanist Linnaeus to name it *Theobroma* which translates as food of the gods in Latin (Lupien, 1999).

From the Americas cocoa was introduced into Europe by the Spanish explorers. To make the cocoa drink more palatable for Europeans, sugar, cinnamon and vanilla were added to the original recipe. During that early phase of cocoa consumption in Europe, cocoa drink was accessible only to the rich and famous. Later its popularity grew and many claims about the medicinal, aphrodisiac, and nutritional values of the cocoa drink were made (Lupien, 1999). As consumption increased, technical advances for the improvement of cocoa drink began to emerge. The first advance was the extraction of cocoa butter in 1828 by Van Houten using a press (Wood and Lass 1985). The fat extraction improved the palatability of the drink and made available cocoa butter used for the development of chocolate. Cocoa butter has a unique characteristic of being solid at room temperature but easily melts at body temperature. So its use for chocolate gives the chocolate candy a desirable mouth feel. Later the addition of milk solid to chocolate by Daniel Peter in Switzerland in 1876, led to the production of the popular milk chocolate. Chocolate became an important element for the candy industry and a key ingredient in the modern snack bar. Beside the chocolate industry, cocoa butter, because of its ability to melt at body temperature is used in the cosmetic and pharmaceutical industry for the manufacturing of lotions, cremes and suppositories. As chocolate became popular

in early 1900s, consumption grew rapidly and cocoa farming began to develop around the world, especially in West Africa where it was newly introduced.

As production and consumption increased, cocoa became not only an important commodity for a multimillion dollar chocolate industry in many industrialized countries, but also a source of cash for the developing countries and their populations that grow it. Since the 1920s cocoa production has shifted from the Americas to Africa (Lass, 1999). Ghana was the world main producer in the 1920s (with 118, 000 metric tonnes per year) until the early and mid 1980s when it was surpassed by Côte d'Ivoire which produced over 1,000,000 metric tonnes per year since 1996 (Lass, 1999). In these African countries, cocoa has contributed to the economic development at both the government and farmer levels. Cocoa plantations are a source of wealth in many villages and are sometimes the only inheritance of economic value passed to the next generation. In addition, cocoa is a source of cash income for many temporary workers that move to Côte d'Ivoire from northern neighboring countries for the cocoa harvest. Although cocoa beans are not used as a currency, in some of these countries, temporary workers are often paid in cocoa beans by plantation owners. For example, in Côte d'Ivoire, after the harvest, the temporary workers get 1/3 of the beans as their payment while the plantation owner keep 2/3 of the production. This arrangement encourages the worker to maintain the plantation and harvest it efficiently, since their payment depends on the overall yield of the

plantation. Today cocoa beans do not have a significant traditional use per se, the production is mainly for the industry.

Botany

Plant growth

Cacao growth and development can be divided into three phases (Wood and Lass, 1985). In the first phase (4 to 6 days after planting) the seeds germinate with a rapid extension of the rootlet. During this phase the hypocotyl vigorously grows vertically to raise the cotyledons to a height of 8 to 12 cm (Mossu, 1992). At that point there is no shoot on the hypocotyl, and that makes this zone a prime target for grafting in order to avoid future shoot development from the rootstock. In the second growth phase the cotyledons open which allows the first stem and leaves to develop and harden. After the hardening of these first leaves, the plant continues to grow vertically producing a new flush of leaves about every six weeks. These first two phases of development exhibit only vertical growth producing an orthotropic stem.

The last phase of development starts when the plant (12 to 18 month old) reaches a height of 1 to 2 m. At that point the apical meristem aborts and vertical growth is terminated. Three to five new axillary shoots appear simultaneously below the aborted apical meristem, to form a crown of fan branches called the jorquette (Greathouse and Laetsch, 1969). From this point on, these branches begin a horizontal or plagiotropic growth to form the main branches of the tree.

The leaves

Cacao leaves exhibit a different phyllotaxy depending on the shoot they originated from (Greathouse and Laetsch, 1969). Leaves from orthotropic shoots have long petioles and show a spiral phyllotaxy while, leaves derived from plagiotropic growth on the fan branches possess shorter petioles and exhibit alternate phyllotaxy (Greathouse and Laetsch, 1969). Leaf size in cacao varies according to the genotype with an average leaf size of 20 cm long and 8 to 10 cm wide (Mossu, 1992). Stomata are present only on the abaxial side of the leaf. Leaf production in cacao is rhythmic with a series of flushes. Based on external morphology, each rhythmic growth is divided into five phases (Greathouse et al., 1971). Each flush begins with a break of the bud dormancy (phase F1) during which the stipules surrounding the bud spread apart and the bud swells, indicating the beginning of growth. Following phase F1 is phase F2, during which the stem elongates, the leaves become visible and start to expand. Leaves from new flushes are soft and depending on genotype, vary in color from a pale green to red. The next 3 phases (I1, I2 and I3) are called "interflush". Interflush 1 (I1), during which the dormant shoot apex is formed, starts when the last formed leaf reaches 1.5 cm and ends when all the leaves have reached the deep green color characteristic of mature cacao leaves. Following I1 is I2 which ends when the stem harboring the leaves turns brown. The last phase, I3 ends at the beginning of F1.

The trunk

The trunk is derived from the first orthotropic growth phase. It measures about 1.5 m and supports 3 to 5 fan branches that constitute the jorquette. During the orthotropic growth phase, the young stem produces leaves with long petiole arranged spirally (Greathouse and Laetsch, 1969). These leaves are lost as the orthotropic stem matures to form the trunk. At maturity, in addition to the mature fan branches, the trunk of a cacao tree bears a large number of fruits (technically a drupe but commonly called pods). These pods are produced from the pollination of a large number of flowers that develop from flower cushions formed on the trunk and mature fan branches. At the base of the trunk, several vertically growing orthotropic shoots called chupons may be produced. These chupons which could be considered as a natural means of vegetative propagation for the tree, are often removed and discarded by farmers (Cheesman, 1936). In recent years, they are being regarded as a means to clonally propagate cacao using orthotropic shoots (Amefia et al., 1985; Bertrand and Agbodjan, 1989; Glicenstein et al., 1990).

Root system

The cacao root system is characterized by the presence of a taproot from which several lateral roots develop. These lateral roots branch to form a network of small roots that can cover an area beyond the canopy of the tree. The presence of the taproot is visible early after germination. The main taproot of a mature cacao tree ranges from 0.8m to 1.5m in length and helps to anchor the tree (Mossu, 1992).

Bertrand and Agbojan (1989) indicated that plagiotropic rooted cuttings that lack a taproot began taproot formation after they were induced to produce orthotropic shoots by bending. This shows a relation between shoot orthotropic growth and taproot formation and explains the lack of taproot on most of the plagiotropic cacao rooted cuttings. That lack of taproot and a canopy training requirement of cacao trees derived from plagiotropic rooted cuttings, have limited their expansion in growing areas such as West Africa (Bertrand and Agbodjan, 1989).

Flower and fruit formation

The cacao tree produces a large number of flowers. Depending on the physiological state of the tree, and the environment, flowering can occur throughout the year or at specific peak times separated by periods of no flowering (Mossu, 1992). The flowers are small (0.5 to 1 cm in diameter), and are produced on the trunk or old branches from axillary buds that develop into an inflorescence with short branches. After several flowering and fruit bearing seasons, the inflorescence which is technically termed a cincinnal cyme, forms a thick region on the stem to produce a zone called a flower cushion (Purseglove, 1987). Each flower is attached to the bark of the tree by a long pedicel and contains; 5 free sepals, 5 free petals, 5 sterile stamens called staminodes, 5 fertile stamens with a tetra-lobed anther, and an ovary with 5 united carpels containing the ovules. At maturity the flowers start to open in the afternoon and remain so until the next morning. The pollen is released and pollination is carry out by midges (small insects that belong to the genus *Forcipomyia* of the Ceratopogonidae family).

There is a complex system of fertilization incompatibility in cacao. Unlike most plants, cacao incompatibility resides at the ovary and ovule level (Mossu, 1992). Most cacao pollen is able to germinate on the stigma and penetrate the style (Wood and Lass, 1985). Although successful pollination can be observed after 3 days with the swelling of the ovary, pod abortion can occur several weeks later due to incompatibility that stops gametic fusion inside the young pod (Wood and Lass 1985). Because of the incompatibility, the short range (50 m) of the pollinating midges and flower anatomy, only 1 to 5 percent of flowers produced by the tree are successfully pollinated to produce pods (Wood and Lass, 1985; Mossu, 1992).

After a successful fertilization, the young pod begins a slow growth for about 40 days after which a faster growth period is initiated. During the slow growth period, the zygote remains a single cell, it starts dividing during the fast growth period. This fast growth period is followed by an embryo development period after which fat accumulation in the cotyledon begins. After pollination, it takes 5 to 6 months for the pod to develop and ripen. Depending on the genotype, each pod can produce up to 60 seeds or beans with varying size, color (white to dark purple) and elliptic or ovoid shape (Purseglove, 1987).

Propagation of cacao

Seeds

Cacao is naturally propagated by seeds. Each fruit or pod contains up to 60 seeds that have no dormancy and germinate upon fruit ripening. Because of the lack

of dormancy in the seed, cacao germplasm is preserved around the world as tree clones. Seed propagation is convenient because of its abundance and low cost. So it is the most used method of propagation around the world. In modern breeding programs, hybrid seeds are produced from a cross between clones that have undergone a genetic selection process over several years. The combining ability of these parents is evaluated and selected parental clones are used for the establishment of seed gardens (Purseglove, 1987). For greater accuracy and control, manual pollination (usually by hands) is used to evaluate selected crosses. In addition to control and accuracy, manual pollination does not require the pollen parent to be in the same plot or area as the seed one. However, natural pollination can be set up by planting clones of pollen parents in the same plot with compatible seed parent (Mossu, 1992). That later system of pollination does not require any labor from a trained pollinator but lacks the accuracy of manual pollination. The production of a large number of individuals from the same clone to be used in breeding programs requires vegetative propagation of that clone.

Vegetative propagation

Rooted Cuttings

Conditions for rooted cutting production in cacao have been clearly defined by Evan (1951). These conditions include almost 100% humidity, the use of semi-hard plant materials with leaves (that can be trimmed to 1/3-1/2 of their original size), a coarse rooting medium and the application of rooting hormone. However Jacob and

Opeke (1967) found that cacao cuttings can be successfully rooted without hormones. Several studies on cacao rooted cuttings revealed a variation in the rooting ability of different cacao genotypes (Evan, 1951; Hall, 1963; Jacob and Opeke, 1967). Cacao rooted cuttings can be produced from chupons or from the fan branches. Because of their large numbers, fan branches, have been the main sources of rooted cuttings in cacao. However, in cacao, the buds on the cutting retain the morphological growth type of the shoot from which they were derived (Cheesman, 1936). Since fan branches exhibit plagiotropic growth, the rooted cuttings produced from them are problematic (Cheesman, 1936). They branch early to produce a bushy plant without a defined canopy so they require pruning in order to produce an acceptable canopy. In addition to the problematic growth habit, plagiotropic rooted cuttings have an adventitious fibrous root system that lacks a taproot (Cheesman, 1936; Bertrand and Agbodjan, 1989). Despite these difficulties, plagiotropic rooted cuttings have been used to establish plantations in Trinidad, Malaysia and Ecuador. However, due to a well-defined and sometimes prolonged dry season, plagiotropic rooted cuttings have not been successful in Africa (Bertrand and Agbodjan, 1989).

Recently more effort was put into the development of an orthotropic rooted cutting system for cacao (Amefia et al., 1985; Bertrand and Agbodjan 1989; Glicenstein et al., 1990). Unlike plagiotropic rooted cuttings, orthotropic ones are derived from orthotropic shoots such as chupons and exhibit cacao dimorphic growth with the formation of a taproot. However, because of their limited number, chupons have not been easily available for rooted cuttings. To increase the number of orthotropic shoots, several methods of inducing a high number of orthotropic shoots were developed (Amefia et al., 1985; Bertrand and Agbodjan, 1989; Glicenstein et

al., 1990). These methods include bending the plants which triggers the development of orthotropic shoots from the axillary shoots (Amefia et al., 1985; Bertrand and Agbodjan 1989; Glicenstein et al., 1990). In addition to bending, decapitation, pruning and pollarding (removal of fan branches) of the cacao tree have been used to induce orthotropic shoot production (Bertrand and Agbodjan, 1989). The subsequent rooting of such cuttings produced plants with the typical cacao dimorphic growth, although the height of jorquette formation remained lower than in seed derived plants (Bertrand and Agbodjan, 1989). In addition to the dimorphic growth these plants developed a functional taproot.

Grafting

Cacao can be propagated using grafting. The grafting method does not require a large investment in material, however its practice requires a skilled worker (Mossu, 1992). Grafting can be performed on chupons growing from a mature tree or on seedlings, which allows the presence of a typical cacao root structure with taproot. When a mature tree is used for grafting, after a successful graft, the tree is cut down to allow a fast growth of the scion. Several methods of grafting in cacao have been developed over time. These include lateral grafting (in which the donor and recipient plant are attached together after an oblique cut is made), the cleft grafting (in which the donor and recipient plant are united by a cleft), and budding (in which a single bud is removed from a bud-wood stock and grafted onto a seedling).

It was believed that grafting on seedlings at the cotyledon stage above the cotyledons would increase the percentage of success because the seed reserve stored

in the cotyledons will be accessible to the scion (Jacob, 1967). However the possibility of chupons growing from such rootstock have resulted in the replacement of the grafting above the cotyledons by grafting below them where there is no shoot (Ascenso, 1968). After grafting, the bud from the donor plant retains the growth characteristics of the shoot from which it was removed. Buds from chupons retain orthotropic growth while those from fan branches exhibit a plagiotropic growth. Because of the pruning required for training a tree derived from a plagiotropic growth in order to form a desirable canopy, shoots from chupons are more desirable for grafting (Cheesman, 1936).

Budding

This method of grafting uses a single bud from the donor plant, increasing the number of plants that can be obtained from a limited amount of bud-wood. There are two major types of budding techniques; patch budding and the inverted T budding. The two types have been used in Saõ Tomé for clonal propagation of high yielding planting material (Ascenso, 1968). In this study, successful budding was obtained on 10 to 12 months old seedlings and chupons from mature trees. To avoid the development of shoots from the recipient plant, budding was done below the cotyledon level where there is no shoot. In order to reduce the time and cost of the budding practice, a nursery based budding, applicable to young seedlings (2 to 12 weeks old) was developed by Giesberger and Coester (1976). In this study, the authors reported 70% to 100% success rates using 2 to 12 week old seedlings as rootstock instead of 10 to 12 month old seedlings as reported by Ascenso (1968).

They also developed a mechanical budding tool to facilitate the budding process (less than 1.5 min per budding operation). This technique is currently used in Asia and South America for propagation of elite cacao plants. However, for unknown reasons, grafting in general and budding in particular have not been very successful in West Africa (Wood and Lass, 1985).

Tissue culture

Somatic embryogenesis

Somatic embryogenesis (SE) can be defined as a process by which cells undergo bipolar development to give rise to a whole plants by means of adventitious embryos that occur without the fusion of gametes. Depending on the origin of the tissue from which the process is initiated, somatic embryogenesis can be of a zygotic origin (immature embryos from seeds) or a somatic origin (leaves, roots, petals, anther filaments, staminodes or ovaries). In cacao both types of somatic embryogenesis have been developed by several authors.

Somatic embryogenesis from zygotic tissue

Somatic embryogenesis from zygotic tissue (SEZ) has first been observed in cacao tissue culture by Essan (1977) who reported buddings of adventitious embryos on immature zygotic embryos when cultured *in vitro*. After that report Pence et al. (1979, 1980) developed a protocol for the production of somatic embryos in cacao

using MS medium (Murashige and Skoog, 1962), sucrose, coconut water, naphthalene acetic acid (NAA), and casein hydrolysate. Early in the development of somatic embryogenesis, Pence et al. (1980) recognized two distinctive morphogenetic processes ("budding" and "non budding"). In the budding process, which is the most prolific, embryos are formed on a suspensor-like stalk originated from smooth green ovoid outgrowths from internal meristematic cotyledonary tissues. In contrast embryos from a non-budding process are formed directly from the swelling and folding of internal meristematic cotyledonary tissue. In addition to the stimulating effect of coconut water and auxins on somatic embryogenesis, Pence et al. (1980) also determined that younger zygotic tissues were more responsive. After these pioneering reports, several protocols for somatic embryos production were later developed using MS basal salt media, sucrose and auxins (Kononowicz and Janick, 1984; Elhag et al., 1987; Adu-Ampomah et al., 1988; Duhem et al., 1989; Wen and Kinsella, 1991). Although embryos could be produced from each of these protocols, their conversion into plantlets was difficult (Adu-Ampomah et al., 1988; Duhem et al., 1989; Wen and Kinsella, 1991). Somatic embryos converted into plantlets with taproots and true leaves were reported by Wen and Kinsella (1991) using liquid media and naphthalene acetic acid (NAA) for two weeks, followed by a transfer to solid media supplemented with abscissic acid (ABA). However no data were reported by the authors on the conversion success rate. Using gibberellic acid (GA3) in combination with cotyledon excision, a total of 4 embryos were successfully converted into plantlets by Adu-Ampomah et al., (1988), but no further development was reported by the author. Duhem et al., (1989) accomplished further development and hardening of somatic embryo derived plantlets with a 25% success rate. In this

study the authors used cytokinins (Zeatine and 2iP (6γ-γ-dimethylallylamino purine)) to promote shoot development, but callus development inhibited their growth. The addition of activated charcoal to the media by the same authors reduced callus growth and 30% of embryo conversion, 25% of plantlet hardening and further development was achieved. In order to improve plantlet development, Anguilar et al. (1992) micrografted somatic embryos onto seedling rootstock and obtained a 30% success rate. Since plants regenerated from zygotic tissue are genetically different from the parent plants, they are from an unproven genetic combination and not desirable for clonal propagation. For this reason, there is a need for vegetative propagation systems that could produce true-to-type plants.

Somatic embryogenesis from maternal (somatic) tissue

Unlike zygotic tissues, somatic tissues have the same genetic make up as the original parent donor. Plants regenerated from elite clones using somatic embryogenesis will theoretically also be elite plants. Somatic embryogenesis in cacao was first reported by Litz (1986) using leaf derived callus and MS media supplemented with high hormone concentrations. However these embryos were developmentally arrested and did not proceed beyond a heart stage. Sondahl et al. (1989) initiated somatic embryogenesis using cacao petal tissue. Embryos from petal tissues were cultured to maturity and germinated plantlets were obtained in a limited number, but no data were reported on conversion and acclimation of the plantlets (Sondahl et al., 1993). This method used MS medium, sucrose, auxin, and coconut water for embryo induction. In the same protocol, a multi-step embryo maturation and

conversion procedure was described using woody plant medium (Lloyd and Mcown, 1980), activated charcoal, and several combinations of growth regulators. Using nucellar tissue from immature pods, and an MS based media formulation, Chatelet et al. (1992) produced embryogenic calli and globular stage cacao somatic embryos. However no embryos were recovered at a later stage. Similarly, Figueira and Janick (1993) produced somatic embryos from nucellar tissue using MS, sucrose, coconut water, 2,4 D (2,4-dichlorophenoxyacetic acid), 2iP, malt extract and vitamins. These embryos were later matured in liquid culture with half strength MS, sorbitol and sucrose, however no plants were recovered. In 1993, Sondahl et al. reported somatic embryo production from petal and nucellar tissue with efficiencies of 4.3% and 2.0% respectively, followed by plant regeneration. These embryos were also produced using MS, sucrose, 2,4 D, and kinetin. Lopez-Baez et al. (1993) developed a simple protocol for cacao somatic embryogenesis using flower parts (petals, staminodes and anther filaments). That protocol used MS medium, sucrose, 2,4 D, KN (kinetin), amino acids, and coconut water to induce embryos. Maturation and conversion was realized using reduced MS concentration, growth regulator, sucrose or glucose, amino acids and activated charcoal. However its application was limited to only 5 genotypes and the percentage of embryo production per explant was around 18%. In an attempt to define limiting factors involved in the inefficient development of cacao somatic embryos, Alemanno et al. (1997) did a comparative study of cacao somatic and zygotic embryogenesis using the same protocol as Lopez-Baez et al. (1993). The authors concluded that adding sucrose and ABA to the maturation medium was effective in increasing reserve synthesis in somatic embryos and resulted in higher germination, conversion and acclimation rates. However only 21.4% of the embryos

could be acclimated from the study. Li et al. (1998) reported somatic embryogenesis in cacao using TDZ (thidiazuron), DKW medium (Driver and Kuniyuki, 1984) and glucose. That protocol showed an improvement over previous ones by its applicability to a wide range of genotypes (19 genotypes) and the high percentage of embryos production (up to 100%). In addition, a large number of plants (over 200) were recovered from that study.

Micropropagation

As an alternative *in vitro* propagation system to cacao somatic embryogenesis, *in vitro* shoot multiplication has been attempted. The first attempt to culture cacao shoots *in vitro* was reported by Orchard et al. (1979). These authors tested shoot apices at various states of flush as described by Greathouse et al. (1971) and indicated that only buds taken at the I-2 stage showed signs of growth when cultured *in vitro*. In the same report, the authors reported that bud break or bud development occurred only when the culture medium was supplemented with KN or GA3 where as ABA inhibited bud development. However, no rooting or plantlets were produced by the study. Essan (1981) attempted shoot organogenesis by inducing callus from cacao embryo axis. Although shoots with leaves or leaf-like structures were obtained by the author, roots were not observed from these shoots and no plants were recovered.

The first successful *in vitro* rooting of cacao shoot explants was achieved by Passey and Jones (1983) by culturing shoot tips or nodal explants on media supplemented with IBA (indole-3 butyric acid) or NAA (naphthalene acetic acid). These authors also indicated that the addition of BAP (6-benzylaminopurine), Z

(zeatin) or ZR (zeatin riboside) separately promoted shoot extension and leaf expansion followed by shoot proliferation. However further development of the rooted plantlets and plant recovery for acclimation was not reported in the study.

Using *in vitro* germinated cacao seedlings as explants, Janick and Whipkey (1987) reported that decapitation and BA (6-benzalmino-purine) treatment alone or in combination increased axillary branching of the cotyledonary node. But when detached for further culture, these axillary branches failed to grow, proliferate or root *in vitro*. Using stem segments from orthotropic or plagiotropic shoots of a six-year-old cacao tree, Flynn et al. (1990) accomplished the first micropropagation that resulted in the recovery of cacao plants. In that report the authors demonstrated that cacao explants grown on woody plant medium (Lloyd and McCown, 1980) were healthier (continue growth without leaf chlorosis and abscission) than those grown on MS medium (Murashige and Skoog, 1962). The same authors indicated that the development of basal buds on the primary explant was promoted by the removal of the shoot apex. They also reported subsequent rooting of the developed shoots *in vitro* and plantlet production, followed by acclimation to greenhouse conditions. Although plants were recovered in that study, a significant proliferation step was not established. In their attempt to improve shoot growth and development Figueira et al. (1991) reported that increased CO_2 and light promoted *in vitro* shoot growth and development. The same authors recorded an average of 6.4 shoots per cotyledonary node explant when TDZ was used. However no plants were recovered in the study.

Another attempt to micropropagate cacao *in vitro* was reported by using gamma ray irradiation to reduce callus growth, however no plant recovery was reported (Adu-Ampomah et al., 1992). Lardet et al. (1998) cultured cacao stem

explants from various positions on the stems and at various morphogenic stages. The authors showed that in addition to the physiological stage of the stems, the position of the bud or node on the stem with respect to the apex affected their response during *in vitro* culture initiation. They indicated that branches taken at stages I1, I2 or I3 exhibited higher *in vitro* responses than those taken at stages F, and that was an agreement with previous findings by Orchard et al. (1979). Lardet et al. (1998) also found that nodes furthest from the apex were more likely to develop into shoots when cultured *in vitro*. Since the report by Flynn et al. (1990) there have been no reports of a successful *in vitro* micropropagation system that resulted in acclimated cacao plants.

Somaclonal Variation

Somaclonal variation can generally be described as phenotypic changes that arise from tissue culture. However the term is mostly used to describe genetic changes that are in nature irreversible and heritable, although phenotypic changes from tissue culture can be epigenetic (non-genetic). Non-genetic changes are reversible, non-heritable and attributed to physiological responses. There is a debate on whether somaclonal variation is the result of preexisting genetic differences or induced by media components during the tissue culture process (Brar and Jain, 1998). Somaclonal variation is influenced by several factors such as the genotype and source of the explant, the media composition, cultural conditions, the culture age and transfer intervals (Deverno, 1995). The mechanism by which these factors influence somaclonal variation is not well understood but higher exposure of the explants to

growth hormone and extended tissue culture period were found to promote somaclonal variation (Deverno, 1995). The genetic changes that produce somaclonal variations are often genomic, but genetic changes in organelle DNA are sometimes responsible for somaclonal variations. The genomic changes can include changes in the karyotype, point mutations, somatic cross-over and sister chromatid exchange, somatic gene rearrangement, DNA amplification, transposable elements, and DNA methylation (Brar and Jain, 1998).

Somaclonal variation can positively or negatively impact a tissue culture system depending on the objectives. When the tissue culture is used for inducing mutation, somaclonal variation becomes a positive input that helps to create genetic variation from which new useful phenotypes can be selected. A list of useful somaclonal variants selected and used in breeding has been reported by Brar and Jain (1998). This list include traits like pest and disease resistance, salt tolerance, frost tolerance, herbicide resistance, solid content, low seed content, etc. However, when the tissue culture system is being used for clonal propagation, somaclonal variation is undesirable since the objective is to produce a large number of true-to-type plants. Since the factors contributing to somaclonal variation are not always easy to control or well understood it is not easy to eliminate or promote somaclonal variation in a particular tissue culture system. Molecular and biochemical approaches have been used to detect the presence of somaclonal variation in tissue culture of various plant species and its presence was found in some systems and not in others (Henry, 1998). In oil palm, somatic embryo derived plants in field evaluation, were found to be sterile due to their abnormal flower production induced by a long exposure to growth hormone (Corley et al., 1986). As has been seen in oil palm, somaclonal variation in

trees can sometimes be detected, years after the tissue culture, during evaluation of the trees in the field. So before any large scale deployment of a tissue culture based clonal propagation method, extensive evaluation of the explants at various stage of culture, and the subsequent regenerated plants is required to check for the presence of somaclonal variation.

Telomere changes during tissue culture

Although plant cloning has been around for over a century, cloning of mammals (Dolly the sheep) was first reported in 1997 (Wilmut et al., 1997). Subsequent study of the cloned mammal revealed that full restoration of telomere length did not occur after the cloning because the animals were produced without germline involvement (Shiels et al., 1999). Telomeric DNA reduction has been linked to cell aging (Harley et al., 1990). In regard to that theory, the reduction of telomeric DNA in the cloned sheep could result in an early aging of the animal. Telomeric DNA change during tissue culture has also been studied in plants (Kilian et al., 1995; Fajkus et al., 1998; Riha et al., 1998). Barley telomeres were found to shorten during differentiation, but to extend in callus culture (Kilian et al., 1995). A similar result was found in ***Melandrium album*** callus culture where telomeres increased in length by 1 to 2 Kb compared to leaves (Riha et al., 1998). In addition to telomere length, a high telomerase activity was reported in meristematic tissue of cauliflower and undifferentiated cells from *Arabidopsis*, soybean, and carrot, whereas telomerase activity in the mature plants was low or not detected (Fitzgerald et al.,

1996). In tobacco an increase in telomerase activity in callus, compared to the source leave, was reported without any change in telomere length. However, several reports in plants indicate the absence of telomere shortening during development (Broun et al., 1992; Riha et al., 1998; Fajkus et al., 1998). The absence of telomere shortening during development in some plants, and the increase of telomerase activity or telomere length during tissue culture explains why the subject of a possible early aging in the first cloned mammal (Shiels et al., 1999) has not been a concern in cloned plants. Although plant cloning by conventional rooted cutting and grafting have been done in species such as apple, geranium, and potatoes for several decades, a search in biological literature databases such as Agricola, Medline and Current-content, has not produced any published research on telomere shorting or early aging of these cloned plants. The observations from the first cloned mammal will probably start further investigations into the possible reduction of telomeric DNA in cloned plants and the possible consequences on their development

Chapter 3

EFFECTS OF CARBON SOURCE AND EXPLANT TYPE ON SOMATIC EMBRYOGENESIS OF FOUR CACAO (*Theobroma cacao* L.) GENOTYPES

INTRODUCTION

In vitro propagation via somatic embryogenesis provides a means to produce large numbers of genetically identical and often pathogen-free plants. It can also be used to develop a genetic transformation system or to preserve germplasm via cryopreservation of somatic embryos. However for these applications to be technically and economically feasible, it is essential to optimize the system variables to obtain high multiplication rates of quality embryos. In most somatic embryogenesis systems, a carbon source is required to provide the necessary energy and building blocks to the process. Sucrose and glucose have been widely employed, however other carbon sources have been reported to be equally effective in supporting somatic embryogenesis in many plants (Brown et al., 1995). For example, the effectiveness of maltose as a carbon source for somatic embryogenesis was demonstrated in petunia (Raquin, 1983), alfalfa (Strickland et al., 1987), pine

(Nagmani et al., 1993), pea (Loiseau et al., 1995) and in apple secondary embryogenesis (Daigny et al., 1996). In addition, maltose has been used to improve somatic embryo maturation in cacao (Lopez-Baez et al., 1993) and in pine (Li et al., 1998a). In maize, Swedlund and Locy (1993), indicated that sorbitol improved embryogenic calli production and plant regeneration. Fructose has been shown to produce large numbers of embryos in cucumber (Ladyman and Girard, 1991), and to produce more embryos than sucrose, glucose or maltose when used at concentrations higher than 252 mM in pea (Loiseau et al., 1995).

In cacao, Elhag et al., (1987) reported a comparative study of sucrose, glucose and fructose effects on somatic embryogenesis using calli induced from zygotic tissues. In that study, the authors indicated that embryogenesis increased when sucrose was replaced by glucose or fructose; however several somatic embryogenesis protocols using tissue from maternal origin have been developed using sucrose (Sondahl et al., 1989; Figueira and Janick, 1993; Lopez-Baez et al., 1993; Alemanno et al., 1997). Recently Li et al. (1998) reported a somatic embryogenesis system using maternal tissue (staminodes and petals), glucose and sucrose. Although cacao somatic embryos could be obtained from floral parts with sucrose and glucose, no comparative studies using various carbon sources and explant types have been reported. The objective of this study was to identify the best combination of explant type and carbon source for efficient somatic embryo production system for cacao.

MATERIAL AND METHODS

Plant Materials

Staminodes and petal explants from immature flowers of four different cacao genotypes (ICS 16, Laranga, Scavina 6 and UF 613) were used. These trees were grown in a greenhouse maintained at 70% to 80% relative humidity and 27°C to 30°C at the Pennsylvania State University, University Park PA. The flowers were surface sterilized for 20 min using a 10% calcium hypochlorite solution. They were then dissected and their staminodes and petals extracted, separated and put into culture on various media contained in 100 x 15 mm petri dishes.

Media formulation

The DKW-based media protocol, according to Li et al. (1998), was used to test five different carbon sources: fructose, glucose, maltose, sorbitol or sucrose. Each carbon source was used at a concentration of 1*M*. The other media components remained as reported by Li et al. (1998).

Somatic embryogenesis

Embryogenic callus was induced from staminode and petal explants by first growing them on primary callus growth (PCG) medium for 14 days after which they were transferred onto a secondary callus growth (SCG) medium for 14 days. Embryo development was initiated by a transfer of the explants from SCG medium to an embryo development (ED) medium. In the first experiment, each of the media contained glucose, fructose, maltose, sorbitol or sucrose, and the explants were consecutively transferred to the next media with the respective carbon source. During that first experiment explant growth was recorded every two weeks by weighing five explants. In the second experiment the different carbon sources were tested only on PCG medium, then the explants were transferred onto SCG containing glucose followed by a transfer on ED containing sucrose and glucose according to Li et al. (1998). Embryo production (percentage of embryogenic explants and average number of embryos per explant) was recorded in both experiments two months after culture initiation.

Data collection and analysis

Each medium and carbon source combination was evaluated with 20 staminodes and 20 petals on each of four replicate plates. Two months after culture

initiation, the data were recorded by counting the number of embryo-producing explants in each treatment and the number of embryos on each responsive explant. Data for explant growth were collected by weighing five staminode or petal explants, every two weeks before a transfer to the next medium. During weighing, a set of five explants was transferred in a previously weighed and clean empty petri dish. Weighing was performed out of the laminar flow hood to avoid balance fluctuation created by the airflow. The data were analyzed using analysis of variance (ANOVA) on the Statview software program (SAS Institute Inc, Cary, NC).

RESULTS

Effect of carbon source on explant growth

In order to test the effectiveness of various carbon sources on explant growth, staminode and petal explants from four different cacao genotypes (ICS 16, Laranga, Scavina 6 and UF 613) were cultured on media containing equimolar concentrations of fructose, glucose, maltose, sorbitol, and sucrose. The explants were weighed every two weeks then transferred onto fresh media. One week after culture initiation, tissue swelling could be observed on petal and staminode explants grown on glucose, fructose or sucrose. Two weeks after culture initiation, visible callus formed on explants cultured in the presence of these three carbon sources but not on maltose or sorbitol (Fig. 3.1). For staminode explants, the fresh weight increase during the eight week period for glucose, sucrose and fructose was 0.4g, 0.6g and 0.9g, respectively

(Fig. 3.2a). In contrast, no growth was observed on staminode explants cultured on maltose or sorbitol containing media. Petal explants exhibited their highest growth on fructose and glucose, producing fresh weights of 0.28g and 0.22g, respectively followed by sucrose and maltose which produced 0.09g and 0.06g, respectively (Fig. 3.2b). No growth was observed for petal explants cultured on sorbitol containing media.

Effect of carbon source on embryo production

Since explant growth does not necessarily translate into high embryo production, the effect of each carbon source on embryo production was evaluated. Embryo development was visible two weeks after explant transfer from SCG medium to ED medium. Similar to their effect on explant growth, fructose, glucose and sucrose supported embryo production in cacao with rates ranging from 6% to 99%, 18% to 98% and 3% to 82% respectively, depending on the genotypes (Fig. 3.3a and 3.3b). For the percentage of embryogenic staminodes, a significant difference was observed between all carbon sources (p=0.001), except between fructose and sucrose (p=0.591). During culture on maltose or sorbitol containing media for up to 4 months, staminode and petal explants did not grow well and did not produce any embryos (Fig. 3.1). When staminodes were cultured for callus induction on PCG media containing glucose, fructose, maltose, or sucrose for two weeks, then transferred onto SCG and ED media according to Li et al., (1998), embryos were recovered (Fig. 3.4a and 3.4b). However embryo production rates were much lower for staminodes induced on maltose containing media (5% to 12%) compared to the

range of 5% to 78%, 7 to 97% and 5% to 99% observed on sucrose, glucose and fructose containing media, respectively (Fig. 3.4a). Sorbitol did not support embryo production. The average number of embryos per responsive staminode produced on glucose, fructose and sucrose containing media were not significantly different ($p=0.247$). In an average, depending on the type of carbon source, 16.9 to 18 embryos per responsive staminode were produced for Scavina 6 and 5.5 to 6.8 for Laranga (Fig. 3.3c). For petal explants, the only carbon source that supported embryo production in all four genotypes was sucrose (Fig. 3.3b). The average number of embryos per responsive petal ranged from 2.5 to 8 embryos per responsive petal.

Influence of genotype on embryo production

Four different cacao genotypes (ICS 16, Laranga, Scavina 6 and UF 613) were evaluated for their interaction with various carbon sources and explant types. Except for ICS16 which did not produce embryo on fructose containing media, all other genotypes tested produced embryos with the 3 responsive carbon sources (fructose, glucose and sucrose). There was a wide variation on embryogenesis rates depending on the genotype used (Fig. 3.3a, 3.3b, 3.3c, and 3.3d). A significant difference was observed between all genotypes ($p=0.001$) except between UF613 and ICS16 ($p>0.096$). The two Forastero genotypes (Scavina 6 and Laranga) produced the highest embryogenesis rates (82% to 99% and 40% to 78% respectively). The Trinitarios (UF 613 and ICS 16) produced significantly lower embryogenesis rates

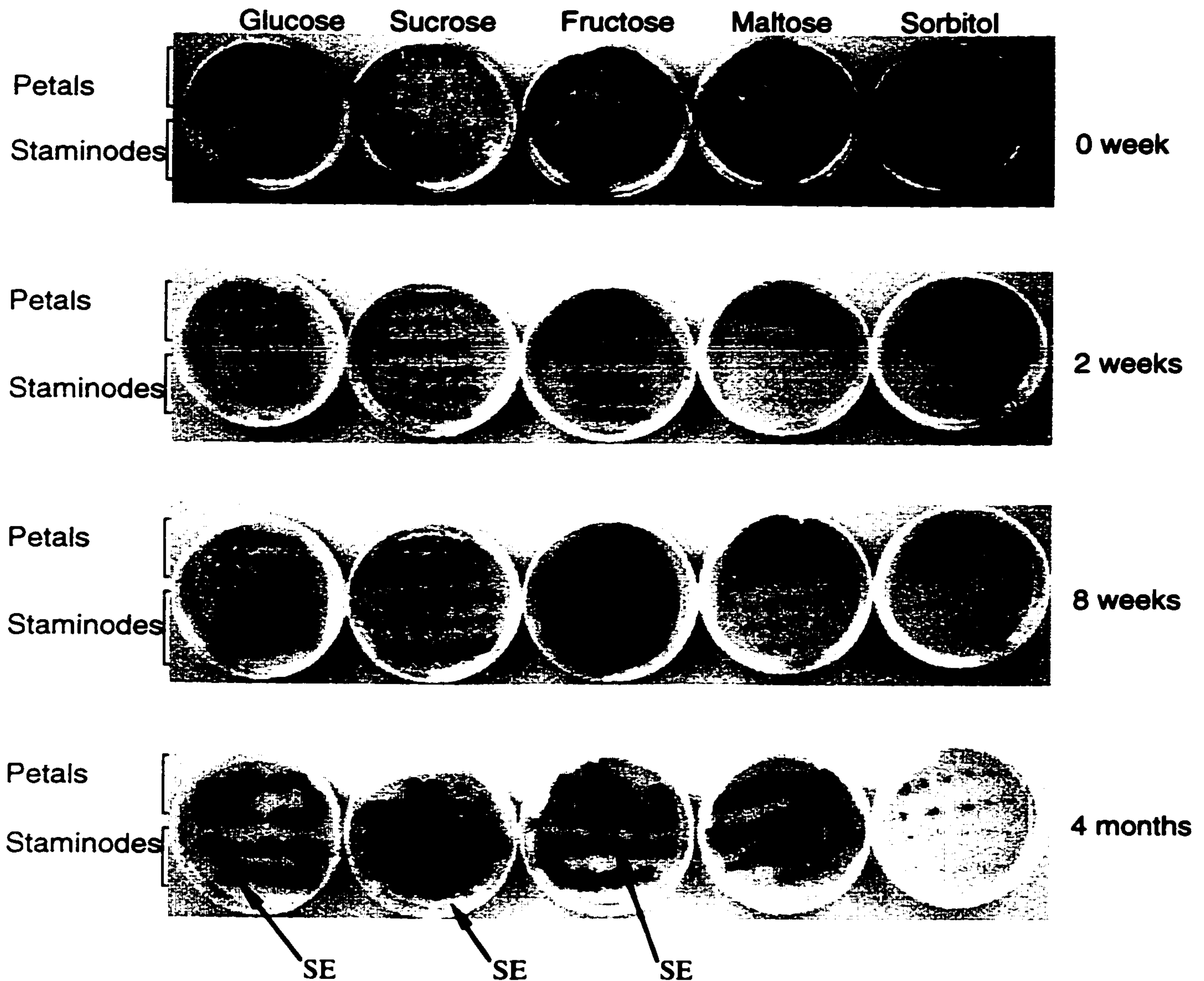

Figure 3.1 Growth of cacao petal explants (top half of plates) and staminode (bottom half of plates) in response to five different carbon sources as indicated at the top. Note somatic embryos (SE) indicated by the arrows. For each combination 15 explants were removed from immature flowers of greenhouse grown cacao plants and cultured using DKW medium-based protocol (Li et al. 1998b). Plates were incubated at 26 °C under 16 hrs of light photoperiod and photographed at intervals as indicated on the right.

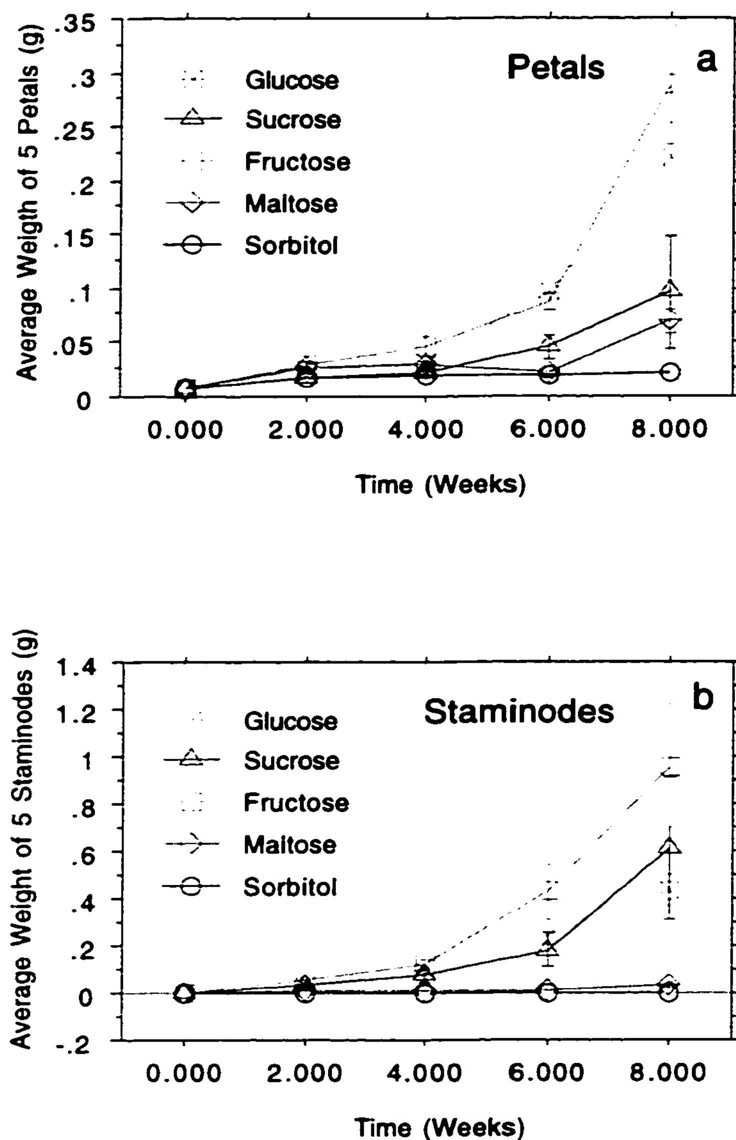

Figure 3.2 Effect of carbon source on growth of Scavina 6 cacao petals (a) and staminodes (b) explants during somatic embryogenesis. Explant fresh weights were recorded every two weeks. The explants were grown for two weeks on primary callus growth medium (PCG) according to Li et al. (1998), supplemented with fructose, glucose, maltose, sorbitol or sucrose. After two weeks the explants were transferred onto secondary callus medium (SCG) for two weeks before a subsequent transfer onto embryo development medium (ED). During each step the explants were transferred onto media containing the same carbon sources. Each treatment contained 15 explants per plate in 3 replicate plates. Each vertical line represents the standard error of the means.

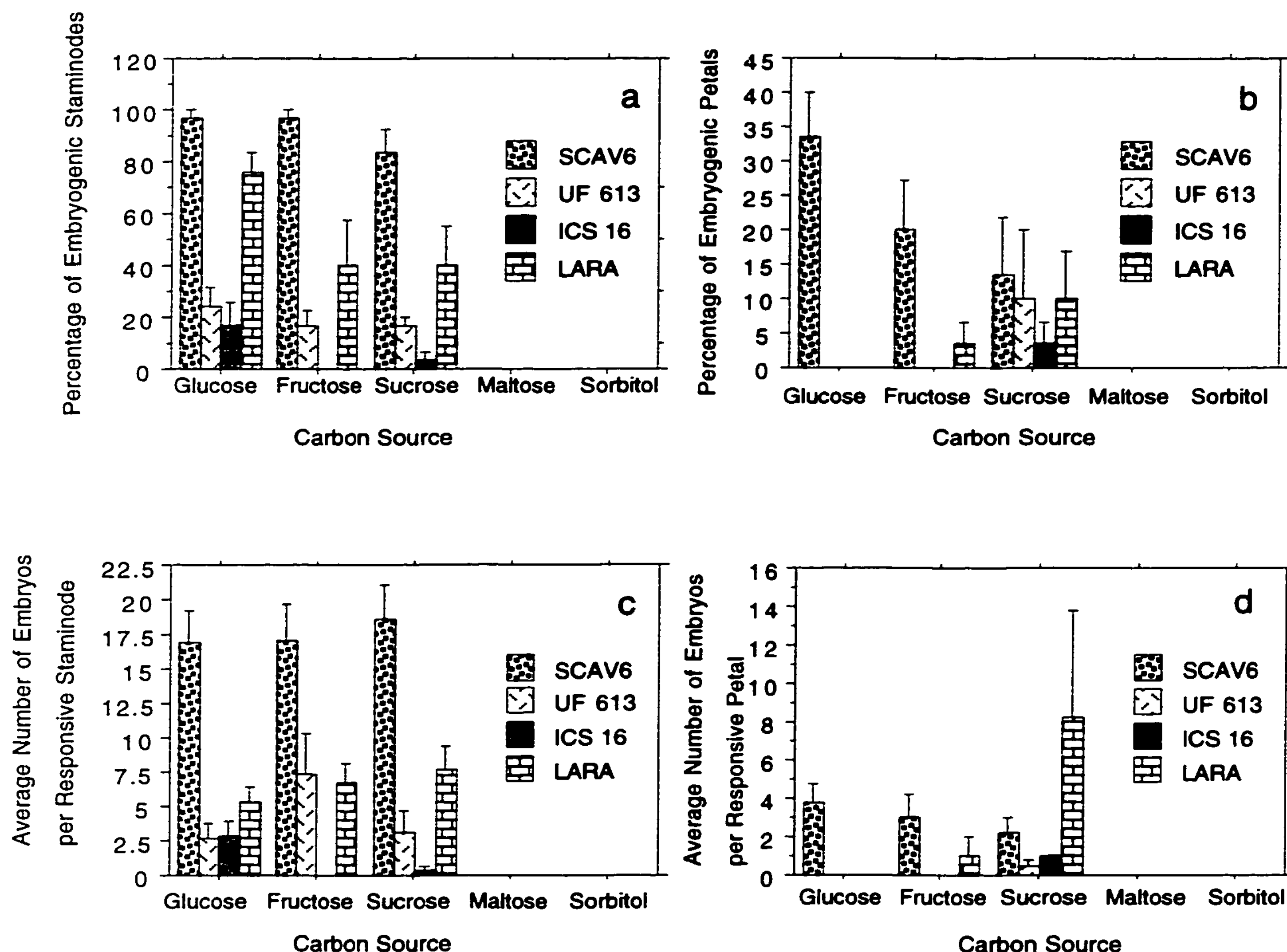

Figure 3.3 Effect of carbon source and explant type on somatic embryo production in four cacao genotypes. The explants were grown for two weeks on primary callus growth medium (PCG) according to Li et al. (1998), supplemented with fructose, glucose, maltose, sorbitol or sucrose. After two weeks the explants were transferred onto secondary callus medium (SCG) for two weeks before a subsequent transfer onto embryo development medium (ED). During each step the explants were transferred on media containing the same carbon sources. a. Effect of carbon source on the frequency of somatic embryogenesis from staminode explants. b. Effect of carbon source on the frequency of somatic embryogenesis from petal explants. c. Effect of carbon source on the average number of primary somatic embryos produced from each responsive staminode explant. d. Effect of carbon source on the average number of primary somatic embryos produced from each responsive petal explant. The data were collected 2 months after culture initiation. Each treatment contained 80 explants from 4 replicate plates containing 20 explants each. Vertical lines represent the standard error of the means.

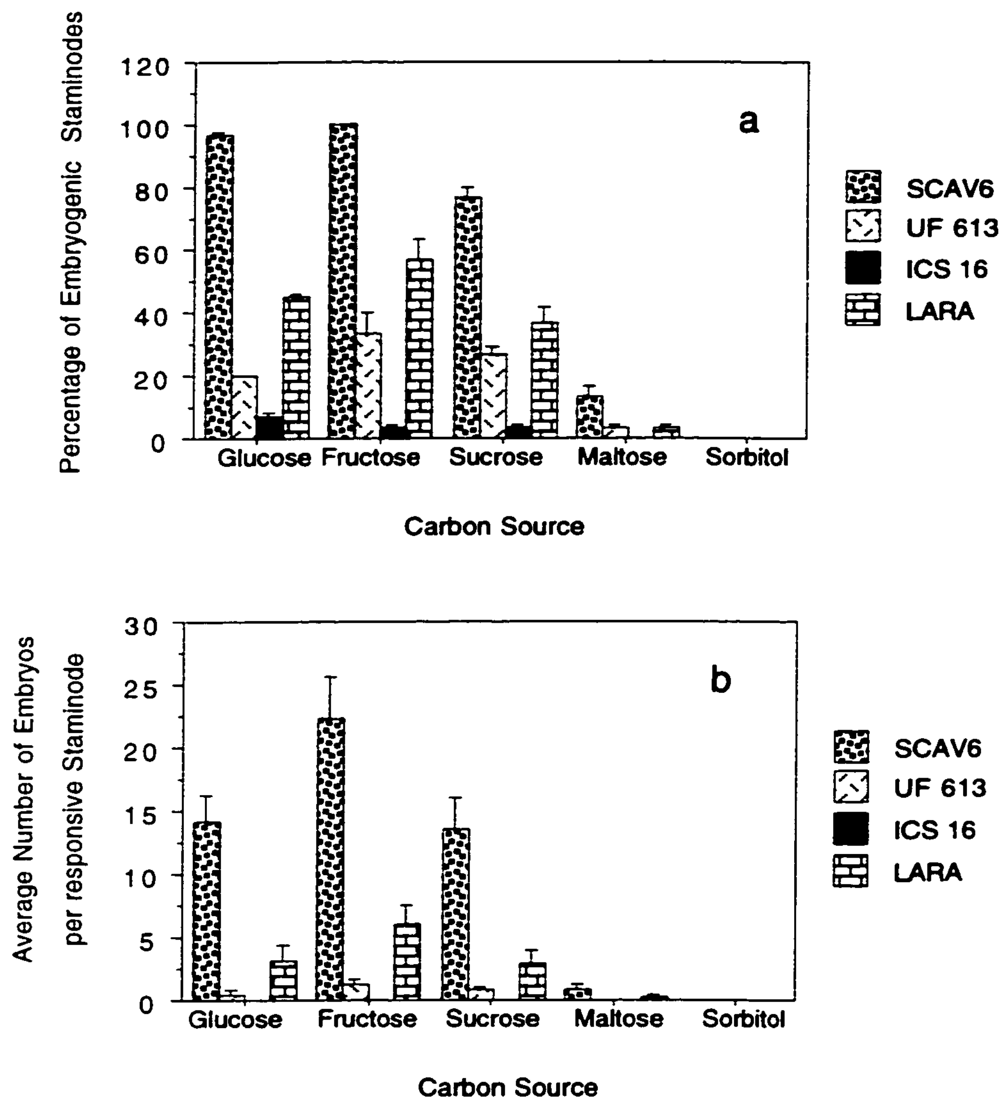

Figure 3.4 Effect of carbon source on somatic embryo induction from 4 cacao genotypes using DKW-based protocol (Li et al 1998). a, Effect of carbon source on the frequency of somatic embryogenesis from staminode explants. b, Effect of carbon source on the average number of primary somatic embryos produced from each responsive staminode explant. Explants were cultured on the 5 different carbon sources containing PCG media for 2 weeks then transferred onto SCG for another two weeks before a transfer onto ED media according to Li et al. (1998). The carbon sources were tested only in PCG. The data were collected 2 months after culture initiation. Each treatment contained 80 explants from 4 replicate plates containing 20 explants each. Vertical lines represent the standard error of the means.

(18% to 22% and 1% to 18% respectively). The same trend was observed with petal explants grown on sucrose containing media (Fig. 3.3b). Similarly, Scavina 6 and Laranga produced higher embryogenesis rates than UF 613 and ICS 16 when staminodes were cultured on PCG with various carbon sources for two weeks before a transfer on a SCG then ED media according to the protocol by Li et al. (1998). Genotypes with higher embryogenesis rates also produced more embryos per responsive staminodes in the following order: Scavina 6 (17 to 18), Laranga (5 to 6), UF613 (2.5 to 7) and ICS16 (1 to 2.5). However the average number of embryos produced per responsive petal did not relate with embryogenesis rates (Fig. 3.3d).

Influence of explant type

In order to investigate explant responsiveness, staminode and petal explants from immature flowers of ICS 16, Laranga, Scavina 6 and UF 613 genotypes were cultured in the presence of various carbon sources. Explant growth was recorded every two weeks by weighing a set of five staminodes or petal explants. Staminode and petal explants swelled one week after culture initiation. By the second week of culture, explant growth and expansion was more visible on staminodes than petals (Fig. 3.1, 3.2, and 3.3.). After eight weeks of culture, staminodes showed a higher growth response (0.99g/5 explants) than petals for which the growth response was 0.29g/5 explants (Fig. 3.2a and 3.2b). In addition to fresh weight increase, staminodes also exhibited a higher percentage of embryo producing explants than petals (Fig 3.1, 3.2, and 3.3). For example: 82 to 99% of the staminodes from the Scavina 6 genotype produced embryos explant compared to 12.5% to 33% for petals

(Fig. 3.3a and 3.3b). The same trend was also observed with the average number of embryos per responsive explant. This responsiveness of staminode explants was consistent across the four genotype tested (Fig. 3.3 and 3.4).

DISCUSSION

In this study, using four cacao genotypes (ICS 16, Laranga, Scavina 6 and UF 613), the embryogenic potential of petal and staminode explants in combination with five different carbon sources (fructose, glucose, maltose, sorbitol and sucrose) was investigated. The best embryogenic responses were obtained when staminode explants were used in combination with either glucose (up to 99%) or fructose (up to 98%); up to 82% of explants produced embryos when grown with sucrose. The effectiveness of glucose, sucrose or fructose for cacao tissue during somatic embryogenesis could be linked to a high level of metabolic enzymes specific for these carbon sources. Since the ability of a somatic cell to undergo embryogenesis relies on reprogramming to embryogenic cells (Merkle et al., 1995), such reprogramming could involve the activation of genes for specific carbon source metabolism. The ability of glucose, fructose and sucrose to support cacao tissue culture was reported by Kononowics and Janick (1984) who indicated that glucose, in combination with sucrose or fructose, was better for cacao embryo growth than sucrose alone. Similarly, using calli from zygotic embryo tissue, Elhag et al., (1987) reported that glucose or fructose increased the rate of cacao somatic embryogenesis. In a previous report (Li et al., 1998), a somatic embryogenesis system for cacao was reported using

glucose either alone or in combination with sucrose, which resulted in higher embryogenic responses than previously reported.

The influence of carbon source on somatic embryogenesis has been reported for other plant species (Strickland et al., 1987, Parrot and Bailey, 1993; Daigny et al., 1996). Although sucrose is by far the most utilized carbon source for plant tissue culture, other sources of carbon such as glucose, fructose, lactose, mannitol, maltose, or sorbitol have been used effectively for somatic embryogenesis in plants (Brown et al., 1995). The effectiveness of a carbon source in plant tissue culture depends on the plant species, the genotype, and the explant used. While maltose was shown to be more efficient than fructose, glucose or sucrose in apple somatic embryogenesis (Daigny et al., 1996), it was found to be toxic for cucumber somatic embryogenesis (Ladyman and Girard, 1992). This study showed that maltose was inefficient for embryo production in cacao. Similarly, sorbitol did not support cacao somatic embryo production or callus growth in this study. Sorbitol was also shown by Daigny et al., (1996) to be inefficient for apple somatic embryogenesis although it was observed to be useful for apple micropropagation (Pua and Chiang, 1983; 1985). In contrast, sorbitol has been reported to support the growth of embryogenic calli in maize and to enhance its regenerative capacity (Swedlund and Locy, 1993). Sorbitol is effectively translocated in apple and related species. Since it can be converted into glucose by sorbitol oxidase or into fructose by sorbitol dehydrogenase, its effectiveness on apple micropropagation and maize somatic embryogenesis was linked to the presence of high levels of these sorbitol metabolic enzymes. The inefficiency of sorbitol for cacao tissue culture might be due to a lack of these enzymes in cacao tissues. Besides a role as an energy source, carbon sources play

important roles in osmo-regulation of plant cells. In addition, interactions between auxin and carbon sources such as sucrose have been shown to promote somatic embryogenesis in soybean somatic embryos (Lazzera et al., 1988).

While flower parts such as anthers, ovules and ovaries have received a lot of attention as explants for somatic embryogenesis in many plant species, other flower parts such as petals and stamens (in particular anther filaments or staminodes) have not. Flower parts other than ovules, ovaries and anthers have been shown to be responsive for embryogenesis in Iris species (Jéhan et al., 1994) in *Lilium logiflorum* (Tribulato et al., 1997), in Cavendish banana (Navarro et al., 1997), and in *Coco nucefera* (Verdeil et al., 1995). In cacao, although somatic embryos have been produced from nucellar tissue (Figueira and Janick, 1993), and leaf explants (Litz, 1986), petal, anther filament, and staminode explants were more successful maternal tissue (Sondahl et al., 1989; Lopez-Baez et al., 1993; Alemanno et al., 1996a, 1996b; Li et al., 1998). This study revealed the higher responsiveness of staminode compared to petal explants for cacao somatic embryogenesis in the genotypes tested.

The influence of genotype on somatic embryogenesis is well established in many plant species (Merkle et al., 1995). A significant genotypic variation in the embrygenesis response was observed in this study. The Forastero genotypes (Scavina 6 and Laranga) were more embryogenic than the Trinitarios (UF 613 and ICS 16). A genotypic effect on cacao embryogenesis efficiency was previously reported (Lopez-Baez et al., 1993; Alemanno et al., 1996b; Li et al., 1998). In their report on cacao somatic embryogenesis using flower parts, Lopez-Baez et al., (1993) obtained embryos from only the Forastero group. Similarly, Alemanno et al., (1996b) tested 25 genotypes from three cacao groups (Forastero, Trinitario and Criollo) and reported

somatic embryogenesis from 5 genotypes, all from the Forastero group. In a previous report (Li et al., 1998), although embryogenesis was observed for all three cacao groups, embryogenesis rates were higher for Forasteros than Criollos, with Trinitarios expressing intermediate rates.

With this study, responsive carbon sources and explants for cacao somatic embryogenesis have been identified or confirmed. Further optimization of carbon source types and amount, along with other variables for specific cacao groups and genotypes is likely to yield even higher embryogenic efficiencies.

Chapter 4

CONVERSION AND ACCLIMATION OF SOMATIC EMBRYOS IN CACAO (*Theobroma cacao* L.)

INTRODUCTION

Conversion can be defined as the transition during which a somatic embryo develops a growing shoot and produces leaves and roots that allow it to independently grow in an *ex vitro* condition, therefore it is the equivalent of germination of zygotic embryos from seeds. Unlike zygotic embryos that germinate after seed formation by utilizing seed reserves stored in the cotyledons, somatic embryos, which lack such reserves, are difficult to convert in many species. Acclimation of somatic embryo derived-plantlets can be defined as their adaptation to *ex vitro* conditions.

Cacao somatic embryogenesis was achieved using zygotic tissue explants (Essan et al., 1977; Passey et al., 1980) however, conversion of these embryos has been problematic (Wang and Janick, 1984, Duhem et al., 1989; Wen et al., 1991). Somatic embryos have also been produced in cacao from different flower parts (Lopez-Baez et al., 1993; Sondahl et al., 1993; Alemanno et al., 1996; Li et al., 1998)

and nucellar tissue (Figueira and Janick 1993). However, conversion and acclimation of these somatic embryos from maternal tissue has remained a limiting step in the practical application of the somatic embryogenesis system.

Several reports on cacao somatic embryogenesis have focused on the use of growth hormones or CO_2 to improve the conversion and acclimation rates of cacao somatic embryos, but in each case plant recovery was limited (Sondahl et al., 1993; Figueira and Janick, 1993; Lopez-Baez et al., 1993; Alemanno et al., 1997). Traditionally MS medium (Murashige and Skoog, 1962) has been used to produce cacao somatic embryos (Essan, 1977; Pence et al., 1980; Wang and Janick, 1984; Adu-Ampomah et al., 1987; Sondahl et al., 1993; Lopez-Baez et al., 1993; Alemanno et al., 1996) but in these studies embryo conversion was limited or problematic. Flynn et al., (1990) reported that woody plant medium (WPM) (Lloyd and McCown, 1980) was more favorable to growth of cacao shoot explants than MS (Murashige and Skoog, 1962) and used it to micropropagate cacao. In the same way, Figueira and Janick (1993) used WPM (Lloyd and McCown 1980) for conversion of somatic embryos from nucellar tissue. Previously, Li et al. (1998) reported the routine recovery of a large number of viable plants from cacao somatic embryos using DKW medium (Driver and Kuniyuki, 1984), and this chapter will discuss the optimization of the conversion step. Although the use of basal salts other than MS to convert cacao somatic embryos was reported by Figueira and Janick (1993), and Li et al., (1998), no comparative studies have been published.

The objective of this study was to investigate the effects of different basal salts on the conversion and regeneration of cacao somatic embryos produced by the method previously reported by Li et al. (1998). We also investigated the characteristics of cacao somatic embryos that influence their ability to convert into normal plantlets. The use of an *in vitro* pruning technique to convert plantlets with abnormal shoot morphology into normal cacao plantlets was evaluated. A growth comparison between somatic embryo derived plants and seedlings during acclimation and early plant establishment is also reported.

MATERIALS AND METHODS

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, or Fisher Scientific, Pittsburgh, PA, unless otherwise specified.

Somatic embryogenesis

Somatic embryos were produced as described by Li et al. (1998). Briefly, staminodes from unopened flowers collected from mature greenhouse grown plants, were used to initiate callus cultures on primary callus growth medium (PCG). After 14 days, explants were transferred onto secondary callus growth medium (SCG) for

another 14 days. Embryo development was then induced and maintained on embryo development medium (ED) by transfer to fresh medium every 3 to 4 weeks.

Media preparation

Four different media formulations were tested for cacao somatic embryo conversion: MS (Murashige and Skoog, 1962), woody plant medium (WPM) (Lloyd and McCown, 1980), DKW (Drivers and Kuniyuki, 1984) and DKW supplemented with potassium nitrate and amino acids (DKWKA). Each medium contained basalt salts (MS, WPM, or DKW), 20 g of glucose per L, 10 g of sucrose per L, 100 mg of *myo*-inositol per L, 2.0 mg of thiamin-HCL per L, 1.0 mg of nicotinic acid per L, 2.0 mg glycine per L and 1.75 g of phytagel per L. In addition, DKWKA contained: 2.5 μM of arginine, glycine, leucine, lysine and tryptophan and 0.3 g of potassium nitrate per L. All media were adjusted to pH 5.7 using KOH before addition of phytagel, then autoclaved at 121°C for 18 min. After cooling, the media were poured into 30 mL plastic petri dishes (100 x 15 mm).

Culture conditions

Mature Scavina 6 somatic embryos 2 cm long or more in size, or embryos that had developed a radicle were transferred from ED medium onto various conversion

media (MS, WPM, DKW or DKWKA) and cultured at 26 °C under a light intensity of 50 µmol.m^{-2}.s^{-1} and a 16 hr. photoperiod. After three weeks of culture, converted plantlets with shoots of at least 5 mm long and roots at least 10 mm long were scored then transferred into magenta box culture vessels containing 150 ml of secondary embryo conversion medium (SEC) for hardening. SEC was composed of 1/2X DKW basal salt, 10 g of glucose per L, 5 g of sucrose per L, 50 mg of *myo*-inositol per L, 1.0 mg of thiamin-HCL per L, 0.5 mg of nicotinic acid per L, 1.2 mg glycine per L, 1.75 g of phytagel per L, supplemented with 2.5 µM of arginine, leucine, lysine and tryptophan per L, and 0.3 g of potassium nitrate per L. The pH was adjusted to 5.5 before phytagel was added and autoclaved at 121 °C for 18 min. The remaining non-converted embryos were transferred onto fresh conversion media containing their respective basal salts, and cultured for three weeks after which they were again scored.

Data analysis

Each treatment comprised four replicates of 10 embryos per experiment and each experiment was replicated three times. Statistical analyses were performed using an analysis of variance (ANOVA) on the Statview software program (SAS Institute Inc., Cary, NC).

In vitro pruning

After six weeks of culture in the hardening medium, plantlets with abnormal shoots (shoots continuously producing cotyledon-like leaves and long internodes) were pruned *in vitro* by cutting the apical shoot 5mm below the apex and removing it. The decapitated plantlets were then transferred onto a fresh SEC medium. A set of non-decapitated plantlets was also transferred onto a fresh SEC medium as controls. Normal shoot development was scored 1 month after the decapitation.

Acclimation and plant development

Somatic embryo-derived plantlets with healthy green leaves at least 4 cm long, with roots longer than 3 cm, were slowly pulled from the SEC medium and rinsed with tap water. The plantlets were transferred into one foot deepot (Hummer International, Earth City, MO) containing sterilized wetted Promix (Premier Horticulture LTEE, Riviere du Loup, Quebec Canada) or Metro-Mix 300 (Hummer International, Earth City, MO) growing medium and placed in a greenhouse. The plantlets were shaded with older plants under 80 % relative humidity (RH) at 27 - 30° C, watered every day for the first three days then watered every two to three days or as needed. After ten days the plants were fertilized every two weeks for the first

two months with a cacao fertilizing solution (see Appendix B), after the two months, they were fertilized weekly.

RESULTS

Effect of different basal salts

In order to optimize the conversion of somatic embryos into plantlets, the effect of different basal salts in conversion media was evaluated. Somatic embryos were placed onto petri dishes containing MS, WPM, DKW or DKW supplemented with amino acids and potassium nitrate. DKW and WPM supported a higher rate of conversion (77% of embryos produced shoots each) than did MS, which resulted in a rate of 59%. DKW and WPM also promoted a higher percentage of normal shoot development than did MS (Fig. 4.1). The addition of potassium nitrate and amino acids to the DKW medium not only increased the conversion rate to 92% but also increased the percentage of normal shoot production to 59%, more than twice that obtained using MS basal salt. Plantlets with green, healthy leaves 4 cm long or more were acclimated to greenhouse conditions after their root systems reached 3 cm long with an average success rate of 94%.

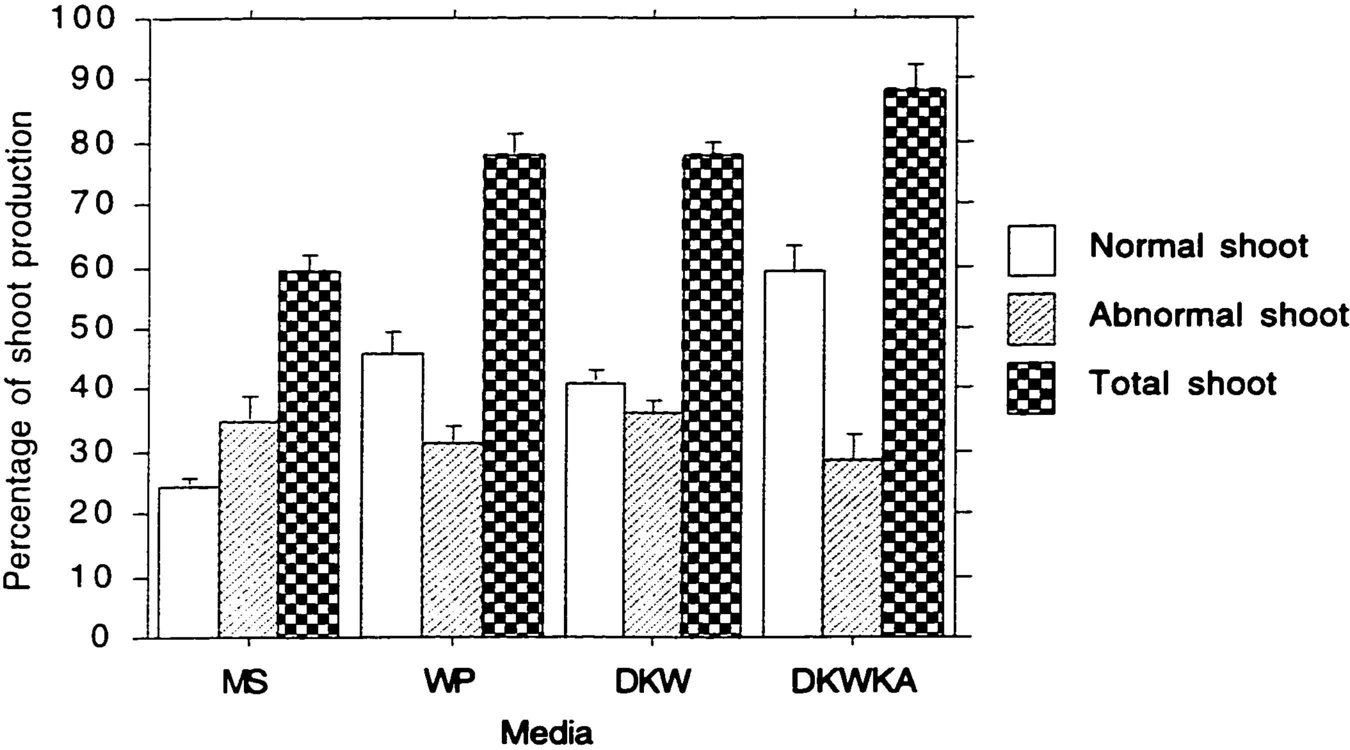

Figure 4.1 Effects of media type on the conversion of Scavina 6 cacao somatic embryos. The percentage of shoot production was evaluated by scoring converted embryos with shoots at least 5mm long, after 3 weeks of culture on the different treatment: MS medium (MS), Woody plant medium (WP), DKW medium (DKW) and DKW medium supplemented with amino acids and potassium nitrate (DKWKA). Each treatment included 10 embryos per plate with 4 replicate plates per experiment. Bar values represent the average percentage of three experiments. Vertical lines represent the standard errors.

Relationship between embryo morphology and conversion

During somatic embryo conversion on DKW medium, a relationship between embryo morphology and their conversion into plantlets was observed. Early in development, two sub-types of somatic embryos can be distinguished based on morphological differences as previously reported (Li et al., 1998). Type I embryos are translucent, tend to have large cotyledons and short embryonic axis that are often not well developed (Fig. 4.2a) and have a lower percentage of conversion than type II (Fig. 4.3 – 4.4). They resemble immature zygotic embryos from pods three months old or younger. These embryos represent 82% of embryos produced during somatic embryogenesis (Table 4.1). At the time of conversion, type I embryos first produce shoots that tend to have leaves of normal morphology (Fig. 4.2c). Later, they produce multiple adventitious roots which often branch during the hardening step and produce a large number of root hairs that are visible in the medium.

In contrast type II embryos have a higher percentage of conversion (Fig. 4.3-4.4) and an opaque or ivory-whitish appearance. They have a very defined embryonic axis and tend to have smaller cotyledons (Fig. 4.2a). These embryos represent 18% of the embryos produced during somatic embryogenesis and initiate roots early during embryo development (Table 4.1). These roots grow straight down into the medium and rarely branch, similar to tap roots produced by zygotic embryos from seeds (Fig. 4.2b, 4.2d and 4.2e). However, type II embryos produce normal

shoots much later during conversion, and have a higher percentage of abnormal shoots with leaves that resemble cotyledons (Fig. 4.2d). A small number of these abnormal shoots can later produce normal shoots.

Somatic embryos with fused axes, folded or extra cotyledons can be observed in either sub-types. During conversion these abnormal embryos also follow a germination path according to their type. Fused type I embryos start germination by initiating shoots first while fused type II embryos first produce taproots. Depending on the extent of the fusion, one embryo or both can develop into plants. Embryos completely fused at the base of the hypocotyl tend to produce a plantlet with one root structure and two shoots. However, during acclimation, one shoot often establishes dominance and grows to produce a single normal plant.

Table 4.1 Morphological characteristics of type I and type II cacao somatic embryos.

Embryo type	I	II
Appearance	translucent	opaque ivory
Percentage (%)	82	18
Cotyledon size (cm)	0.7 – 2	0.3 – 0.8
Embryonic axis	not well developed	distinctive
Conversion indicator	shoot initiation	taproot formation

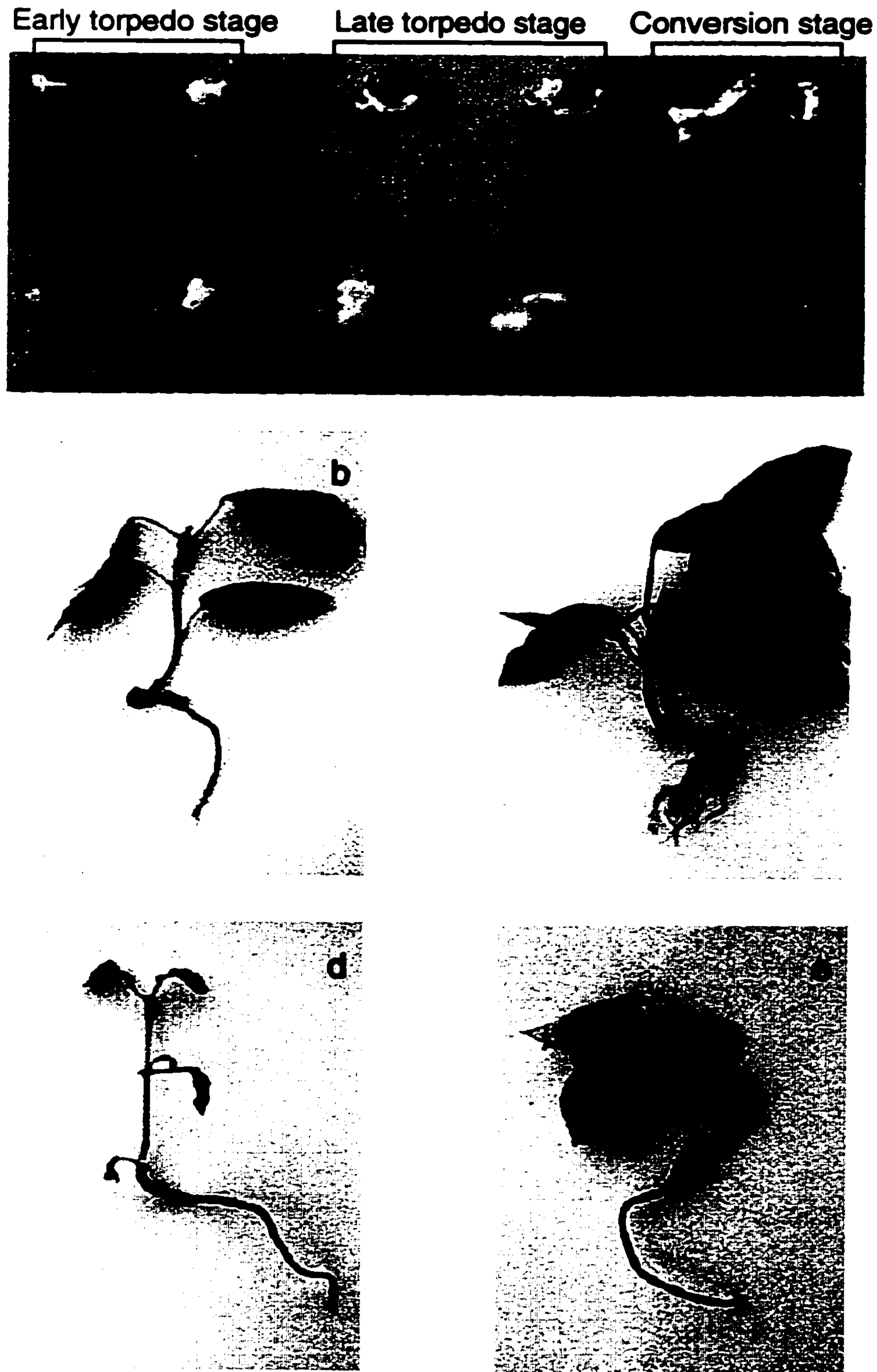

Figure 4.2 Somatic embryo conversion and plant regeneration in cacao. a. Type I and Type II cacao somatic embryos during maturation. b. Cacao plantlet with normal shoot and tap root. c. Cacao plantlet with normal shoot and adventitious roots. d. Cacao plantlet with abnormal shoot before decapitation. e. Emergence of normal shoot from axillary buds of a plantlet with abnormal shoot 3 weeks after decapitation.

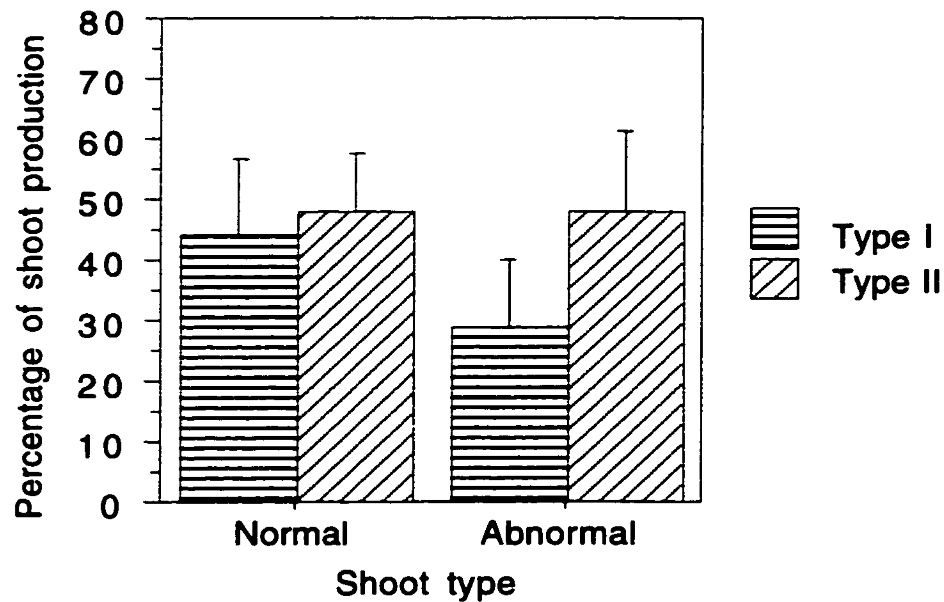

Figure 4.3 Relationship between embryo type and shoot production during conversion of Scavina 6 cacao somatic embryos. Each treatment included 10 embryos per plate with 4 replicate plates per experiment. Bar values represent the average percentage of three experiments. Vertical lines represent the standard errors.

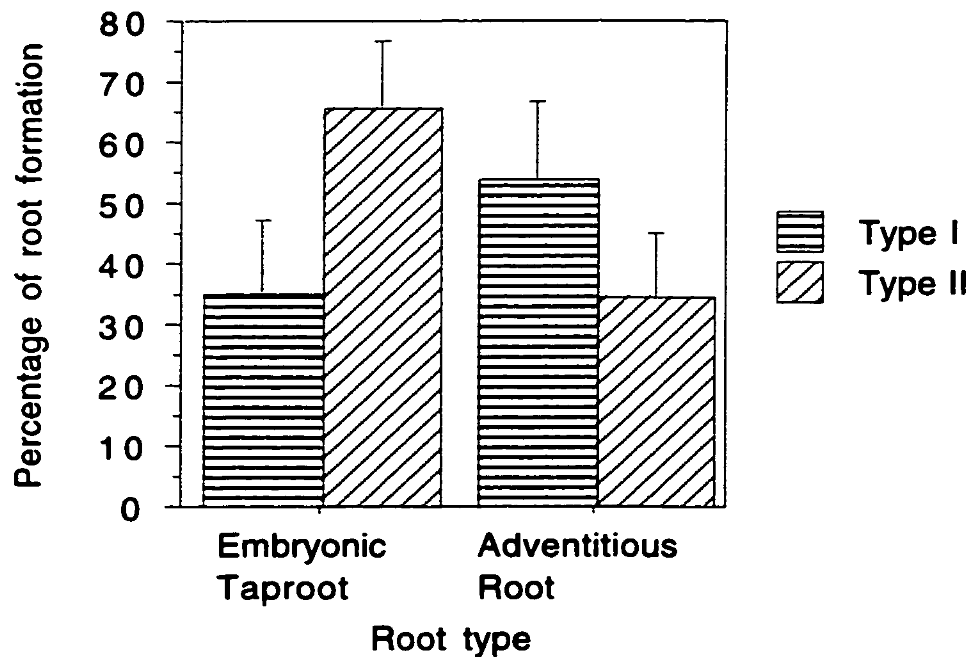

Figure 4.4 Relationship between embryo type and root production during conversion of Scavina 6 somatic embryos. Each treatment included 10 embryos per plate with 4 replicate plates per experiment. Bar values represent the average percentage of three experiments. Vertical lines represent the standard errors.

Effect of *in vitro* pruning

Plantlets with cotyledon like shoots (Fig. 4.2d) elongate during conversion and hardening, but only 5% of them produce normal shoots after 8 weeks of culture (Table 4.2). When transferred to the greenhouse for acclimation, these abnormal plantlets do not acclimate well nor do they produce a normal shoot and most of them die within 4 weeks after transfer to the greenhouse. To recover healthy plants from such plantlets, removal of the apex was used as a means to promote normal lateral shoot development. The removal of the apex on these plantlets allowed the development of lateral meristems that form normal shoots (Fig 4.2e). Plantlets that undergo decapitation produced a normal shoot 79% of the time (Table 4.2), and normal shoot development can be observed one week after pruning. Axillary buds from each node on the plantlet tend to develop a new shoot, however, one of these grows quickly, establishes apical dominance, and stops the growth of the other shoots. Normal plantlets from the *in vitro* pruning acclimate well to produce normal plants in the greenhouse.

Table 4.2. Effect of *in vitro* pruning on conversion of plantlets with abnormal shoots.

Treatments	Number of plantlets	Plantlets with normal shoots	Percentage of normal shoots
Control	39	2	5.1
Decapitated	38	30	78.9

Acclimation and plant development

During acclimation somatic embryo-derived plants started growth 1 month after transplanting (Fig. 4.5) and that lag time corresponds to a period during which the new plants adjust to the *ex vitro* conditions. After the 1 month period, slow growth began, followed by more active growth and the production of new leaves, indicating their establishment in the *ex vitro* conditions (Fig. 4.6a). No difference in growth was observed between type I and type II somatic embryo-derived plantlets. In contrast, germinating seedlings supported by large cotyledonary reserves grew very fast during the first 2 weeks (Fig. 4.5). However, after one month, the somatic embryos derived plants and seedlings exhibited similar growth rates, although the overall height of the seedlings remained higher due to the initial elongation of the hypocotyl during germination.

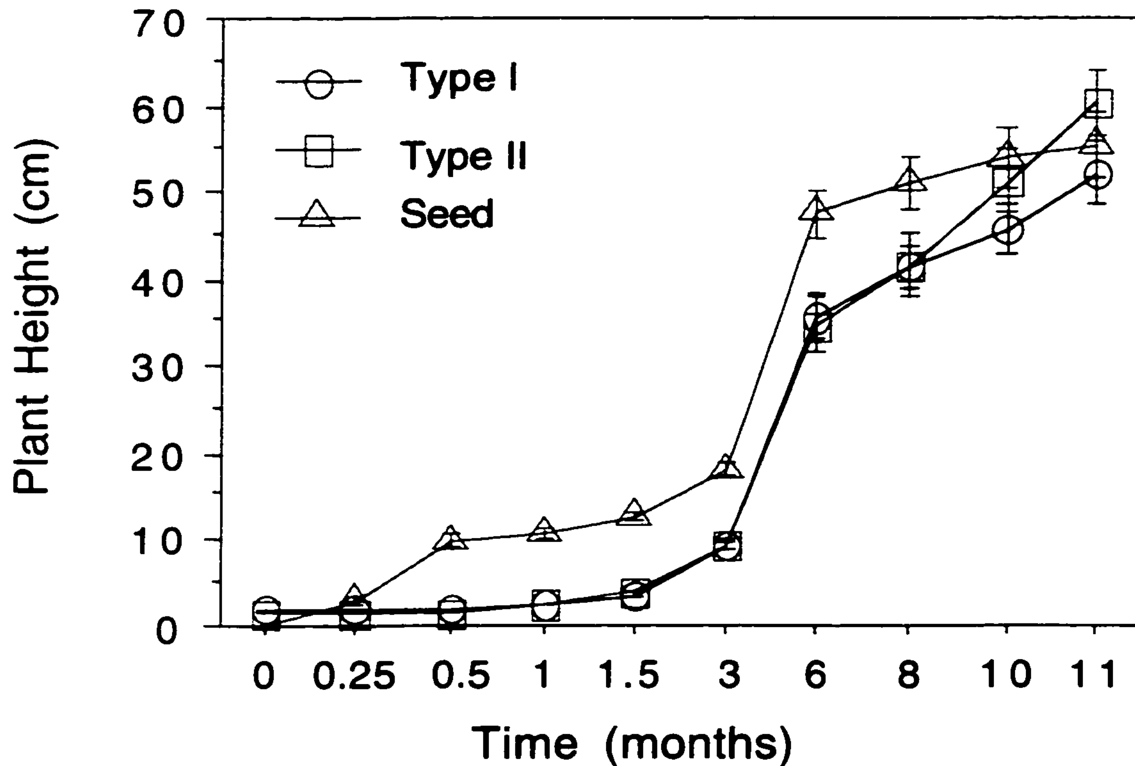

Figure 4.5 Growth of cacao seedlings and somatic embryo derived-plantlets during acclimation. Each plantlet type and seedling was planted in rows of eight plants in six replicate trays that contained 3 rows per tray. Somatic embryo derived plants were from the Scavina 6 genotype and seedlings from a cross Scavina 6 x West African Amelonado. Vertical lines represent the standard errors of the means.

Seven different clones have been successfully acclimated in the greenhouse and their respective rates are summarized in Table 4.3. When plantlets with roots smaller than 1 cm were acclimated, the success rate was lower and plantlet mortality higher than those with longer roots. Over-watering, lack of shade and low humidity were also found to reduce the acclimation rate. The overall health of the plantlets after the hardening period, which occurred *in vitro*, is important for faster establishment during acclimation in the greenhouse. Plantlets with yellow leaves or leaves smaller than 4 cm started growth later and had a higher mortality rate.

Somatic embryo-derived plants developed similar to seed grown plants in the greenhouse by exhibiting orthotropic growth and producing a vertical stem with spiral phyllotaxy which developed into the main trunk of the tree. Approximately ten months after transfer to soil in a greenhouse, in a developmental change common to seed derived plants, the apical bud branched to produce a crown of 3 to 5 lateral branches called the jorquette. These lateral branches exhibit plagiotropic growth, producing horizontal fan branches with alternate leaves. The jorquette, on somatic embryo trees was located at an average height of 1.35 m, similar to those on seed grown plants (1 to 2m). Fourteen months after their transfer to the greenhouse, all juvenile somatic embryo derived plants produced flowers and manual reciprocal pollination tests were conducted. Somatic embryo derived plants were successfully pollinated by pollen from seed grown plants to produce pods (Fig. 4.6d). Similarly,

pollen from somatic embryo derived trees were used to successfully pollinate seed grown plants also resulting in pods.

Table 4.3. Response of seven different cacao clones to acclimation.

Cacao clones	Number of plantlets	Number of acclimated plantlets	Percentage of acclimated plantlets (%)
Laranga	23	21	91.3
RB 48	5	4	80.0
Scavina 6-1	30	26	86.6
Scavina 6-2	80	78	97.5
Scavina 6 x ICS1	10	8	80
TSH 516	32	32	100
UF 613	4	4	100
TOTAL	184	173	94

Figure 4.6 Development of somatic embryo derived-plant during and after acclimation. a. Cacao plantlets during acclimation. b. Juvenile cacao somatic embryo derived plant with jorquette. c. Somatic embryo derived cacao plant with jorquette and flowers. d. Pod from S.E. derived cacao plant

DISCUSSION

Successful plant regeneration using somatic embryogenesis relies on efficient conversion and acclimation steps. The production of a high number of somatic embryos does not always translate to higher plant production. Conversion of cacao somatic embryos into plantlets has been mainly attempted using MS (Murashige and Skoog, 1962) medium and basal salt as source of nutrients (Adu-Ampomah et al., 1988; Duhem et al., 1989; Pence et al., 1991; Wen et al., 1991; Lopez-Baez et al., 1993; Sondahl et al., 1993; Alemanno et al., 1997). Early reports of conversion into plantlets of cacao somatic embryo from zygotic tissue showed a low number of plantlets obtained on MS medium alone (Wen et al., 1991), in combination with cotyledon excision (Adu-Ampomah et al., 1988), activated charcoal (Duhem et al., 1989; Pence et al., 1991) or by micrografting (Anguilar et al., 1992). The results obtained here indicate that although plantlets can be obtained using MS based medium, it is less efficient than WPM (Lloyd and McCown, 1980) or DKW (Driver and Kuniyuki, 1984) media. Flynn et al., (1990) indicated that WPM was more favorable for cacao shoot culture than MS, and similarly, Figueira et al. (1993) used WPM to promote shoot development during cacao somatic embryo conversion under elevated CO_2 atmosphere. During hardening, cacao plantlets grown on MS medium or WPM medium tend to turn yellow and require a constant transfer into fresh medium every two to three weeks in order to stay green. Plantlets cultured on DKW

medium stay green and can be maintained in the same medium for more than two months, which allows for a hardening step without disturbance of the root system caused by frequent transfer required when other basal salts are used. Woody plant medium (WPM) and DKW which have higher salt concentrations than MS were optimized for woody plants and walnut tissue culture, and thus it is not surprising that these two basal salts support a higher conversion rate than MS.

The addition of potassium nitrate and amino acids to DKW medium improved the conversion rate by increasing the overall shoot production rate and the percentage of normal shoot production. Amino acids may promote protein accumulation during the maturation and conversion of somatic embryos. Stuart et al., (1984) observed that the incorporation of amino acids during alfalfa somatic embryogenesis improved the embryo quality and conversion rates. Similarly, Lopez-Baez et al., (1993) reported conversion of cacao somatic embryos using amino acids. Accumulation of protein and starch reserves in cacao somatic embryos cultured on high sucrose and ABA containing medium improved their conversion into plantlets (Alemanno et al., 1997). Potassium nitrate is not present in DKW basal salt and based on this study, its addition to that medium seems to be beneficial to cacao somatic embryo conversion.

Some of the difficulties encountered during conversion are related to the aberrant forms of embryos produced during somatic embryogenesis in many species (Tissera et al., 1979; Bucheim et al., 1989; Wetzstein and Baker, 1993). Somatic embryos similar to the type I embryos described in this study have been previously

reported by Adu-Ampomah et al. (1988) who observed embryos with large cotyledons and arrested developmental embryonic axis in liquid culture. Only four plantlets were obtained from such embryos after the removal of the cotyledons.

The results presented indicated in this chapter that type I embryos have a lower germination rate than type II. Somatic embryos from zygotic tissue with description similar to type II embryos were successfully converted into plantlets (Duhem et al., 1989). These authors also reported aberrant plantlets with cotyledon-like leaves, however these were not converted into normal plants. Our characterization of somatic embryo derived plantlets indicate similar abnormalities on a fraction of the plants (Fig. 4.2d). Their conversion into normal plantlets was accomplished using *in vitro* pruning (Fig. 4.2e).

Sondahl et al. (1989) reported that ivory colored cacao somatic embryos are most effective for achieving differentiation and that translucent embryos do not proceed on a normal developmental pattern. In this study, translucent type I embryos were successfully converted into normal plants, but their conversion rate remained lower than that of the ivory colored type II embryos. Cacao zygotic embryos, which are translucent during the early phase of embryogenesis, turn opaque during their growth phase and accumulate lipid and starch reserves during maturation (Alemanno et al., 1997). Somatic embryos grown in presence of high concentrations of sucrose and ABA accumulate protein and carbohydrate reserves, and show higher percentages of opaque embryos with higher conversion rates (Alemanno et al., 1997). The higher

conversion rate of type II embryos in this study could be due to higher protein and carbohydrate reserve content. After decapitation of cacao plantlets with abnormal apical shoots, normal shoots developed from axillary meristems as a result of a release from apical dominance. This indicates that abnormal apical shoots possess normal axillary buds. These results are in an agreement with the previous report by Janick and Whipkey (1985), who induced adventitious shoots from the axillary buds of cotyledonary nodes in germinating cacao seedlings after decapitation.

The slow growth of somatic embryo derived plantlets during acclimation was not surprising. Somatic embryo-derived plantlets require an acclimation period during which they slowly adapt to the change from the *in vitro* to the *ex vitro* conditions. Unlike seed, somatic embryo-derived plantlets do not have stored seed reserves that can be utilized during acclimation. Lipids and proteins reserves that are abundant in cacao seeds provide the energy and nutrient to fuel the rapid growth observed during seed germination. At the end of the germination these reserves are exhausted and the growth rate of the seedlings are considerably reduced and similar to that of somatic embryo derived plantlets. However, due to the hypocotyl elongation that occurred during seed germination, seedlings remain 5 to 12 cm taller than somatic embryo derived plant for 10 months.

In cacao the development of the jorquette occurs at a height of one to two meters, and flowering begins between 12 to 18 months (Wood and Lass, 1985). The somatic embryo-derived plants produced in this study flowered and developed a

jorquette in these ranges, indicative of a normal development. Furthermore, the stems of these trees have a normal brown color as in seed-derived plants and produce flowers that are fertile. Pollination of these flowers has yielded numerous cherelles (young cacao pods) and mature pods on several trees. Seeds from these pods were successfully germinated and normal plants produced.

Prior to this study, conversion and acclimation steps remained a block for plant production using cacao somatic embryogenesis. The improvements shown here by the use of media adapted to tissue culture of woody plant species, indicate that cacao tissue culture can be greatly improved by optimizing the basic nutrient content of the medium. In this study, abnormal embryos not fit for conversion were able to produce normal plants. This approach to conversion and acclimation of cacao somatic embryos by optimizing the basic medium, identifying and broadening the use of all embryo types, and simplifying the acclimation protocol, has opened a way for the practical application of somatic embryogenesis to clonal propagation in cacao. Field evaluation in producing countries of plants produced by these methods is necessary in order to establish conformity and production data and to compare them with plants produced by other methods of propagation currently used in cacao.

Chapter 5

A MICROPROPAGATION SYSTEM FOR CACAO (*Theobroma cacao* L.) USING SOMATIC EMBRYO DERIVED PLANTLETS

INTRODUCTION

Cacao is mainly seed propagated, but because of genetic variability among seed derived plants, a proportion of cacao trees in a plantation show less than desired productivity. Thus there is a need for an efficient system for clonal propagation of superior trees selected in breeding programs or identified in the wild or in plantations. Seed derived cacao trees are characterized by two types of growth. First a vertical or orthotropic growth is initiated just after germination to form the main trunk of the tree. Approximately one year after germination a horizontal or plagiotropic growth is initiated with the formation of a crown of three to five fan branches called the jorquette. Clonal propagation in cacao using rooted cuttings and grafting is used in growing regions such as Trinidad, Ecuador and Malaysia. However, because of the lack of a taproot and the absence of a typical cacao dimorphic growth (orthotropic and plagiotropic) in the resulting plants, these methods have not been successful in other growing regions like Africa. Plants produced from plagiotropic rooted cuttings exhibit a bush-like growth habit that requires pruning and they lack a taproot making them susceptible to drought (Bertrand and Agbodjan, 1989). During orthotropic

growth, young cacao plants have the ability to produce new orthotropic shoots from the axillary buds when the death or excision of the apical orthotropic shoot occurs (Wood and Lass, 1985). This is also true in decapitated mature trees as demonstrated by Bertrand and Agbodjan (1989) who used this technique to produce orthotropic rooted cuttings. Vegetative propagation methods capable of producing cacao plants with dimorphic growth have been demonstrated in the past by the bending of mature trees, (Glicenstein et al., 1990), or by bending of young orthotropic and plagiotropic stems (Amefia et al., 1985; Bertrand and Agbodjan, 1989; Bertrand and Dupois, 1992).

As an alternative to vegetative propagation, *in vitro* shoot multiplication has been attempted in cacao with limited success. (Orchard et al., 1979; Essan, 1981; Passey and Jones, 1983; Janick and Whipkey, 1985; Flynn et al., 1990, Figueira et al., 1991; Adu-Ampomah et al., 1992; Lardet et al., 1998). Recent advances in the development of cacao somatic embryogenesis as a way to clonally propagate superior genotypes has permitted the production of hundreds of aseptically grown cacao plantlets from staminodes and petals cultures. However, the production cost of these plants remain high and the efficiency of embryogenesis in certain genotypes low (Li et al., 1998). The development of a quick and relatively inexpensive micropropagation system using somatic embryo derived plantlets as stock plants would increase the efficiency of *in vitro* plant production in cacao. The objective of this study was to develop a micropropagation protocol for cacao using somatic embryo derived plantlets as stock material. We report methods for shoot tip and internodale stem culture using explants from somatic embryo derived plantlets. Shoot multiplication using *in vitro* pruning technique of plantlets is evaluated and the

integration of these methods as means to increase *in vitro* clonal propagation efficiency of cacao is discussed.

MATERIALS AND METHODS

Chemicals were obtained from Sigma Chemical Co., St Louis, MO, or Fisher Scientific, Pittsburgh, PA. The pH for all media was adjusted to 5.5 with 1*M* KOH before autoclaving at 121 C for 20 min.

Plant material

Somatic embryo derived plantlets from the genotypes Scavina 6, TSH 516 and TSH 565 were produced according to Li et al. (1998). These plantlets were maintained in the secondary conversion medium according to Li et al. (1998) until they reached 2 to 3 cm in height with roots and leaves 3 cm long or more, at which time they were used for micropropagation.

In vitro punning

Selected plantlets were decapitated 1 to 2 cm below the apex. The leaves on the apical explant were trimmed to 1/4 of their size before transfer into G 7 magenta vessels containing 50 Ml of root induction medium (RI).

Root induction

Explants were cultured in root induction (RI) medium composed of; 1/2 strength DKW basal salts (Driver and Kuniyuki, 1984) supplemented with 10 g/L of glucose, 5 g/L of sucrose, 50 mg/L of myo-inositol, 1.2mg/L of glycine 1 mg/L of thiamin-HCL, 0.5 mg/L of nicotinic acid, 0.3 g/L of potassium nitrate, 3 g/L of IBA indole butyric acid, 1.75 g of phytagel, 2.5 μM of arginine, leucine, lysine and tryptophan. The explants were induced in RI media for 1, 2, 4, 8, or 16 days at 25°C under 16 hr photoperiod with a light intensity of 50 $\mu mol.m^{-2}.s^{-1}$.

Root development and plantlet maintenance

After induction in RI medium the explants were transferred into G 7 magenta culture vessels containing 150 mL of root development (RD) medium (RI media without IBA) and cultured until roots had grown at least 4 to 5 cm. Explants without roots were subcultured every 4 weeks and maintained on RD medium until roots developed. After 8 weeks of culture, explants without roots were induced again in RI media.

Effect of TDZ on internodal and apical explants

RD media supplemented with various concentrations of thidiazuron (TDZ, a shoot promoting synthetic cytokinin) were poured into 15 mm petri dishes. Apical and internodal TSH 516 explants containing 2 leaves, trimmed to 1/4 of their size,

were placed horizontally into the medium. The explants were cultured for five days after which they were transferred onto a TDZ free RD medium. Bud break was scored 8 days after culture initiation by counting buds that were swollen and extended. Shoot development was scored 6 weeks after culture initiation, by counting buds that developed leaves at least 1 cm long.

Plant acclimation

When newly induced roots reached 4 cm long or more, and explants produced 3 to 5 hardened leaves, the new plantlets were transplanted into pots containing a wetted mixture of equal amounts of promix (Premier Horticulture LTEE, Rivière du Loup, Quebec, Canada G5R4C9), and sand. Plantlets were transferred to a greenhouse, maintained at 27-30°C, 80% relative humidity, and were watered every day for the first three days, after which they were watered as needed and fertilized once a week with a cocoa fertilizer (see appendix C).

Shoot proliferation

After decapitation, the remaining mother plantlets were subcultured onto fresh RD medium. Extra care was taken during the transfer by breaking the surface of the medium to facilitate the immersion of the roots into the medium without damaging them. The decapitated plantlets were cultured under 16 hr photoperiod with a light intensity of $50\mu mol.m^{-2}.s^{-1}$ at 25°C. After 10 days, newly released axillary buds developed into shoots. When 2 to 3 cm in height and after their leaves had hardened

(approximately 3 weeks after their release), the shoots were used for rooting experiments.

RESULTS

Effect of TDZ on Axillary Bud Development of Internodal and Apical Explants

Cacao bud development is characterized by bud swelling, bud opening followed by leaf expansion and shoot elongation. In the preliminary experiments, a single shoot developed from each axillary bud of internodal explants. In an attempt to increase the number of shoots that developed per bud, the effect of TDZ was studied on bud break and leaf expansion. Apical and internodal explants from TSH 516 somatic embryo derived-plants (SEPs) were excised and cultured on media with various concentration of TDZ. Five days after culture initiation on TDZ containing media bud break was observed on both apical and internodal explants (Fig. 5.1a). Explants on TDZ containing media developed callus growth even after a transfer onto TDZ free media (Fig. 5.1b). Callus growth was extensive when higher TDZ concentrations were used. Only a small amount of callus developed on the wound site of explants grown in absence of TDZ. TDZ did not significantly affect bud break on internodal explants, but at higher concentrations, it increased bud break in apical explants ($p=0.068$) (Fig. 5.2a). Although bud break occurred in both types of explants when cultured in the presence of TDZ (Fig. 5.2a) only buds from internodal explants developed further to produce leaves (Fig. 5.1c and 5.2b); however at high TDZ concentrations that development was reduced (Fig. 5.2b). In the absence of

TDZ, internodal explant exhibited five times more bud break than apical explants (86% and 16% respectively). Swollen buds from apical explants did not proceed to open and produce leaves, instead the primary apical bud restarted growth and continued to elongate and grow as a single shoot (Fig. 5.1e). Approximately two months after culture initiation, sporadic root formation can be observed at the base of some of these apical explants in the absence of exogenous auxin in the media (Fig. 5.1e).

Shoot Proliferation

A shoot proliferation step was achieved by culturing decapitated plantlets (Fig. 5.3a). The removal of the shoot tip allowed the release of axillary buds from apical dominance (Fig 5.3b, 5.3c and 5.3d). An average of 2 to 3 new and actively growing shoots were obtained per explant (Table 5.1). As each of the axillary buds was harvested for rooting, subtending buds were released, creating several branches on the plantlet (Fig. 5.3d). Shoot tips from each branch were harvested for rooting. Internodal explants cultured on RD medium released most of their axillary buds which were further harvested for rooting (Fig. 5.1e). However if a rapidly growing shoot is not quickly harvested, it establishes dominance and inhibits the growth and development of other subtending buds reducing proliferation (Fig. 5.1d).

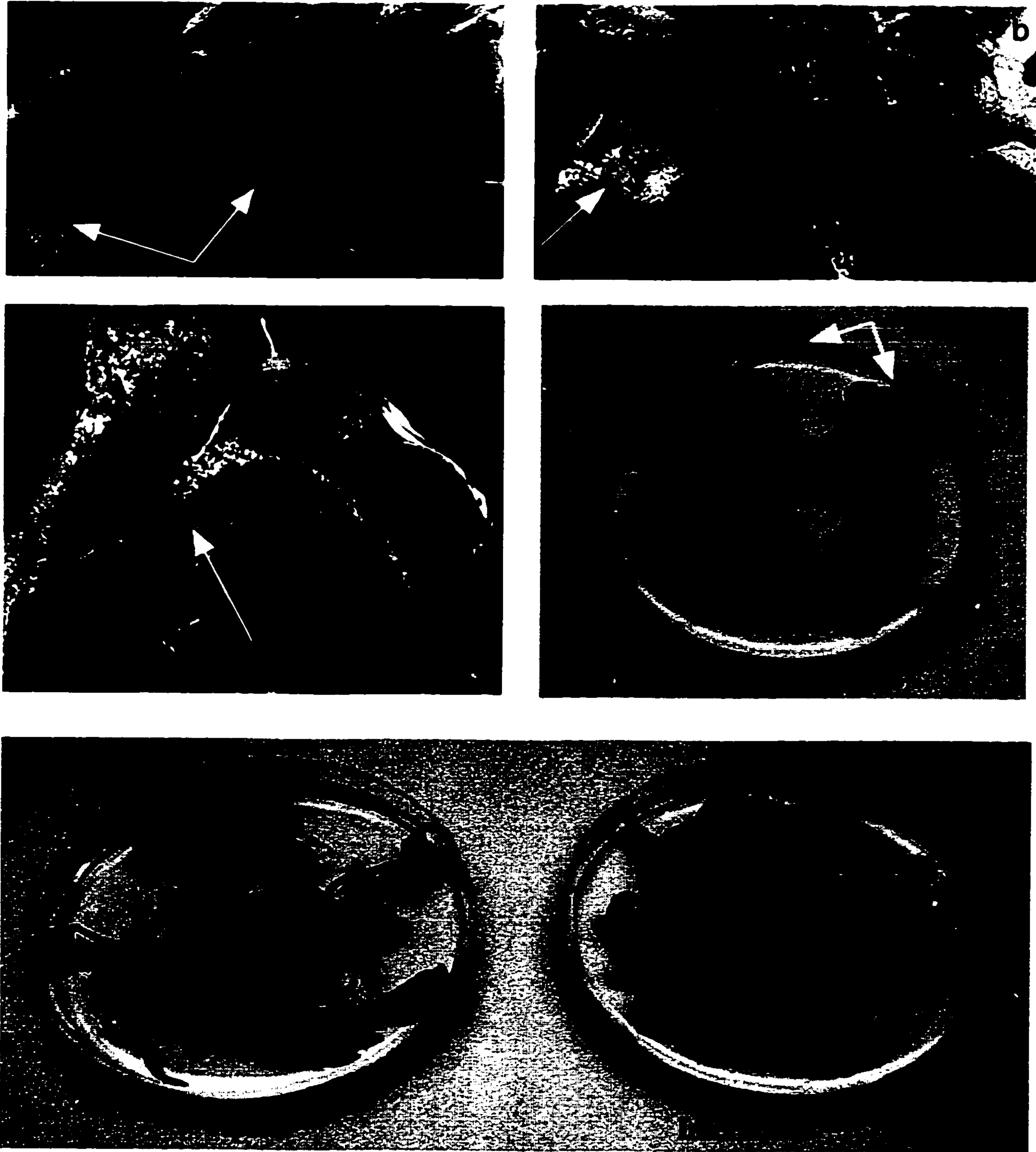

Figure 5.1 Internodal and apical shoot culture of cacao somatic embryo derived-explants. a. Bud break 8 days after culture initiation. b. Callus growth around the developing axillary shoot from an internodal explant 8 days after culture initiation. c. Elongating axillary shoot from an internodal explant 10 days after culture initiation. d. Establishment of apical dominance by fast growing axillary shoot from internodal explant 3 weeks after culture initiation. e. Apical and internodal cacao explants 3 weeks after culture initiation. *Bar* = 1mm

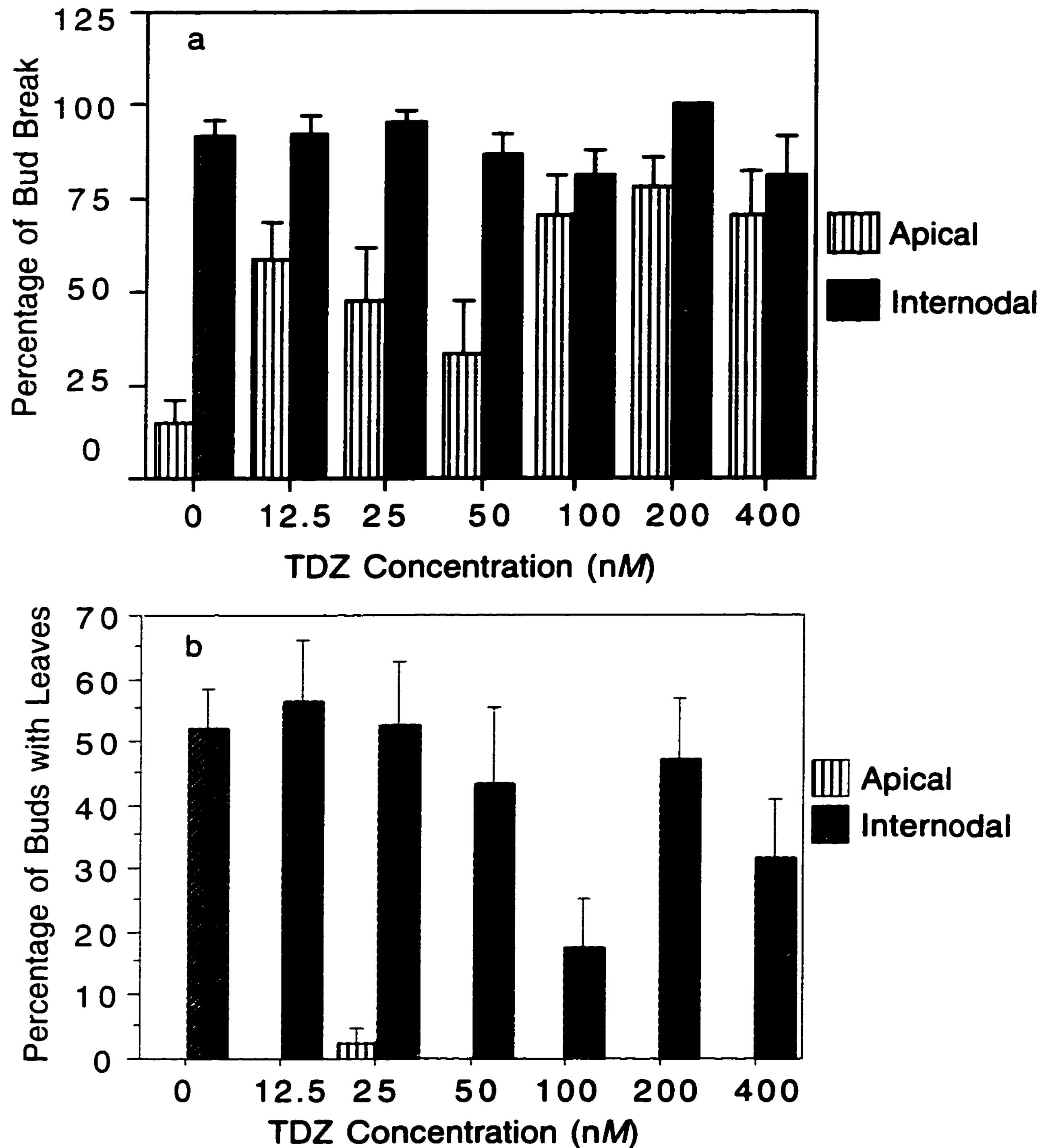

Figure 5.2 Effect of TDZ on cacao axillary bud development of internodal and apical explants from TSH 516 somatic embryo derived plantlets. a. effect of TDZ concentrations on bud break. b. effect of TDZ concentration on leaf production. Bud break was scored 8 days after culture initiation by counting buds that have swollen and extended. Shoot development was scored 6 weeks after culture initiation, by counting only buds that have developed leaves one cm long or more. Each treatment contained 3 replicate plates of 12 to 18 explants each. Vertical lines represent the standard errors of the means.

Figure 5.3 Shoot proliferation after *in vitro* pruning of cacao somatic embryo derived plantlets: a. 5 days after *in vitro* pruning. b. 10 days after *in vitro* pruning. c. Initial branching three weeks after the first *in vitro* pruning. d. Multiple branch formation four weeks after several rounds of *in vitro* pruning.

Table 5.1. Response of three different cacao clones to shoot proliferation after *in vitro* pruning.

Cacao clones	Number of plantlets	Number of plantlets with shoots	Percentage of plantlets with shoots (%)	Average number of growing shoots per after decapitation
Scavina 6-2	56	56	100	2.1
TSH 516	29	29	100	2.9
TSH 565	12	12	100	2.3
TOTAL	97	97	100	2.4

Effect of IBA on rooting

Cacao shoot tips from SEPs can sporadically produce roots in the absence of exogenous auxin. However, to increase rooting percentage and the number of roots per explant, rooting of harvested shoot tips was achieved in a two phase-system using IBA. Roots were first induced by culturing the explants on IBA containing media for varying lengths of time (Fig. 5.4a) followed by a transfer onto media without IBA (Fig. 5.4b). Root formation was visible as early as one week after culture initiation. In some cases the formation of adventitious roots was preceded by callus formation at the base of the shoot tip (Fig. 5.4b). The effect of IBA exposure time on the number of roots produced by the explants was evident after one month. A significant effect of IBA exposure time on the number of roots produced was observed ($p=0.0001$) Prolonged exposure of the shoot tip to IBA before a transfer onto media without IBA

resulted in larger callus production at the base of the explant and also in a higher number of roots per explant (Fig. 5.5b and 5.6b). However, a slight reduction of rooting percentage resulted (Fig. 5.5a) and some of these plants were stunted. A continuous culture of shoot tips in IBA containing media resulted in inhibition of root development and plantlet growth. A short root induction time (24 hr or 48 hr) produced plantlets with one or two roots (Fig. 5.5b). The most significant increase in root number (approximately 5 per explant) was observed after an IBA induction time of 16 days which was more than twice the number of roots obtained for 1 or days of IBA induction. The adventitious roots grew in a manner similar to that of cacao embryonic taproots, they grew straight down into the media without branching and occasionally produced root hairs (Fig. 5.4c and 5.4d). This is different from rooting of plagiotropic shoots, which form fibrous root systems. The percentage of rooting (≈90%) was not significantly affected by various IBA induction times (p=0.062) (Fig. 5.5b), however, a significant difference was observed between the contrcl and all IBA treatments (p=0.0002). While no significant difference was observed between the rooting of Scavina6 and TSH 516 or TSH 565 (p=0.585 and p=0.4189 respectively), a significant difference was observed between TSH 516 and TSH 565 (p=0.013). As indicated in table 5.2, rooting percentage varied between 83% and 92% with in average of 2 to 3 roots per explant following an 8 day IBA induction treatment.

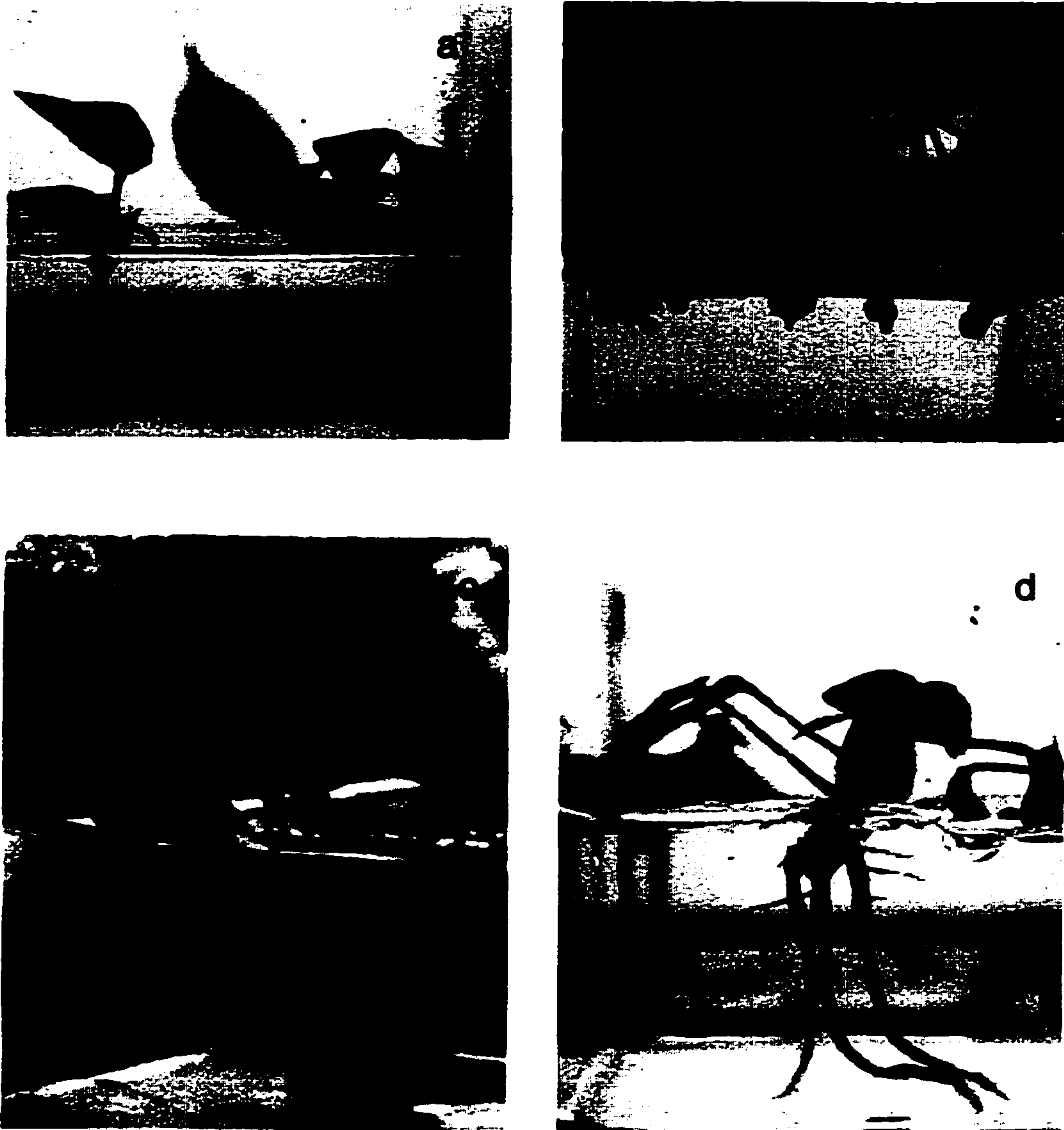

Figure 5.4 Rooting of somatic embryo derived cacao shoot tips. a. Freshly cultured shoot tip. b. Shoot tip explants after 8 days of IBA induction and two days in IBA free medium. c. Root initiation on shoot tip explant 3 days after a transfer onto IBA free medium. d. Rooted micropropagated cacao plantet 2 weeks after culture initiation.

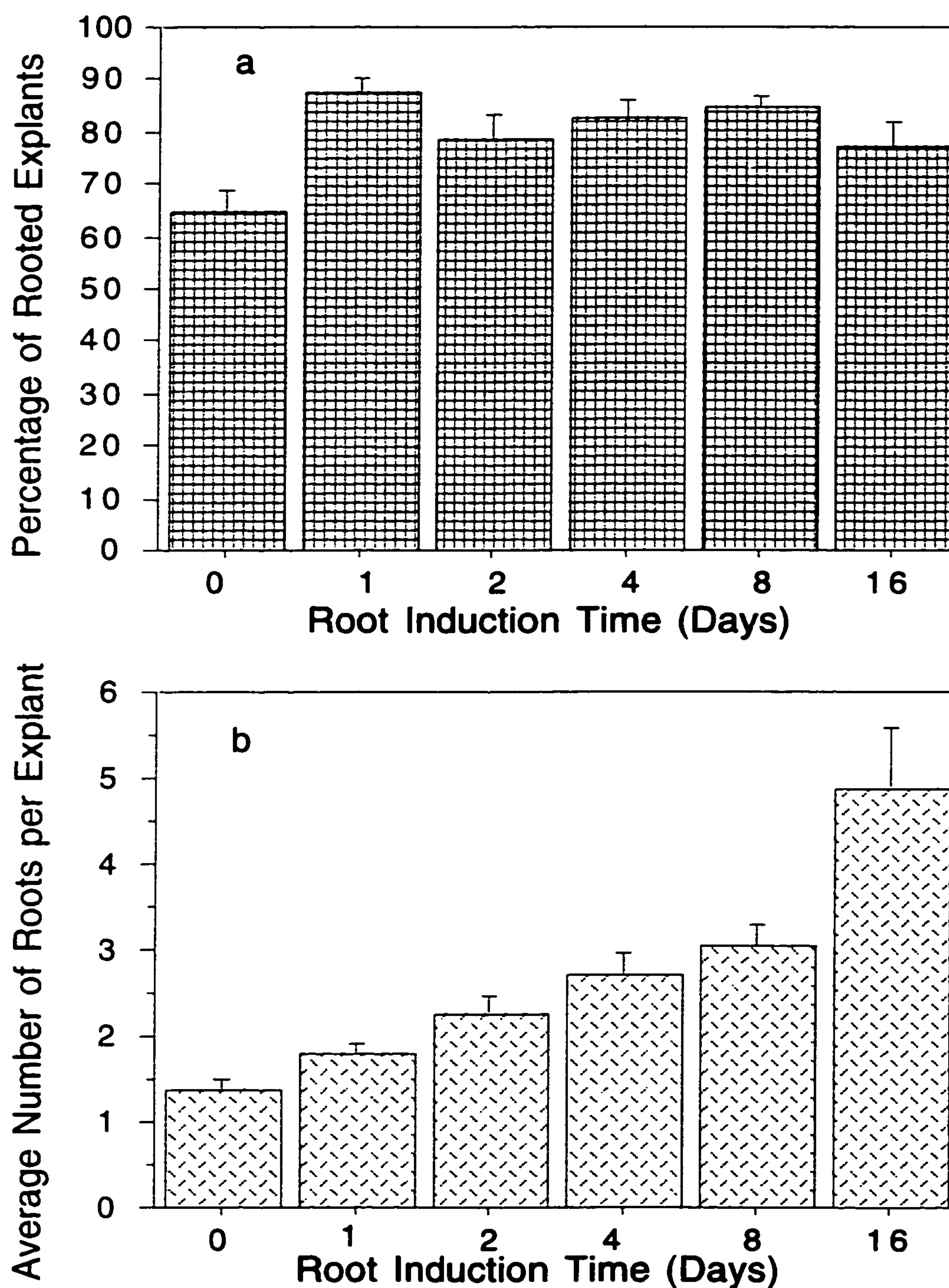

Figure 5.5 Effect of IBA induction time on rooting of cacao shoot explants from the Scavina 6 genotype: a. Percentage of rooted explants. b. Average number of root per explant. Explants were cultured in magenta vessel. Each vessel contained 5 plantlets with 4 replicate vessels per experiment, and each experiment was repeated 3 times. Vertical lines represent standard errors of the means.

Table 5.2. Response of three different cacao clones to *in vitro* rooting of apical shoots.

Cacao clones	Number of plantlets	Number of rooted plantlets	Percentage of rooted plantlets (%)	Average number of roots per plant
Scavina 6-2	50	44	88	3.1
TSH 516	29	24	83	3.6
TSH565	12	11	92	4
TOTAL	91	79	86	3.4

Acclimation of Micropropagated Plantlets

Approximately 3 weeks after transfer onto media without IBA, the shoot tip explants, which had produced roots and resumed growth with the production of new leaves, were transferred into soil and maintained in the greenhouse for acclimation (Fig. 5.6a and 5.6b). After a one week period of acclimation, new leaves were observed on these plants indicating their successful establishment in the *ex vitro* condition. Depending on the genotype, 84% to 92% acclimation rate was achieved in the greenhouse (Table 5.3). Micropropagated plants continued a normal dimorphism growth similar to that of cacao seedlings (Fig. 5.6c). Similar to seed derived-plants, the presence of functional taproots was observed in the root system of all plants investigated (Fig. 5.6d). In addition to normal growth and root system development, these plants produced normal cacao leaves and a normal brown colored stem (Fig. 5.6d). As of January 2000, close to 80 micropropagated cacao plants 4 to 10 months in age are growing in the greenhouse and the oldest ones have formed a jorquette.

Table 5.3. Response of three different cacao clones to acclimation.

Cacao clones	Number of plantlets	Number of acclimated plantlets	Percentage of acclimated plantlets (%)
Scavina 6-2	44	37	84
TSH 516	26	24	92
TSH 565	14	12	86
TOTAL	84	73	87

Figure 5. 6 Acclimation of micropropagated cacao plantlets. a, Six week old single rooted micropropagated cacao plantlet ready for a transfer into soil. b, Six week old multiple rooted micropropagated cacao plantlet ready for a transfer into soil. c. Two month old micropropagated cacao plants after acclimation in the greenhouse. d. An 8 months old micropropagated cacao plant showing a functional tap root.

DISCUSSION

In previous reports, micropropagation of cacao has been met with limited success (Orchard et al., 1979; Passey and Jones, 1983; Flynn et al., 1990; Adu-Ampomah et al., 1992; Lardet et al., 1998). Factors such as media, explant type and age, culture conditions and sterilization of the vegetative shoot explants have been implicated to be limiting in achieving an efficient micropropagation system. By using somatic embryo derived plantlets from *in vitro* culture, the procedure described here eliminated the need for sterilization, resulting in minimal explant loss and healthier explant growth. In previous reports, MS medium (Murashige and Skoog, 1962) was used for micropropagation in cacao (Orchard et al., 1979; Essan, 1981; Passey and Jones, 1983; Janick and Whipkey, 1985; Flynn et al., 1990; Adu-Ampomah et al., 1992; Lardet et al., 1998), however Flynn et al. (1990) found that woody plant medium (Lloyd and McCown. 1980) was an improvement over MS medium. These authors indicated that explants continued growth for a longer period of time without leaf chlorosis and abscission when woody plant medium (WPM) was used instead of half-strength MS salts. Similarly, Figueira et al. (1991) utilized WPM and a high CO2 environment to promote *in vitro* shoot growth of cacao. More recently, Li et al. (1998) demonstrated that the use of DKW medium (Driver and Kuniyuki, 1984) in combination with TDZ stimulated embryogenic callus growth and efficiently induced cacao somatic embryos and plantlet development. The use of DKW media in this protocol promoted healthy shoot and root development. DKW, which was developed for woody plants, contains higher concentrations of elements such as calcium, sulfur and magnesium than does MS medium.

TDZ has been used to promote shoot development in many woody species (Carl et al., 1993; Lu, 1993; Murphy et al., 1998). In cacao it was shown to be a potent inducer of somatic embryo production (Li et al., 1998). Figueira et al. (1991) used TDZ and BA to promote shoot proliferation from cotyledonary nodes of decapitated cacao seedlings however the shoots failed to root *in vitro* and to develop into normal plants. To the contrary, the use of TDZ in our experiments did not improve shoot development. This could be due to the short TDZ exposure time implemented in order to avoid extensive callus growth. Similar results have been reported in other plant species (Ashok et al., 1996; Jusaitus, 1997; Kim et al., 1997; Ben Jouira et al., 1998; Huang et al.,1998). Cacao shoot explants growing in TDZ containing media tend to quickly produce callus growth that later interferes with bud development. Extensive callus growth has been found to be a limiting factor in healthy cacao shoot tip culture (Adu-Ampomah et al., 1992). The tendency of TDZ to promote callus growth have been recently reported for other woody plants (Lakshmanan et al., 1997; Ben Jouira et al, 1998; Huang et al., 1998). Our result indicated that axillary buds on internodal explants were more responsive to bud development than those on shoot tips. This difference in response was due to the presence on shoot tip explants of the apical meristem that continues to exercise apical dominance on axillary buds present on these explants. In contrast, axillary buds from nodal explants are released from apical dominance, so several develop quickly into shoots with healthy growing leaves. A similar response was previously reported in cacao (Adu-Ampomah et al., 1992; Lardet et al., 1998) and in other woody plants species (Lakshmanan et al., 1997; Huang et al., 1998). The release of axillary buds from apical dominance in decapitated plantlets is a very important factor for the proliferation steps in which 2 to

3 buds develop into new shoots at each decapitation cycle. An earlier report by Flynn et al. (1990) indicated that the removal of the shoot apex of micropropagated cacao explants promoted basal bud development, however, a proliferation step was not developed in that protocol. Shoot proliferation from cotyledonary nodes of decapitated cacao seedlings was previously shown, but such shoots could not be rooted *in vitro* for plant production (Janick and Whipkey, 1985; Figueira et al., 1991). A similar approach has been used with *Ixora coccinea* L. which possesses a strong orthotropic growth pattern (Lahshmanan et al., 1997). In that study the authors indicated a higher proliferation rate from stem cuttings, where as cultured shoot tip explants (which contained the apical meristem) did not proliferate and continued growth as a single shoot. The apical meristem maintains an apical dominance on the axillary buds below, by preventing their growth, limiting a proliferation step when the shoot tips were used.

In this study, rooting of developed axillary shoots from decapitated plantlets of nodal explants was readily achieved after IBA treatment. Although sporadic rooting was observed on some shoot tip explants in the absence of exogenous auxin, IBA treatment greatly promoted rooting in cacao. Previously, *in vitro* rooting of cacao shoot tips was performed by a continuous culture of the explants on auxin containing media until rooting (Flynn et al., 1990; Adu-Ampomah et al., 1992). In our study this approach resulted in massive calli formation at the base of explant and an inhibition of plantlet growth and root development. By inducing rooting with IBA containing media, followed by a transfer onto IBA free media, we were able to achieve fast and vigorous root development. With this protocol, plantlets can be produced and ready for acclimation in the greenhouse in as little as 3 weeks.

The lack of a dimorphic growth pattern and taproot formation in plagiotropic cacao rooted cuttings have been a concern in the past (Bertrand and Agbodjan, 1989). Previously, Bertrand and Agbodjan (1989) have demonstrated that orthotropic rooted cuttings produce a dimorphic growth pattern and a functional taproot. Our work indicated that micropropagated plants using somatic embryo derived-plantlets (which are still in the orthotropic growth phase) possess a functional taproot in addition to a characteristic cacao dimorphic growth.

Cacao plants have previously been produced *in vitro* using somatic embryogenesis (Sondahl et al., 1989, 1993; Lopez-Baez et al., 1993; Li et al., 1998). Despite this progress, several cacao genotypes exhibited a low somatic embryogenesis rate (Lopez-Baez et al., 1993; Alemanno et al., 1996; Li et al., 1998). In addition, the cost of plant production using these systems is high and time consuming (8 months from flower parts to plantlet). A micropropagation protocol using somatic embryo derived plantlets offers an additional multiplication step. With a turnover time of 5 weeks, plantlet numbers can double or triple every month. Somatic embryogenesis is also an important regeneration system for the production of transgenic cacao plants. However transgenic plant production in a woody species such as cacao is still a low frequency event. An integration of somatic embryogenesis and micropropagation will allow a rapid multiplication of transgenic plants. Unlike secondary embryogenesis or other tissue culture based methods, micropropagation from axillary buds does not involve a callus phase, thus reducing the risk of somaclonal variation during multiplication. A combination of somatic embryogenesis for initiation of a propagation stock, combined with micropropagation for a rapid and

inexpensive multiplication step is a potentially valuable tool for clonal propagation of elite germplasm.

Chapter 6

FACTORS LIMITING THE GENETIC TRANSFORMATION OF CACAO (*Theobroma cacao* L.)

INTRODUCTION

Genetic transformation is a reality in many plants. However, attempts to genetically transform cacao has yielded little success. Genetic transformation of cacao has been attempted using the biolistic method (Perry et al., 1998) and Agrobacterium (Sain et al., 1994). Although transient expression was obtained using the biolistic method (Perry et al., 1998) no stable transformed tissue was reported. Sain et al. (1994) reported stable transformation of callus cells using Agrobacterium but no plant regeneration was obtained from these cells. Guiltinan et al. (1997) reported the first genetically transformed cacao somatic embryos using Agrobacterium. However, the regeneration of a large number of genetically transformed cacao plants has remained difficult. Previously, Li et al. (1998) has reported a somatic embryogenesis and plant regeneration system for cacao. The system showed up to 100% embryo production in certain genotypes. However, ongoing work in our laboratory on genetic

transformation of cacao has yielded a large number of stable transformed callus cells, without their regeneration into plants. There are several steps involved in the transformation protocol used in our laboratory (Fig. 6.1). Since the embryogenesis protocol shows a high efficiency when genetic transformation was not involved, factors limiting embryo production during genetic transformation attempts probably reside in one or several of the transformation steps. In order to determine those limiting factors, we individually tested the influence of some of the variables in the transformation steps on explant growth and development during somatic embryogenesis. Factors limiting explant growth could be linked to embryogenesis inhibition. In addition, the biolistic method was evaluated using callus from staminode and cotyledons from cacao somatic embryos.

MATERIALS AND METHODS

Immature flowers were collected from Scavina 6 plant growing in greenhouse conditions at the Pennsylvania State University, University Park, PA. The flowers were dissected and staminodes were extracted and put into culture according to Li et al. (1998). After seven days of culture on primary callus growth (PCG) medium the explants were transferred into 50 mL tube containing *Agrobacterium tumefaciens* EHA 105 suspended in ED medium (Li et al., 1998) at 1.0 OD (optical density). The *Agrobacterium tumefaciens* carried a disarmed Ti plasmid (PCP108) containing *npt II*

and *gfp* (green florescent protein) genes respectively for kanamycin selection and fluorescent microscopy scoring. The explants were sonicated for 60 sec in a liquid bath sonicator. After sonication the explants were cocultivated in the same tube with Agrobacterium for 10 min. The explants were taken out of the tube, the excess liquid was removed by a sterile paper towel and placed on petri dishes containing a fresh PCG medium with Whatman filter paper on top. Cocultivation was carried out for 48 hr in the dark at 25 - 27 °C followed by a transfer of the explants onto media containing 400 mg/L of the antibiotic cefotaxime (Hoechst-Roussel Pharmaceuticals Inc. Somerville, NJ) for Agrobacterium counterselection.

In order to identify limiting steps in the transformation protocol, some of the steps involved in the transformation process (explant transfer to liquid media, Agrobacterium treatment, cocultivation on filter paper, antibiotic cefotaxime) were individually tested one at a time. For example, the antibiotic cefotaxime was tested in the absence of Agrobacterium and filter paper treatments. In addition, a positive control (standard somatic embryogenesis protocol) and a negative one (the standard transformation protocol) were included in the experiment.

Each treatment had a total of 60 explants from 3 replicate plates containing 20 explants each. The data were recorded 4 weeks after culture initiation (3 weeks after the transformation) by weighing the staminode explants in groups of five. The data were analyzed using analysis of variance (ANOVA) on the Statview software program (SAS Institute Inc. , Cary, NC).

For the biolistic method, an embryonic cell suspension culture was initiated by grinding embryogenic calli in a cell dissociation sieve (Sigma CD-1). The callus was induced according to Lopez-Baez et al. (1993). The cell suspension was cultured in liquid media for two weeks before plating on solid media for particle bombardment. Cotyledon explants from somatic embryos and embryogenic calli produced according to Li et al. (1998), were also bombarded using the biolistic. Particle bombardment was performed using an helium-driven PDS-1000/He system with 1100 psi rupture discs and 1µ gold particles. The vacuum inside the bombardment chamber was set to 27 Hg and the culture plates placed at the second level from the bottom. Particles for bombardment were coated with a plasmid (PBI 221) DNA containing a ß-glucuronidase (GUS) gene according to Heiser (1992). Transient expression was assayed 72 hrs after bombardment by immersing the explants in X-Gluc histochemical stain (Jefferson et al., 1987) for 24 hrs.

RESULTS AND DISCUSSION

For genetic transformation using the biolistic method, actively dividing suspension cell cultures were established (Fig 6.2a). Suspension cells were spread on petri dishes containing solid medium and bombarded using the biolistic system. Somatic embryo development from the cells was sporadic and only 3 plates out of 40 produced embryos (Fig 6.2b, 6.2c and 6.2d). Three days after bombardment,

transient expression was observed on cotyledon and callus tissue but no stable transformants was observed (Fig 6.2e and 6.2f).

One week after the different treatments, a visible difference in tissue growth could be observed. Tissue treated with Agrobacterium looked dark with no apparent growth. The tissue growing on antibiotic cefotaxime started to produce a hard and whitish non-embryogenic calli, while sonicated tissue and positive control (standard embryogenic tissue) showed normal growth and friable embryogenic calli. The average weight of 5 staminodes showed a negative effect of Agrobacterium treatment on explant growth (Fig. 6.3). The average weight of 5 staminodes treated with Agrobacterium was only 0.011g while staminodes from the control embryogenesis weighed 0.051g. In addition to the Agrobacterium treatment, the filter paper and the antibiotic cefotaxime influenced explant growth negatively (0.039g and 0.033g, respectively). Somatic embryos were later observed on explants from each treatment except those treated by Agrobacterium and or cefotaxime (400 mg/L). Transgenic calli and a proembryonic structure expressing GFP were later obtained from the transformed explants (fig. 6.4), however, no embryo development was observed.

This result clearly indicates that Agrobacterium, the antibiotic cefotaxime and the filter paper have negative effects on explant growth during somatic embryogenesis. The development of explants into a non-embryogenic calli demonstrates that the antibiotic cefotaxime at 400 mg/L interferes with cacao somatic embryogenesis. Cefotaxime was previously shown to reduce embryo development in

Sitka spruce (Sarma et al., 1995). These results open new avenues into the investigation of genetic transformation in cacao. It is now clear that a large number of cacao somatic embryos can be regenerated from staminodes and that Agrobacterium can successfully infect and transform cacao tissue. A future identification of antibiotics capable of eliminating Agrobacterium without interfering with cacao somatic embryo production may increase the efficiency of transgenic embryo production and plant regeneration in cacao.

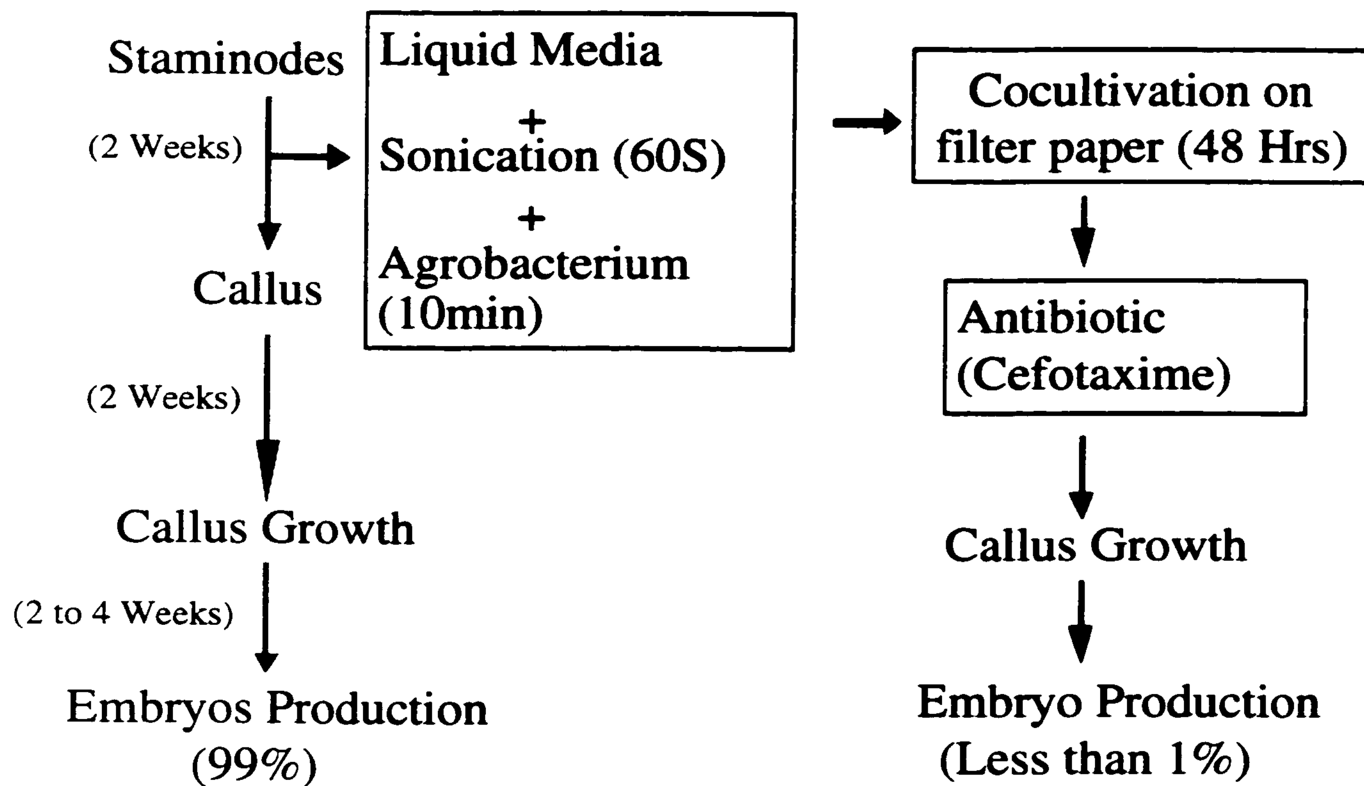

Fig. 6.1 Simplified diagram of Agrobacterium mediated genetic transformation and somatic embryogenesis of cacao using staminode tissue

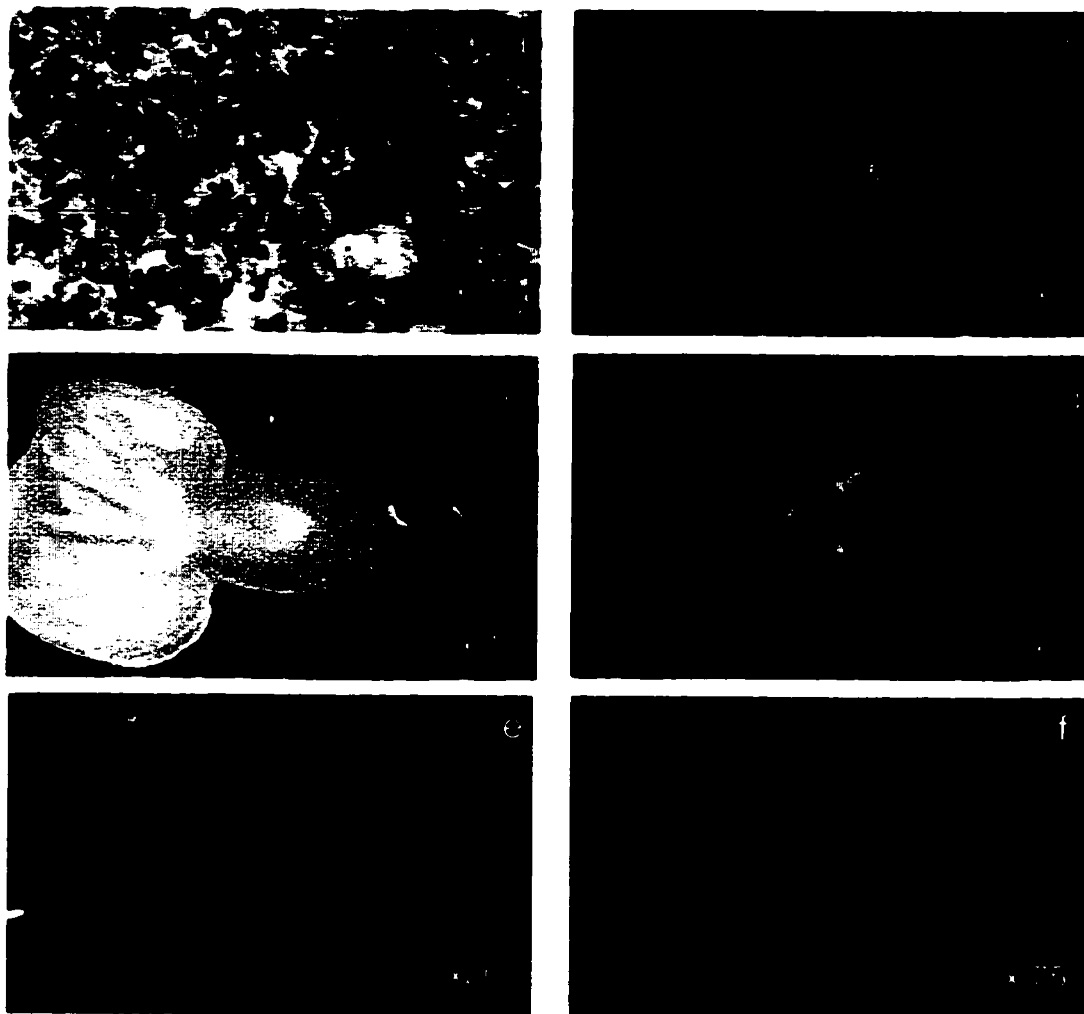

Fig 6.2 Somatic embryogenesis from cell culture and genetic transformation using biolistic method. a. cacao cells cultured in liquid media. b. c and d. somatic embryos developed from cell culture after plating on solid media. e. GUS expression on cotyledon of cacao somatic embryo one week after particle bombardment. f. GUS expression on cacao callus from staminode one week after particle bombardment.

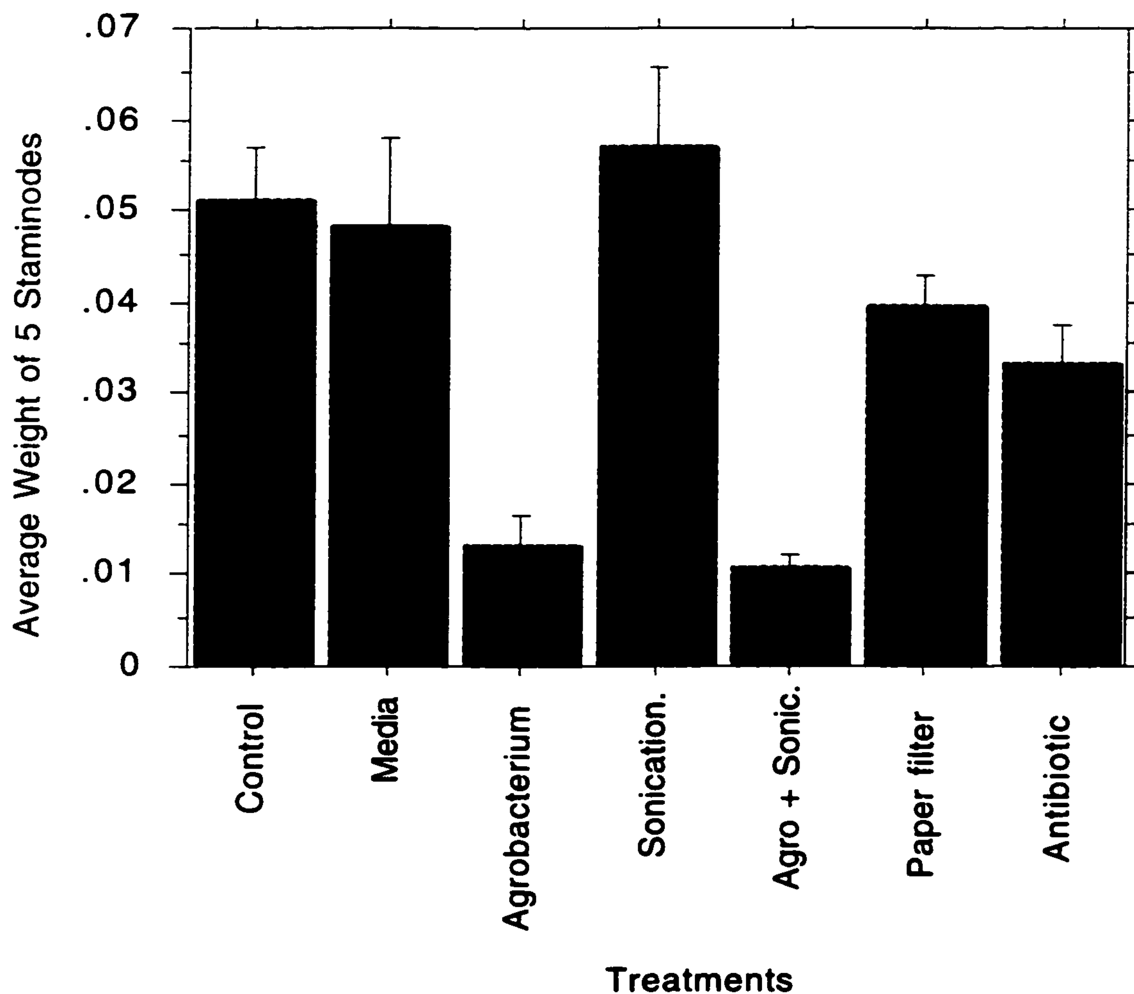

Fig 6.3 Factors affecting somatic embryogenesis during genetic transformation of cacao staminode tissue with Agrobacterium. Each treatment contained 60 explants from 3 replicate plates. Staminode explants were weighed 4 weeks after culture initiation (three weeks after the transformation). Vertical lines indicate standard errors of the means.

Fig 6.4 GFP expression in transgenic cacao tissue. a. cacao proembryonic structure from staminode callus under normal light. b fluorescent cacao proembryonic structure expressing GFP under blue light. c. callus tissue from cacao staminode under normal light. d. GFP expressing cacao callus tissue from staminode under blue light. *Bar* = 0.5 mm.

Chapter 7

CONCLUSION AND FUTURE PERPESPECTIVES

In this research, carbon sources capable of supporting somatic embryo production in cacao have been identified. Glucose, sucrose and fructose can effectively support somatic embryo production in cacao. This response was consistent in both staminode and petal explants although staminode explants showed a higher embryogenic response than petals. The combination of staminode and responsive carbon sources resulted in up to 99% somatic embryo production in cacao, which varies widely according to the genotype used. In general, Forasteros (Scavina 6 and Laranga) showed better embryogenesis response than Trinitarios (UF 613 and ICS 16).

The comparative study of MS, WPM and DKW media indicated that the later two were better adapted for cacao embryo conversion than MS. The addition of amino acids and potassium nitrate to the DKW medium improved the rate and quality of embryo conversion. In addition, the use of an *in vitro* pruning technique has allowed the conversion of embryos that could not be converted in previously published protocols. Cacao somatic embryogenesis produces different types of embryos. In the past, most of these embryos were not converting into plantlets. The combination of *in vitro* pruning with media better adapted to cacao *in vitro* culture,

and the addition of amino acids and potassium nitrate to the media has improved cacao somatic embryo conversion. This improvement resulted in a large number of somatic embryo derived plants being produced from various genotypes and the conversion of transgenic cacao plants. At the moment these plants are being tested in the field in St Lucia at the Union Valley Estate. This conversion protocol has successfully been tested in France by Dr. Alemanno at CIRAD and in Brazil at the Amarante farm where a large number of plants have been produced by a team from M&M Mars company. This optimized protocol for cacao somatic embryo conversion has opened the way for a practical application of somatic embryogenesis in cacao.

The use of the *in vitro* pruning technique for the development of normal shoots during the conversion of abnormal cacao somatic embryo has led to the development of a proliferation step for a micropropagation system in cacao. By removing the apical shoot, somatic embryo derived plantlets can be induced to produce more shoots from the axillary buds. The removed apical shoot can, in turn, be rooted to produce a new plant. The subsequent proliferating shoots, produced by the decapitated plantlet, can also be harvested for rooting every three to four weeks. The addition of this micropropagation step to the somatic embryogenesis system increases the speed and the efficiency of somatic embryo derived plant production. A one month turn over time of the micropropagation offers 4 to 7 months time saving over embryogenesis which require 5 to 8 months for an embryo to form from a staminode and develop into a plantlet ready for acclimation.

The acclimation step starts with fully grown plantlets that possess leaves at least 4 cm long and roots longer than 3 cm. This has greatly improved the survival rate of the plantlets after their transfer from *in vitro* to *ex vitro* conditions in the

greenhouse. During acclimation and plant development we observed that somatic embryo derived-plants showed a lag time during which they adapt to the *ex vitro* conditions, before starting new growth. In contrast, because of their seed reserve stored in the cotyledons, seedlings germinated and started an immediate and fast growth. Somatic embryo derived-plants eventually catch-up with seed derived plants by the end of the first year of development. The plants we observed showed normal cacao characteristic like brown stems and a dimorphic growth with the production of a jorquette at an average height of 1.35 M. In addition, fruits were obtained from some of these plants after pollination, which indicate that cacao somatic embryo derived plants produced using this protocol were fertile. Seeds from these pods were germinated and normal plants were obtained from them.

An optimized cacao somatic embryogenesis and plant regeneration system allows a high probability of regenerating a transgenic cacao plant after genetic transformation. However, genetic transformation using biolistic or Agrobacterium also required an optimization of the gene delivery system. The investigation of some of the factors that affect somatic embryogenesis during genetic transformation using Agrobacterium tumefaciens indicated that Agrobacterium and the antibiotic cefotaxime used to reduce Agrobacterium overgrowth greatly affected cacao somatic embryogenesis. This observation showed that optimized parameters for gene delivery could have an adverse effect on somatic embryogenesis and plant regeneration.

The results obtained here are preliminary, but highlight the importance of attention to the somatic embryogenesis as effected by the protocol. For further research, various concentrations of Agrobacterium will need to be tested for a reduced impact on somatic embryogenesis. In addition, various concentrations of

cefotaxime will need to be tested in order to balance Agrobacterium elimination with somatic embryo production. Other antibiotics capable of eliminating Agrobacterium without inhibiting somatic embryogenesis may eliminate this problem.

For the future an extensive field test of a large number of somatic embryo derived-plants from various genotypes needs to be conducted in order to evaluate the cost of the system and ensure that the system produces true-to-type plants without somaclonal variants. Since the efficiency of somatic embryogenesis varies according to the genotype, a custom optimization of the system for a particular genotype of interest will be necessary. Such optimization could include: the increase or reduction of the growth hormone during embryo induction, or shoot explant rooting, a longer or shorter exposure of the explants to the growth hormone or a variation in amino acids and potassium nitrate concentration during conversion.

The development of cell suspension culture and the adaptation of the system for bioreactor usage could greatly reduce the cost of embryo production and reduce the extensive labor required for embryo transfer. For an immediate cost reduction, somatic embryogenesis, micropropagation and an orthotropic vegetative propagation can used together to produce an integrated propagation system. A diagram of such a system is depicted in figure 7.1. Using an integrated system, somatic embryogenesis and micropropagation will first allow the production of several hundred or even several thousands elite plants from selected clones for further evaluation. After evaluation and approval, these selected clones produced via somatic embryogenesis and or micropropagation, will be utilized for the establishment of a bent-wood garden that will continuously produce orthotropic shoots, for rooting cuttings at a relatively low cost. In addition, farmers will be able to purchase several dozen or few hundred

selected clones and establish their own bent-wood garden for low cost multiplication of planting material. To avoid a possible breakdown in the case of disease or pest resistance, clonally propagated planting material should be from several selected clones with different genetic make up. The two tissue culture systems (somatic embryogenesis and micropropagation) will also allow an exchange of clean disease free plantlets between growing regions without a long quarantine period, while the orthotropic vegetative propagation will produce a low cost elite planting material. Considering the losses in cocoa plantations due to diseases, pests and genetic variability, an integrated propagation system for cacao can be implemented today at a reasonable cost.

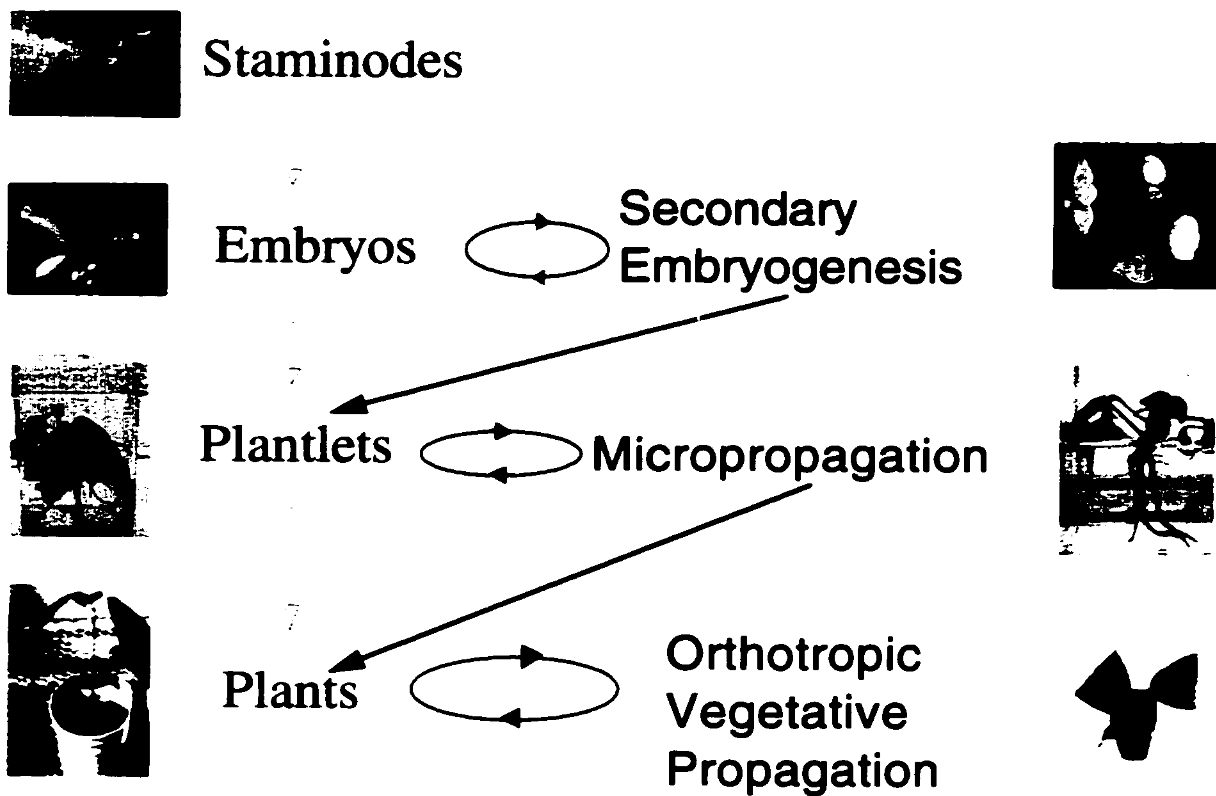

Fig. 7.1 Integrated system for clonal propagation of cacao (*Theobroma cacao* L.)

APPENDIX A

MEDIA FORMULATIONS

Primary Callus Growth Medium (PCG)

	1 L	2 L
DKW macro A (10X)	100 ml	200 ml
DKW macro B (10X)	100 ml	200 ml
DKW micro (100X)	10 ml	20 ml
DKW vitamins (1000X)	1 ml	2 ml
Glucose	20 g	40 g
Glutamine	250 mg	500 mg
myo-Inositol	100 mg	200 mg
2,4-D (1 mg/ml stock)	2 ml	4 ml
TDZ (0.25 mg/ml stock)	20 ul	40 ul
pH 5.8 (1 M KOH)		
Phytagel	2 g	2 X 2 g (split into two 1 L bottles)
Autoclave	18 min	20 min

Note:

1. DKW macro A, DKW macro B, DKW micro stock solutions are stored at 4°C made fresh each month.
2. DKW vitamins solution is stored at - 20°C as aliquots, made fresh every 3-4 months.
3. 2,4-D and TDZ stock solutions are stored at 4°C and remade every two months.

Secondary Callus Growth Medium (SCG -1)

	1 L	2 L
McCown's salts (Sigma M-6774)	2.3 g	4.6 g
B5 vitamins (1000X stock)	1 ml	2 ml
Glucose	20 g	40 g
Coconut water	50 ml	100 ml
2,4-D (1 mg/ml stock)	2 ml	4 ml
Kinetin (1 mg/ml stock)	300 ul	600 ul
pH 5.7 (1 M KOH)		
Phytagel	2.2 g	2 x 2.2 g (split into two 1 L bottles)
Autoclave	18 min	20 min

Note:

1. McCown's salts (Sigma M-6774) are stored at 4°C in a desiccator.
2. B5 Vitamins solution is stored at -20°C as 1 ml aliquots and made fresh every 3-4 months.
3. Kinetin is stored at -20°C as 0.3 ml aliquots and made fresh every 3-4 months.
4. 2,4-D stock solution are stored at 4°C and remade every two months.

Secondary Callus Growth Medium (SCG -2)

	1 L	2 L
McCown's salts (Sigma M-6774)	2.3 g	4.6 g
B5 vitamins (1000X stock)	1 ml	2 ml
Glucose	20.0 g	40.0 g
2,4-D (1 mg/ml stock)	2 ml	4 ml
BA (1 mg/ml stock)	50 ul	100 ul
pH 5.7 (1 M KOH)		
Phytagel	2.2 g	2 x 2.2 g
		(distribute in two 1 l bottles)
Autoclave	18 min	20 min

Note:

1. McCown's salts (Sigma M-6774) are stored at 4°C in a desiccator.
2. B5 Vitamins solution is stored at -20°C as 1 ml aliquots and made fresh every 3-4 months.
3. BA and 2,4-D stock solutions are stored at 4°C and remade every two months.

Embryo Development Medium (ED)

	1 L	2 L
DKW macro A (10X)	100 ml	200 ml
DKW macro B (10X)	100 ml	200 ml
DKW micro (100X)	10 ml	20 ml
DKW vitamins (1000X)	1 ml	2 ml
Sucrose	30 g	60 g
Glucose	1 g	2 g
pH 5.7 (1 M KOH)		
Phytagel	2 g	2X2 g
Autoclave	18 min	20 min

Note:

1. DKW macro A, DKW macro B, DKW micro stock solutions are stored at 4°C, made fresh each month.
2. DKW vitamins solution is stored at - 20°C as aliquots, made fresh every 3-4 months.

Primary Embryo Conversion Medium (PEC) or DKWKA

	1 L	2 L
DKW macro A (10X)	100 ml	200 ml
DKW macro B (10X)	100 ml	200 ml
DKW micro (100X)	10 ml	20 ml
DKW Vitamins (1000X)	1 ml	2 ml
$KN0_3$	0.3 g	0.6 g
Amino acids solution (1000X)	1 ml	2 ml
Glucose	20.0 g	40.0 g
Sucrose	10.0 g	20.0 g
Adjust pH to 5.7		
Phytagel	1.75 g	2X1.75 g (distribute in two 1 l bottles)
Autoclave	18 min.	20 min.

Note:

1. DKW macro A, DKW macro B, DKW micro stock solutions are stored at 4°C made fresh each month.
2. DKW vitamins solution is stored at - 20°C as aliquots, made fresh every 3-4 months.
3. Amino acid (AA) 1000X solution is stored at -20C as 1 ml aliquots, made fresh every 3-4 months.

Secondary Embryo Conversion Medium (SEC)

	1 L	2 L
DKW macro A (10X)	50.00 ml	50.0 ml
DKW macro B (10X)	50.00 ml	50.0 ml
DKW micro (100X)	5.00 ml	5.0 ml
DKW vitamins (1000X)	0.5 ml	0.5 ml
KNO_3	0.30 g	0.4 g
Glucose	10.00 g	10.0 g
Sucrose	5.00 g	5.0 g
Amino acids solution (1000X)	1 ml	2 ml
Adjust pH to 5.5		
Phytagel	1.75 g	2 x 1.75 g (distribute in two 1 L bottles)
Autoclave	18 min	20 min

Note:

1. DKW macro A, DKW macro B, DKW micro stock solutions are stored at 4°C made fresh each month.
2. DKW vitamins solution is stored at - 20°C as aliquots, made fresh every 3-4 months.

Root Induction Medium (RI)

	1 L	2 L
DKW macro A (10X)	50.0 ml	100 ml
DKW macro B (10X)	50.0 ml	100 ml
DKW micro (100X)	5.0 ml	10 ml
DKW vitamins (1000X)	0.5 ml	1 ml
KNO_3	0.30 g	0.60 g
Glucose	10.00 g	20.00 g
Sucrose	5.00 g	10.00 g
Amino acids solution (1000X)	1 ml	2 ml
IBA (1 mg/ml stock)	3 mg (3 ml)	6 mg (6 ml)
pH 5.8 (1 M KOH)		
Phytagel	1.75 g	1.75 X 2 g (distribute in two 1 l bottles)
Autoclave	18 min	20 min

Note:

1. DKW macro A, DKW macro B, DKW micro stock solutions are stored at 4°C made fresh each month.
2. DKW vitamins solution is stored at - 20°C as aliquots, made fresh every 3-4 months.
3. IBA stock solution is stored at 4°C and remade every two months.

Root Development and Maintenance Medium (RD)

	1 L	2 L
DKW macro A (10X)	50.0 ml	100.0 ml
DKW macro B (10X)	50.0 ml	100.0 ml
DKW micro (100X)	5.0 ml	10.0 ml
DKW vitamins (1000X)	0.5 ml	1.0 ml
KNO_3	0.3 g	0.6 g
Glucose	10.0 g	20.0 g
Sucrose	5.0 g	10.0 g
Amino acids solution (1000X)	1 ml	2 ml
pH 5.8 (1 M KOH)		
Phytagel	1.75 g	2 x 1.75 g
		(distribute into 2 one liter bottles)
Autoclave	18 min	20 min

Note:

1. DKW macro A, DKW macro B, DKW micro stock solutions are stored at 4°C made fresh each month.
2. DKW vitamins solution is stored at - 20°C as aliquots, made fresh every 3-4 months.

STOCK SOLUTIONS FORMULATIONS

DKW l0X Macro Elements Solutions A & B

Solution A	**1L**	**2L**
NH_4N0_3	14.16 g	28.32 g
$Ca(N0_3)_2 .4H_20$	19.69 g	39.36 g

Solution B	**1L**	**2L**
$CaCl_2 .2\ H_20$	1.49 g	2.98 g (always add first!)
K_2SO_4	15.59 g	31.80 g
$MgS0_4 .\ 7\ H_20$	7.40 g	14.80 g
KH_2PO_4	2.65 g	5.30 g

DKW macro A and DKW macro B stock solutions are stored at 4°C. Make fresh each month. Use 100 ml of each for 1 L of medium.

DKW 100X Micro Elements Solution

	1 L	**0.5 L**
$Zn(N0_3)_2.6\ H_20$	1.700 g	0.8500 g
$MnS0_4\ .\ H_20$	3.340 g	1.6700 g
$CuS0_4\ .\ 5\ H_20$	0.025 g	0.0125 g
H_3BO_3	0.480 g	0.2400 g
$Na_2MoO_4\ .\ 2\ H_20$	0.039 g	0.0200 g
$FeS0_4\ .\ 7\ H_20$	3.380 g	1.6900 g
Na-EDTA	4.540 g	2.2700 g

Store solution at 4°C. Make fresh each month. Use 10 ml for 1 L of medium.

DKW 1000X Vitamins Stock Solution

	100 ml	50 ml
myo-Inositol	10.0 g	5.000 g
Thiamine-HCL	0.2 g	0.100 g
Nicotinic acid	0.1 g	0.050 g
Glycine	0.2 g	0.100 g

B5 1000X Vitamin Solution

	50 ml
Myo-Inositol	5 g
Nicotinic Acid	50 mg
Pyridoxine	50 mg
Thiamine	500 mg

Store solutions at -20°C as 1 ml aliquots. Make fresh every 3-4 months. Use 1 ml per 1L of medium.

Plant Growth Regulators Stock Solutions

According to specifications for chemicals purchased from Sigma Chemical Co.

TDZ 0.25 mg/ml stock solution - Dissolve and store in DMSO at 4°C, remake every two months.

2,4-D 1 mg/ml stock solution - Dissolve and store in 100% or 95% EtOH, store at 4°C, remake every two months.

Kinetin 1 mg/ml stock solution - Dissolve in 1/20 of the final volume in 1N NaOH and adjust the final volume with H_2O, aliquot, store at -20°C remake every 3-4 months. Thaw before adding to medium.

BA 1 mg/ml stock solution - Dissolve in 1/20 of the final volume in 1N NaOH and adjust the final volume with H_2O, store at 4°C, remake every two months.

IBA 1 mg/ml stock solution - Dissolve in 1/20 of the final volume in 1N NaOH and adjust the final volume with H_2O, store at -20°C, remake every two months.

Amino Acid 1000X Stock Solution

	100 ml
Arginine	43.55 mg
Glycine	18.76 mg
Leucine	32.80 mg
Lysine	45.65 mg
Tryptophan	51.05 mg

Store solution at - 20°C as 1 ml aliquots, remake every 3-4 months. Use 1 ml per 1L of medium.

APPENDIX B

COCOA GREENHOUSE FERTILIZER

100X FERTILIZER SOLUTIONS:

1. For 95 L total fertilizer solution in tank A, dissolve:

   a. Peters 5-11-26 Hydro-Sol hydroponic fertilizer 9.25 kg

   b. FeEDTA 140g (=150ml tapped vol)

   c. $MgSO_4$-H_2O (epsom salts) 2.0kg

2. Adjust pH of warm solution to 1.9 with concentrated HNO_3 (about 700 ml) or H_2SO_4

3. For 95 L total fertilizer solution in tank B, dissolve:

   a. Ca (NO3)2 12.25 kg

   b. Solution of 0.25 g/L $CoCl_2$, 28.9g/L H_3BO_3, 18.0g/L $CuSO_4$, 15.4g/L $MnSO_4$-H_2O, 27 g/L KCl, 74.4 g/L $ZnSO_4$, 0.2 g/L $NiCl_2$-$6H_2O$ 950 ml

4. Adjust pH of warm solution to 1.4 with about 200ml concentrated HNO_3

5. 1X nutrient solution at plant should have a pH of 5.9 (5.5-6.5) adjusted with phosphoric acid

6. 1X working solution :98 parts water plus 1 part 100X from tank A and 1 part 100X from tank B

Final nutrient composition (PPM) of 1X working solution:

Tank	A	+	B
Nitrogen as N	50	+	200
Phosphorus as P	48		
Potassium as K	210		
Magnesium as Mg	50		
Sulfates as SO_4	117		
Calcium as Ca			258
Iron as Fe	5.0		
Zinc as Zn	0.15	+	1.69
Copper as Cu	0.15	+	0.45
Boron as B	0.50	+	0.50
Manganese as Mn	0.50	+	0.50
Molybdenum as Mo	0.10		
Chlorides as Cl	0.04	+	1.29
Sodium as Na	3.62		
Cobalt as Co			0.00618
Nickel as Ni			0.005

Notes:

a. H_3PO_4 causes phosphate salt precipitates to form if used in tank B.

b. pH of greenhouse tap water is 7.8

c. Total dissolved solids (TDS) of the final nutrient solution should not exceed 3500 PPM (mg fertilizer/L). This solution is 2500 PPM.

d. PPM=mg element /L

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VITA

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Abdoulaye Traoré was born in Grand Bassam, Côte d'Ivoire (Ivory Coast) in West Africa, where he attended elementary and secondary schools. He earned his B.S. in Natural Sciences from the National University of Abidjan in 1988 and a M.S. in Plant Genetics from the same University in 1990. In 1991 he was awarded a scholarship by the Ministry of Scientific Research of Côte d'Ivoire to study in the United State. In 1994 he earned his M.S. in Crop Science from Oregon State University in the USA. His advisor was Dr. Patrick Hayes and the subject of his thesis was QTL mapping of yield and yield components in barley.

Abdoulaye then joined the American Cocoa Research Molecular Laboratory at the Pennsylvania State University in 1994 where he entered a Ph.D. program in the Interdepartmental Graduate Degree Program in Genetics. In 1995 he transferred into the Food Science Program and continued a Ph.D. Program working on the development of somatic embryogenesis and genetic transformation of cacao, first under the direction of Dr. Douglas Furtek, then Dr Mark Guiltinan.