ENHANCEMENT OF STORAGE LIFE OF COCOA (*Theobroma cacao* L.) SEEDS THROUGH ENCAPSULATION AND GERMINATION INHIBITION

By

SHIRAN KALAPPURAKKAL

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THESIS

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COLLEGE OF FORESTRY
KERALA AGRICULTURAL UNIVERSITY
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DECLARATION

I hereby declare that the thesis entitled **"Enhancement of storage life of cocoa (*Theobroma cacao* L.) seeds through encapsulation and germination inhibition."** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Place: Vellanikkara

Date: 31-10-2012

SHIRAN KALAPPURAKKAL

(2010-17-105)

Dr . A.V. Santhoshkumar
Associate professor and Head
Dept.of Tree Physiology and Breeding,
College of Forestry,
Kerala Agricultural University,
Vellanikkara, Thrissur-680 656

Date: 31-10-2012

CERTIFICATE

Certified that this thesis entititled "**Enhancement of storage life of cocoa (*Theobroma cacao* L.) seeds through encapsulation and germination inhibition.**" is a record of research work done independently by **Mr. Shiran Kalappurakkal.** (2010-17-105) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

Place: Vellanikkara
Date: 31-10-2012

Dr.A.V.Santhoshkumar
Chairman
Advisory committee

CERTIFICATE

We, the undersigned members of the Advisory Committee of **Mr. Shiran Kalappurakkal** (2010-17-105), a candidate for the degree of Master of Science in Forestry, agree that the thesis entitled "**Enhancement of storage life of cocoa (*Theobroma cacao* L.) seeds through encapsulation and germination inhibition.**" may be submitted by **Mr. Shiran Kalappurakkal** (2010-17-105) in partial fulfillment of the requirement for the degree.

Dr . A.V. Santhoshkumar
Associatte professor and Head
Dept.of Tree Physiology and Breeding,
College of Forestry,
Kerala Agricultural University,
Vellanikkara, Thrissur
(Chairman)

Dr. P. K. Ashokan
Professor ,
Dept. of Tree Physiology and Breeding,
College of Forestry,
Kerala Agricultural University,
Vellanikkara.
(Member)

Dr. K. Sudhakara,
Professor and Head
Dept. of Silviculture and Agroforestry
College of Forestry,
Kerala Agricultural University,
Vellanikkara.
(Member)

Dr. J.S. Minimol,
Assistant Professor (SS), CCRP,
College of Horticulture,
Kerala Agricultural University,
Vellanikkara.
(Member)

EXTERNAL EXAMINER

Dr. REKHA R. WARRIER
SCIENTIST
Institute of Forest Genetics and Tree Breeding
P.B. No: 1061, R.S. Puram, (Post)
COIMBATORE - 641 002.

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Shiran Kalappurakkal

CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURES

INTRODUCTION

INTRODUCTION

Seeds are the dispersal units consisting of embryos which are usually supplied with food reserves and having some form of protective structures. All over the world, they are used as main propagule in majority of plant species. The nature and storage behavior of seeds varies with species as an adaptation to their environment. Development of human civilization has identified the storage behavior of various seeds and classified it into different categories. One of the earliest classifications of longevity in seeds was that proposed by Ewart (1908), in which seeds were grouped into micro biotic (short longevity), meso biotic (medium longevity) and macro biotic (high longevity). But this classification has been criticized for ignoring the fact that longevity is also a function of environment. Later, based on the storage behavior, seeds were broadly classified into two groups as orthodox and recalcitrant (Roberts, 1973).

Orthodox seeds include majority of dry zone species and humid zone pioneers, with lower moisture content tolerance. The low level of moisture content (6-8 percent) assures safe storage for long time at low temperatures. Orthodox seeds will be shed from the parent plant at a lower moisture content after undergoing maturation drying. In the dehydrated state, the seeds can resist the changes of the environment, and unless dormant, will resume full metabolic activity, growth and development when favorable conditions for germination are provided (Roberts and King, 1980). Due to these properties they can be stored for long periods.

Recalcitrant seeds, which prevail among humid zone climax forests like tropical evergreen forests, do not tolerate much desiccation and can only be stored in high moisture content for a short period. They do not undergo maturation drying and are shed at relatively high moisture content. Storage of recalcitrant seed has always been a challenge to the seed technologists and conservationists. Due to various complex problems, the storage becomes difficult in different species. Recalcitrant seeds are highly susceptible to desiccation injury and are killed if the moisture content is reduced below some relatively high critical value (Roberts and King, 1980). Recalcitrant seeds are produced by two types of plants - by those growing in aquatic environments and by perennial plants confined to tropical and temperate wet evergreen forests, where seeds

are unlikely to be exposed to seasonal drying and where the environment is suitable for seedling growth throughout the year (King and Roberts, 1980). In addition to many fruit and timber species of humid tropics, some of the economic crops like cocoa, coffee, oil palm, coconut, rubber etc. also show recalcitrant behavior. Their seed production and maturation ceases with the commencement of the monsoon. Usual nursery preparation starts at the end of monsoon season. This result in the lack of availability of the viable seeds for nursery raising of these recalcitrant species.

The major factors responsible for short longevity of recalcitrant seeds are desiccation injury, chilling damage and problems associated with the storage of seeds having high moisture content such as microbial contamination and germination during storage (King and Roberts, 1980). Even though several methods have been employed to improve the longevity of many recalcitrant seeds, a perfect method for long-term storage still does not exist. Since recalcitrant seeds are desiccation sensitive, storing the seeds under moist conditions is suggested to avoid desiccation injuries (Chin, 1988). But this has only been successful for short term storage. Furthermore, storing seeds with high moisture content at sub-ambient or ambient temperatures will lead to fungal contamination and germination of the seeds while being kept for storage. Storing the seeds under low temperature has been suggested to prevent fungal contamination and germination during storage (King and Roberts, 1980).

Embryonic axes have higher desiccation tolerance than intact recalcitrant seeds (Pammenter and Berjak, 1999). Because of its organized small structure and its ability to produce a whole plant from the meristematic tissues, embryonic axes are preferred for storage in recalcitrant species (Fu *et al.*, 1990). Introduction of synthetic seeds by using excised embryonic axes is one of the methods by which the longevity of recalcitrant seeds can be increased (Sudhakara et al. 2000). This is because the recalcitrant nature is supposed to be due to seed coat and storage tissue. Synthetic seeds, otherwise called artificial seeds, are the manmade counter part of the true seeds. It comprises of an embryo separated from its accessory structures and encapsulated in a hydrated gel capsule, which will permit natural development to the embryo. The term synthetic seed was first proposed by Murashigae in 1977. It was then defined as an embryo entrapped in a bio- degradable synthetic coating which act as an artificial seed coat. It is capable of developing in to an entire plant once favourable conditions are

provided. Encapsulation and addition of inhibitors in synthetic seeds can be useful in developing new storage techniques for recalcitrant seeds making them viable over a longer duration of time.

Theobroma cacao is a tropical evergreen tree native to deep tropical regions of Amazon basin. It is widely domesticated for commercial purposes and grown as a plantation crop or agroforestry species. As like most of the tropical species, recalcitrant nature of the seed is a limiting factor in plantation raising. Usually the viability of the cocoa seed lasts only for a maximum of two weeks. The synthetic seed technology can help to ease storage problem of cocoa seeds (Sudhakara et al. 2000). It will also be useful for the germplasm conservation of the species, germplasm transfer, and transfer of planting materials to far non-conventional areas.

All over the world research to extend the storage life of cocoa seeds by various methods viz., storing in moist conditions, sealed containers, perforated polythene bags, burying in soil etc., have been tried at different temperatures to find out an ideal storage method for long term storage of recalcitrant seeds, but in vain. Suitable storage techniques have to be devised to achieve long term storage of these seeds without any loss of their viability and longevity to make forestry programmes as well as plantation programmes involving *Theobroma cacao*, a success. Under these circumstances a novel method of storing cocoa seeds through synthetic seed production and storing in inhibiting medium was attempted.

The present study was carried out during 2010-2012 in the College of Forestry, Vellanikkara with the objectives

1. To find out the effect of germination inhibitors on the excised embryonic axes as well as synthetic seed
2. To find out the effect of different osmotica on the embryonic axes as well as synthetic seed
3. To check the possibility of enhancing storage life of synthetic seeds using germination inhibitor and osmotica
4. To find out best desiccation protocol for storing synthetic seeds

REVIEW OF LITERATURE

REVIEW OF LITERATURE

The importance of seed storage has been recognized ever since humans began to domesticate plants. Since the advent of agriculture, farmers have learnt how to maintain viable seeds from one growing season to the next. Seed storage is widely regarded as the most efficient and cost-effective means of *ex situ* plant germplasm conservation, being used for the majority of accessions maintained world-wide (IBPGR, 1976; Withers, 1988; Bonner, 1990; Vertucci and Roos, 1990; Hong and Ellis, 1996; Engels and Engelmann, 1998). The duration of successful storage depends upon both the objectives and the species concerned. As each seed represents a genetically unique individual, stored seeds are ideal for re-establishing wild populations of threatened taxa, especially when the sample size and collection methods employed ensure a genetically representative sample (Frankel, 1990). However, all seed storage methods cannot be applied to all plant species with the same efficacy, since, post-harvest behaviour of the seeds, determines the most suitable method of conservation.

The duration of successful storage depends upon both the objectives and the species concerned. Seed longevity varies greatly among species. Seeds of species with orthodox seed behaviour can be maintained satisfactorily *ex situ* over the long term in appropriate environments. Short term storage is usually the best that can be achieved with seeds, which shows recalcitrant storage behaviour. Medium term storage is feasible for seeds of species with intermediate storage behaviour provided the storage environment is well defined and well controlled. Roberts (1973) defined two categories of seed storage behaviour: orthodox and recalcitrant. A third category intermediate between the orthodox and recalcitrant categories has since been identified (Ellis et al., 1990). Recalcitrant seeds cannot be dried beyond critical moisture content without damage. When fresh recalcitrant seeds begin to dry, viability also get reduced. Loss of viability get increased as the moisture content get reduced and at a certain moisture content termed as the "critical moisture content" (King and Roberts, 1979, 1980) or "lowest safe moisture content" (Tompsett, 1984a) viability get completely reduced. If drying continues further, viability is eventually reduced to zero. Moist storage for recalcitrant seeds should be at the moisture content levels between the "lowest safe

moisture content" and the "fully imbibed" level at the coolest temperature, which is not damaging the seed viability.

2.1 Recalcitrant Seed storage

Recalcitrant seeds are usually short-lived and very sensitive to desiccation and low temperatures, making it very difficult to store them on a long term basis (Chin et al., 1981). Unfortunately, seeds of many economically important tree species in the tropics are recalcitrant in nature and cannot be stored for long periods. Their requirement for high moisture content reduces their storability as they can only, be stored in wet medium to avoid dehydration injury at warm temperature (Come and Corbineau, 1996). The current view is that recalcitrant seeds are metabolically active and undergo germination-associated changes in storage. Some of these changes such as extensive vacuolation and increase in cell size imply a requirement of water addition to that present in the seed at the time of shedding (Pammenter et al., 1994).

The quick loss of viability may also be due to desiccation sensitivity, chilling injury, microbial contamination and germination during storage (King and Roberts, 1980). Mature orthodox seeds survive desiccation to a low moisture contents, upto 2-6 percent depending on the species. Above this value, there is a negative logarithmic relation between seed moisture content and longevity (Ellis and Roberts, 1980). Depending on the species, hydrated, intact recalcitrant seeds can be stored only for periods from days to months (Chin and Roberts, 1980).

Current methods for conservation and storage of recalcitrant seeds are based upon high moisture content maintenance, because of the fact that recalcitrant seeds cannot be dried without damage (Roberts, 1973). The values of the "lowest safe moisture content" vary between extremes of 23% for cocoa (*Theobroma cacao*) (Mumford and Brett, 1982) and 61.5% for *Avicennia marina* (Farrant et al., 1986).

Great progress in storage of recalcitrant seeds was made by the work of Farrant et al. (1986) and Pammenter et al. (1993). They proposed that water stress can occur in hydrated stored recalcitrant seeds, because recalcitrant seed do not stop growing at the end of its formation and maturation, they only reduce their levels of metabolism. The moisture status of recalcitrant seeds has been extensively studied in *Avicennia marina*

(Berjak et al., 1990), *Quercus robur* (Finch-Savage et al., 1994) and many other species (Pammenter et al., 1993).

One of the major difficulties in the short term storage of recalicitrant seeds, is that the high relative humidity conditions necessary to prolong storage life of the seeds, are also conducive to the proliferation of the micro-organisms, especially as chilling is precluded in many instances (Berjak, 1996). As the vigour of the wet stored recalcitrant seeds declines as a result of inherent changes (Pammenter et al., 1994), it has been suggested that natural defense mechanisms fail, facilitating fungal invasion of the debilitated seed tissues. As per Harrington (1963), at seed moisture contents in excess of 10 to 13 percent fungal invasion can rapidly diminish seed viability. Since recalcitrant seeds should be stored in a moist condition, microbial contamination could be an important constraint to reckon with in the conservation of recalcitrant seeds. Microbial growth can be reduced to some extent by lowering the temperature of the storage environment (King and Roberts, 1980) but in the case of recalcitrant seeds, being chilling sensitive, this may not be applicable.

2.2. Role of abscisic acid (ABA) in germination

Seed dormancy is influenced by the inhibitor present in the surrounding tissues of embryo. Abscisic acid is a plant hormone ubiquitously present in higher plants; it plays a vital role in seed dormancy regulation, embryo development, and adaptation to various environmental stresses, most notably drought (Qin et al., 2008). It is found in most of the dormant embryo. It can be postulated that the inhibitor especially those in the cotyledons control the growth of the axis. Inhibitors have been isolated from dormant embryos of several species and most prominent member was abscisic acid (ABA). It is found in the embryos and in the covering tissues (Sondheimer et al., 1974). It has been proposed by several authors that abscisic acid is probably related directly or indirectly to seed desiccation tolerance (Farrant et al., 1993).

Abscisic acid plays an important role in seed dormancy, embryo development and adaptation to environmental stresses. Culture studies with exogenous ABA application suggest that ABA is important in preventing germination. Correlations can be seen between the depth of embryo dormancy and the concentration of abscisic acid.

Probably, washing away of ABA and other inhibitors can induce germination in dormant embryos of *Elaegnus umbellate* (Hamilton, 1976). The addition of abscisic acid (ABA) to mature non-dormant seeds inhibits their germination (Garciarrubio et al., 1997). This effect of ABA might be related to its natural function as an endogenous inhibitor of precocious germination during seed formation. Endogenous ABA level is a determinant of these physiological processes, and ABA-deficient mutants exhibit reduced seed dormancy and reduced drought tolerance (McCarty, 1995). Conversely, exogenous application of ABA results in delayed germination (Guo et al., 2008) and increased tolerance to a variety of abiotic stresses (Li and Pan, 1996).

In plants, abscisic acid is synthesized through the cleavage and oxidation of carotenoids, and is catabolized via hydroxylation or by conjugation to glucose (Nambara and Marion-Poll, 2005). Dormant seeds treated with fluridone (a compound which inhibits carotenoid and, thus, ABA synthesis) often have similar germination characteristics to non-dormant seeds, indicating that the continued synthesis of ABA is required for dormancy maintenance in imbibed seeds of several species (Ali-Rachedi et al., 2004; Kusumoto et al., 2006; Feurtado et al., 2007). Similarly, Millar et al. (2006) and Gubler et al. (2008) showed that dormancy maintenance in seeds of arabidopsis and barley depends upon the balance of ABA synthesis and catabolism being shifted towards the former.

It has been shown that abscisic acid (ABA) plays a role in the induction and maintenance of seed dormancy, whereas gibberellins (GAs) are associated with dormancy breaking and germination (Kucera et al. 2005). Also, application of paclobutrazol (an inhibitor of GA biosynthesis) depressed seed germination indicating that *de novo* biosynthesis of GA is required during imbibition (Karssen et al. 1989; Bradford and Nonogaki 2007). Benech-Arnold et al. (2006) reported that fluctuating temperature stimulated germination of immature dormant sorghum caryopses by reducing embryo sensitivity to ABA. Huarte and Benech- Arnold (2010) found that fluctuating temperature decreased ABA concentration prior to radical emergence, and exogenous GA_3 enhanced seed germination at constant temperature. This implied that ABA and GA were involved in seed germination and the dormancy response to fluctuating temperature. In *Coffea arabica* seeds, Valio (1976) found that endogenous

ABA-like substances and exogenous ABA cause inhibition of germination by preventing embryo growth. Da Silva et al. (2004) reported a transient rise in endogenous ABA content during germination in the embryo cells, suggesting that ABA inhibits cell wall extensibility by not permitting an increase in cell turgor.

2.3. Role of coumarin in germination

Coumarins form a large class of allelochemicals widely distributed in both natural plant communities and crops (Zobel and Brown, 1995). Being localized on the leaf, seed surface, and pollen wall, and released into the environment by living plants or by decomposing plant material, coumarins are involved in ecological interactions in both managed and natural plant communities (Rice, 1984; Zobel et al., 1991; Bertin et al., 2003; Bais et al., 2004).

Coumarins (1, 2-benzopyrones) are secondary metabolites often found in roots of higher plants where they originate from the general phenylpropanoid pathway (Bourgaud et al., 2006). While their exact biological function in plants remains unclear, historically they are known to be involved in plant defense (Baumert et al., 2001; Carpinella et al., 2005; Brooker et al., 2007) along with flavonoids, phenolics, and alkaloids. Unlike most other secondary metabolites, coumarins appear to specifically inhibit seed germination and root growth (Svensson, 1971; Abenavoli et al., 2001, 2004, 2006, 2008; DeBolt et al., 2007). They also influence membrane transport in the root tip (Lupini et al., 2010) and nitrogen uptake and metabolism (Abenavoli et al., 2001, 2003). Coumarin (2H-chromen-2-one), the simplest compound of this class, affects root form and function (Abenavoli et al., 2001, 2004), decreases respiration and photosynthesis (Moreland and Novitzky, 1987), and influences nitrogen uptake and metabolism (Abenavoli et al., 2001, 2003).

Coumarins are one of the chemical substances that widely exist in natural and farming plants communities, and they are considered as strong inhibitor for seed germination. Being a strong inhibitor of seed germination, coumarin may confer an advantage to the producing plant species by reducing competition in its immediate environment, and/or by delaying germination of its own seeds under unfavorable conditions (Zobel and Brown, 1995). To explain this inhibitory action, coumarin has been hypothesized to be either a blocker of the cell cycle (Zobel and Brown, 1995),

uncoupler of oxidative phosphorylation (Ulitzer and Poljakoff-Mayber, 1963; Khan and Zeng, 1985), or to interfere with amino acid transport and protein synthesis (Van Sumere et al., 1972). Aliotta et al. (1992, 1993) reported that coumarin is an inducer of coat-imposed dormancy through the inhibition of water uptake during seed imbibition. Such a multiplicity of proposed roles suggests that the key mechanism(s) by which coumarin affects seed germination remains to be identified.

In different species, it is observed that the higher concentration of coumarin inhibits the seed germination (Aliotta et al., 1992, 1993). Coumarin is a compound that inhibits seed germination and seedling growth (Peal and Williams 2002). In durum wheat seeds, coumarin inhibited seed germination at concentrations above 200mM (Abenavoli et al., 2006). This inhibitory effect may confer a competitive advantage for the plants that secrete coumarin into the environment. Coumarin solutions of 10^{-5} to 10^{-3} M had a negative effect on radish seed germination and radicle growth. The greatest inhibitory effect of 10^{-3} M coumarin was also observed in seed germination and radicle elongation (Peal and Williams, 2002; Williams and Bartholomew, 2011).

2.4. Role of Cycocel (CCC) in germination

(2-chloroethyl) -trimethylammonium chloride (CCC/ cycocel) has a growth retardant property on seeds (Aliotta et al., 1993; Abenavoli et al., 2001). Cycocel, inhibits plant growth (Rademacher, 2000). The combination of coumarin and CCC has been noticed to inhibit seed germination (Knype, 1967). Application of CCC inhibited seed germination of non-dormant groundnut cultivar (Sengupta et al. 1979). Growth retardant property of cycocel was utilised in different ways by the scienctific community. CCC inhibited main axis growth, reduced the level of endogenous GA and caused a marked release of lateral buds from apical dominance (Okoloko and Lewis, 1968). Cycocel has been found to give good results in height control (Pearse, 1974). Ornamental crops that are effectively controlled by cycocel include herbaceous crops, such as Pointsettias and Geraniums, as well as woody flowering crops such as Hibiscus and Azaleas (Olympic Horticultural Product, 2005).

Application of cycocel decreased plant height in sunflower (Savmell, 1972; Dorell, 1973; Bhattacharjee and Gupta, 1981). Ogilvy (1985) indicated that, foliar application of cycocel (chloremequat) at the start of the stem extension resulted in

retarded vegetative growth in rape, but none of the growth regulators affected the branching pattern. Singh and Brar (1999) reported that application of cycocel at 125 ppm significantly reduced the plant height compared to water sprayed crop in upland cotton. CCC was also found effective in decreasing plant height (Rowland et al., 1974). Ramprakash and Mangalprasad (2000) noticed that foliar spray of CCC at 50 and 100 ppm reduced plant height significantly over control in cotton. CCC strongly inhibit GA biosynthesis (Kende et al., 1963;; Zeevaart, 1965). In *Fusarium moniliforme* gibberellin production is suppresed by adding CCC to the culture medium. A concentration of 0.1 mg/l of CCC causes 50% inhibition whereas 10 mg/l and higher concentrations fully suppress GA production (Ninnemann et al. 1964).

2.5. Osmotica

Osmotic agents such as sucrose, mannitol and sorbitol not only act as a common source of carbon in cell culture media and energy but also as an osmotica during organogenesis (Al-Khayri and Al-Bahrany, 2002). Osmotic agents accumulated in many plant tissues in response to environmental stress, including water deficit (Ramos et al., 1999) play a role in osmoregulation and cryoprotection.

In experiments sorbitol used as high osmoticum had supressed germination and maintained the storage protein synthesis. Similar results have been obtained with mannitol and sucrose also (Finkelstein and Crouch, 1986). Seong et al. (1988) reported that the moisture content and the seedling length decreased when the mannitol concentration increased. Germination of sorghum seeds in 0.75 M mannitol, was considerably lower compared to in distilled water (Gill et al. 2002). Regeneration frequency in Super Basmati decreased by the addition of either mannitol or sorbitol (Naqvi et al. 2005).

Salts can affect seed germination either by restricting the supply of water (osmotic effect) or causing specific injury through ions to the metabolic machinery (ionic effect). Studies have been carried out on the effect of various chloride and sulfate salts on the germination of halophytes where all the salts exhibited some osmotic effects but no specific ion effect (Mohammed and Sen, 1990; Egan et al., 1997; Agboola, 1998; Pujol et al., 2000) while others reported both osmotic and ionic effects (Mohammed and

Sen, 1990). Isotonic solutions of PEG 6000 and NaCl had effect on the seed germination of halophytes (Myers and Couper, 1989; Naidoo and Naicker, 1992; Ungar, 1995; Bajji et al., 2002).

Seeds stored in concentration of eight gL^{-1} of NaCl corresponds to the limit of salinity beyond which germinated faculty of the three different species of *Atriplex* decrease appreciably (Souhail and Chaabane, 2009). Germination decreased with increase in salinity. Seed germination percentage was lower in NaCl than in iso-osmotic PEG solutions at osmotic potential less than -0.5 MPa. Non-germinated seeds under various salt treatments when transferred to distilled water recovered completely, indicating little ionic effect of salinity on seed germination and viability. Germination inhibition, therefore, appears to be osmotic. Similar recovery response was noted when seeds from PEG solution were transferred to water (Duan et al., 2004). High recovery germination percentages consequent to transfer to water would indicate that previous seed germination was inhibited by an osmotic effect, whereas, low germination would indicate specific ion toxicity (Khan, 2002; Pujol et al. 2000).

2.6. Synthetic seed

Production of synthetic seeds by using excised embryos is one of the recent methods by which the longevity of recalcitrant seeds can possibly be increased. Synthetic seed is a novel analog to botanic seed capable of developing to an entire plant body which comprises of meristematic tissue, encapsulated free from botanical accessory structures in a hydrated gel capsule (Redenbaugh et al., 1986). The gel acts as a synthetic seed coat and protects embryo from injuries. The synthetic seed coat must be non-damaging to the embryo, should protect the embryo from mechanical damage during handling and allow germination and conversion to occur, without inducing undesirable variation.

The embryo of the zygotic seed is surrounded by nutritive and protective tissue that enable the embryos to retain their metabolic activities for a very long time. Beyond protection and nutrition, such tissues also create barriers to regulate gas exchange. Oxygen availability, which is limited by these tissues, influences embryo respiration, regulates embryo development and prevents precocious germination (Carman, 1988). In

zygotic seed, the protective tissue (seed coat) besides providing protection from the disease causing organisms and mechanical stress, also exerts a physical influence or pressure that may also contribute to the normal development of embryo, attainment of maturity and onset of dormancy. In addition to these, the seed coat is possessed with some degree of impermeability to water and gases, including oxygen and exerts regulatory influence on metabolism and growth of the embryo (Bewley and Black, 1994).

The concept of synthetic seeds, which consists of an embryo surrounded by an artificial layer called capsule, was put forward by Murashige (1977) and the first report of successful synthetic seed production was published in 1982 (Kitto and Janick 1982). Encapsulation of alfalfa and celery somatic embryos with natural hydrogels such as calcium alginate was done by Redenbaugh et al. (1987).

Kitto and Janick (1985) reported coating of clumps of carrot somatic embryos and shoots in a wafer disk, but presented data only on embryo "survival". Subsequently, synthetic seed technology was applied to several plants especially agricultural crops, ornamental plants and medicinal plants and recently there has been an increased interest in the production and utilization of synthetic seed technology (Pattnaik and Chand 2000; Ganapathi et al. 2001; Nyende et al. 2003, 2005; Huda et al. 2009).

The production of hydrated synthetic seeds is through a simple direct process, where in the somatic embryos are mixed with sodium alginate and dropped into complexing bath containing calcium chloride using a dropper or pipette. Each drop containing an embryo at the center will form into a calcium alginate capsule or bead around the embryo. The capsule hardness of 0.5 to 2.0 kg breaking pressure per capsule was found to allow germination, while providing sufficient integrity so that the capsules could be handled on a routine basis without damage or breakage. The size of capsule can be controlled by the viscosity of the sodium alginate and by the inside diameter of the nozzle used to form the drops (Redenbaugh, 1993).

Fujii et al. (1988) reported that artificial seeds of alfalfa consisting of somatic embryos encased in a hydrogel have the potential to be an economic means for rapid propagation of elite plant lines. Use of artificial seeds would eliminate *in vitro* plantlet

growth and acclimatization step, thereby reducing costs and labour for tissue culture plant propagation.

Janick et al. (1989) developed the coat for somatic embryo of *Datura carota* which is known as synthetic seed coat such as polywax WSR-N 750 to prevent drying, hardening and prevent precocious germination. Senaratna et al. (1990) produced artificial seeds of alfalfa by inducing desiccation tolerance in somatic embryos. Mathur and Ahuja (1991) have detailed different areas of synthetic seed technology. They have listed the crops in which encapsulation has been documented. They have reported that among various hydrogels used, sodium alginate is widely employed because of its easy complexation with calcium chloride, biologically non-damaging, biodegradability and low price.

Chen et al. (1992) lists the propagules used for making artificial seeds. Their study included the induction of both adventitious buds and embryoids in nine species of seven families including *Populus*, *Eucalyptus*, *Mycetia*, *Picea*, *Brassica* and *Camellia*. It was found that the conversion percentage of artificial seeds produced using adventitious buds as propagules was significantly higher in comparison with those from embryoids. Bapat (1993) produced the synthetic seeds of sandalwood by somatic embryogenesis and by multiple bud formation in mulberry. Alginate was used as encapsulating matrix. Redenbaugh et al. (1993) reported synthetic seed technology developed based on the use of somatic embryos as functional seeds. George and Eapen (1995) reported encapsulation of small clusters of somatic embryos of *Eleusine caracana* with alginate matrix to prepare synthetic seeds. The highest frequency of germination and the highest average number of plants per capsule were obtained by encapsulating the embryo clusters in three per cent alginate and germinating the capsules on MS medium. Timbert et al, (1995) encapsulated carrot somatic embryos in alginate gel beads. To improve the quality of synthetic seed coating, the rheology and dehydration properties of different matrices were tested. Increasing alginate concentration, calcium chloride concentrations, additional mineral elements, resulted in increased resistance to rupture and depressed the germination of somatic embryos.

Redenbaugh et al. (1993) reported that the synthetic seed coat used in encapsulation must serve as synthetic endosperm containing carbon sources, nutrients,

growth regulators, antimicrobial agents etc. Literature revealed that during the production of artificial seeds, usage of $CaCl_2$ concentration beyond 75 mM adversely affected shoot emergence. Best results in terms of bead quality and per cent shoot emergence were obtained when the beads were formed using a combination of five per cent sodium alginate solution with 75 mM $CaCl_2$ $2H_2O$. Studies conducted on encapsulation of zygotic embryo of cocoa (*Theobroma cacao*) showed that a combination of 14 per cent sodium alginate and 75 mM $CaCl_2$ $2H_2O$ are appropriate (Sudhakara et al., 2000).

Another area of future development in relation to synthetic seed is to prepare a suitable synthetic endosperm that could act as energy reservoir during storage and planting. Mariani (1992) carried out research on egg plant somatic embryogenesis and synthetic seed technology. He added GA_3 at 1, 2 and 8 mg per litre or sucrose at 20 ml per litre to the sodium alginate solution to evaluate their effect on germination. GA_3 and sucrose showed negative effects on synthetic seed germination. Machii (1992) added nutrient components and plant hormones during encapsulation of adventitious buds in mulberry (*Morus alba*) and then the synthetic seeds were transplanted to an appropriate medium. When the capsules contained the same nutrient components as MS medium, the effect of encapsulation was great and synthetic seeds grew vigorously. Sakamoto et al. (1992) reported that embryos with an artificial endosperm showed a higher germination compared with seed without an endosperm. Toruan and Sumaryono (1994) reported that incorporation of mycorrhizae, pesticides and fertilizers into the capsule enhance germination rates and seedling growth. Encapsulation in nutrient enriched alginate capsules of bud excised from *in vitro* proliferated shoots of olive can be considered as an important contribution to micropropagation, for germplasm preservation and/or for synthetic seed technology (Micheli et al.,1998).

Arunkumar et al. (2005) revealed that the synthetic seeds with synthetic endosperm constituents of MS nutrient; sucrose (3%), IAA (0.5 mg/l), NAA (0.5 mg/l), BA (0.5 mg/l) and charcoal (1.25%) gave a maximum germination (30%) and conversion (27%) of synthetic seeds in hybrid rice. The most important requirement for a synthetic seed to be used for clonal mass propagation of plants is high and uniform

conversion under practical sowing condition such as nursery bed in a greenhouse or in the field (Onishi et al., 1994).

2.7. Storage and viability of synthetic seed

The encapsulated embryos of carrot, pretreated with ABA and chilling survived 16 days of storage in darkness at 4°C (Kitto and Janick 1985). Enhanced storage of 45 days was obtained from encapsulated axillary bud of mulberry in alginate stored at 4°C with no loss of viability (Bapat et al., 1987). Gupta and Durzan (1987) reported that somatic embryos of pines were encapsulated and stored in an alginate gel or in liquid nitrogen successfully. Datta and Potrykus (1989) reported that artificial seeds in barley maintained their germination capacity for atleast six months, whereas non-encapsulated embryos did not survive for more than two weeks in storage. Artificial seed thus probably provide a simple and universal delivery system of *in vitro* plantlets to greenhouse or field. Singh (1991) reported that the beads of orchids (*Spathoglottis plicata*) could be stored for 180 days at 4°C without much loss of viability. The beads (*Dendrobium wordeanum*) could be stored for up to 180 days at 4°C, but regeneration declined as storage duration increased (Sharma et al., 1992).

Machii (1992) reported that when synthetic seeds of mulberry (*Morus alba*) were stored in water at 4°C for 30 and 80 days, germination was poor. However, they could be successfully stored in MS liquid medium at 4°C for the same period. Chetia and Devi (1996) observed that there was decline in germination of orchid species with increase in duration of storage. A low germination (10 – 20%) was observed after 45 days of storage at room temperature. However, about 50 per cent germination can be maintained upto 60 days by storing at 4°C which will facilitate transportation of germplasm and thereby help in commercial propagation of orchid. Embryogenic cell suspensions of sandalwood which were encapsulated and stored at 4°C for 45 days produced embryos when recultured as suspensions (Bapat and Rao, 1988, 1992). Sushmita et al. (1998) reported that the germination rate in Phalaenopsis hybrid declined as storage duration was extended and germination was faster following storage at 4°C than at 25°C.

Micheli et al. (1998) reported that the encapsulated buds of olive could be conveniently stored at 4°C, but not for longer than 45 days. Datta et al. (1999) reported that synthetic seeds of *Geodorum densiflorum* has shown 100 per cent germination when these seeds were stored at 4°C for 120 days whereas non-encapsulated embryos showed no viability after 30 days at 4°C. The encapsulated buds of *Ocimum* species could be stored for 60 days at 4°C (Jaydip et al., 2000). Bekheet et al. (2002) from experiment conducted on synthetic seed system of date palm reported that the storage of encapsulated embryos at low temperature (15°C) showed highest percentage of conversion in different maturation stages of somatic embryos compared with incubation at 25°C. Ipekci and Gozukirmizi (2003) reported that storage of synthetic seed at 4°C for 30 days or 60 days significantly reduced survival and germination frequencies of both encapsulated and non-encapsulated embryos relative to those of non-stored somatic embryos from *Paulownia elongata*. However, the survival and germination rates of encapsulated embryos increased following storage at 4°C. Reddy et al. (2005) conducted an experiment on synthetic production in *Rauvolfia serpentina* and reported that the artificial seed was stored successfully for three months at 4°C. The synthetic seeds of *Pinus patula* trees could be stored at 2°C for 120 days without reduction in germination as opposed to non encapsulated somatic embryos which showed only 9 per cent germination after 20 days at 2°C (Ravindra and Staden, 2005). Kavyashree et al. (2006) reported that the synthetic seed of mulberry var. S54 stored at 4°C for one to four months resulted in maximum conversion response under both *in vitro* and *in vivo* conditions.

Sunilkumar et al. (2000) made an attempt to improve the storage life of *Hopea parviflora* seeds through synthetic seed production. Storage at temperatures of 10°C and 4°C retained viability for upto one and four weeks for the intact and synthetic seeds, respectively. On the other hand, synthetic seeds and intact seeds stored at room temperature retained viability for only upto one week with approximately 30 percent germination. Synthetic seeds obtained from seeds pre-treated with 2 and 3 mgl^{-1} of abscissic acid showed tolerance to low storage temperature and retained higher percentage germination.

2.8. Dessication tolerance

Recalcitrant seeds are those that undergo little, or no, maturation drying and remain desiccation sensitive both during development and after they are shed. In reality, however, the situation is far more complex than this perception implies, because of the wide range of variability among recalcitrant seeds of different species (Berjak and Pammenter, 1997). Farrant et al. (1993) proposed a continuum of recalcitrant seed types mainly based on the sensitivity towards water loss and low temperature. Variation in desiccation sensitivity between species has been reported in many genera such as *Dipterocarpus* (Tompsett, 1984), *Acer* (Olsen and Gabriel, 1974) and in *Araucaria* (Tompsett, 1984). According to King and Roberts (1980), variation in the moisture content at fully imbibed state is the main reason for apparent variation in susceptibility to desiccation in different species.

Recalcitrant seeds are shed in a hydrated condition, and the water content can be anywhere in a wide range. Shedding water content is partially species characteristic, depending on the degree of dehydration that occurs late during seed development; this goes hand-in-hand with the degree of desiccation tolerance developed by individual species (Finch-Savage et al., 1994). Desiccation tolerance in recalcitrant seeds increases during seed development on the mother plant; however, unlike orthodox seeds, maturation drying to low moisture contents does not occur (Hong and Ellis, 1990). Fresh recalcitrant seeds have high levels of moisture contents at maturity, for example, 36 per cent for rubber (Chin et al., 1981) and 90 per cent for Choyote (*Sechium edule*) (Ellis, 1991).

There are marked differences in the rate at which water will be lost from seeds of various species under the same dehydrating conditions (Farrant et al., 1989). There are other factors too, that influence the post-harvest responses of recalcitrant seed, e.g. developmental status (Berjak et al., 1993; Finch-Savage and Blake, 1994) and chilling sensitivity (Berjak and Pammenter, 1997).

Recalcitrant seeds are sufficiently hydrated at shedding and germination without any additional water (Berjak et al., 1989; Farrant et al., 1988). Desiccation sensitivity of recalcitrant seeds is intimately associated with their persistent state of metabolic

activity. Generally, the axes of the recalcitrant seeds arc at considerably higher water content than the cotyledons (Berjak etal., 1989; Maithani etal., 1989; Fu et al., 1993). Finch-Savage (1992) has demonstrated that for *Quercus robur*, there is a higher proportion of matrix bound water in the cotyledons, which may underlie the greater desiccation sensitivity of the cotyledons relative to the axes.

Hanson (1985) has described methods of drying seeds using silica gel. Dry (newly regenerated) silica gel is in equilibrium with about 5 per cent moisture content at 20°C. The first change of the colour of the indicator (diminishing intensity of blue) occurs when the silica gel moistens to equilibrium with about 12-13 per cent relative humidity. By the time it has become pale blue it is in equilibrium with about 49 per cent relative humidity (and thus very moist): 100 g of regenerated silica gel (in equilibrium with about 5 per cent relative humidity) can absorb about 7 g of moisture from seeds until the first colour change is apparent (in equilibrium with 13.5 per cent relative humidity). At this point the silica gel should be regenerated. It should be done by drying in an oven maintained at about 130°C for 3-4 hours. It should be then stored in a sealed container for overnight to cool to ambient temperature before being used to dry the seeds (Hong and Ellis, 1996).

Drying excised embryonic axes by silica gel or an aseptic air current allowed excised embryonic axes to survive desiccation to a lower value than that achieved by the vacuum method (Fu et al., 1993). For example, although the vacuum drying method provided more rapid drying, no excised embryonic axes of *Artocarpus heterophyllus* survived desiccation to 44 per cent moisture content, while the excised embryonic axes dried with an aseptic air flow and silica gel tolerated desiccation to 26 per cent and 16 per cent moisture content respectively (Fu el al., 1993).

The use of somatic embryos from cell culture systems in the clonal propagation of plants would be greatly facilitated if the somatic embryos could be dried and stored in a dormant state in a similar way to true seeds (Senaratna et al., 1990). Abscisic acid, heat shock, high sucrose concentration (Mc Kersie et al., 1990), water or nutrient stresses (Mc Kersie et al., 1989) applied to the embryoids at the cotyledonary stage of development resulted in acquiring desiccation tolerance. The embryoids could be subsequently air dried slowly (over seven days) or rapidly (over one day) to moisture

contents of less than 15 per cent and retained viability up to eight months (Mc Kersie et al., 1989).

The rate of drying also affects survival of somatic embryos. Senaratna et al. (1995) reported that slow drying (1.2 g H_2O/g/day over six days) gave higher and more consistent embryoid survival, compared to fast drying (6 g H_2O /g/day over one day). Desiccation tolerance was also induced by exposing somatic embryos to sub lethal levels of low temperature. However, some of the stress pre-treatments had other deleterious effects on embryoid maturation and seedling vigour after inhibition. Treating the embryos with ABA for ten days before encapsulation is also reported to be enhancing germination even when the embryo is dehydrated to five per cent moisture content (Liu et al., 1994).

Anandarajah and Mc Kersie (1990) investigated the effect of sucrose concentration in the maturation medium in combination with a heat shock treatment at 36°C to improve the vigour of seedlings grown from dry somatic embryos of *Medicago sativa*. It was found that with three percent sucrose in the maturation medium the somatic embryos germinated precociously and were unable to survive desiccation. At higher sucrose concentration germination was delayed and after 13 days on 6 per cent sucrose medium, the somatic embryos tolerated drying to 12 per cent moisture content without exposure to exogenous ABA. Heat shock, which presumably stimulated endogenous ABA synthesis, improved the desiccation tolerance of somatic embryos if applied prior to 27 days after sieving.

Redenbaugh (1990) observed that desiccated artificial seeds had other problems that needed resolution. The desiccation process itself damages the embryos. One exception to this was reported by Gray (1987), who found that desiccation appeared to release grape somatic embryos from a quiescent state and increase conversion (20% to 28%) for desiccated embryos stored for 7 to 21 days and 5 per cent for non-desiccated embryos. Hydrogel encapsulated or hydrated synthetic seeds are the most commonly produced synthetic seeds and the most commonly used hydrogel is sodium alginate because of its gelatin properties, ease of use, less viscosity and low cost (Redenbaugh et al., 1987).

2.9. Storage of cocoa seed

Cocoa pods reach ripening stage after 150-170 days of effective pollination. The pods will reach physiological maturity two weeks before ripening. September- October is the peak period for production of cocoa seed. Usually nursery period starts in Dec-Jan. and the planting is done during May-June. Prasannakumari et al. (2009) reported that cocoa seed germination was not influenced by their position in the pod or size of the pod. The difference in germination depended on the sowing month. Higher germination percentage was seen during March. Low germination was obtained in December, January and April.

The earlier storage studies done in cocoa shown that cocoa seed had shorter longevity of 2 weeks in "in the pod" condition. In extracted condition longevity was less than seven days (Nair, 1987). In refrigerated condition, seeds lost viability and no germination was observed. Seeds stored in room temperature showed decreased germination with increasing time of storage. Conversion of cocoa embryonic axes to synthetic seed and storing was found effective. This is because the recalcitrant nature is supposed to be due to seed coat and storage tissue (King and Robert, 1980).

In an experiment conducted in College of Forestry, Vellanikkara, encapsulated zygotic embryo of cocoa were stored at 4°C on dry cotton and wet cotton and was found that synthetic seeds stored on wet cotton retained viability for longer period (Sudhakara at al., 2000). Seventy one percent of synthetic seeds stored on wet cotton regenerated to complete plantlets after 25 days while synthetic seeds stored on dry cotton showed a gradual decline in regeneration after 15 days of storage and only 50 per cent germination was obtained after 25 days of storage.

Recalcitrant seeds storage is still lacking a technique to improve storage period without loss of viability. The encapsulation of embryo provides increased longevity and inhibitors influence can be used for checking the continuous growth of the embryo. The influence of growth inhibitors and osmotica on synthetic seeds should be analysed for effective storage. Moisture level near the embryo has an important role in maintaing viability in recalcitrant seeds, so the influence of desiccation on encapsulated seeds should be analysed for developing a new technology.

MATERIALS AND METHODS

MATERIALS AND METHODS

The present investigation titled "Enhancement of storage life of cocoa (*Theobroma cacao* L.) seeds through encapsulation and germination inhibition" was undertaken during the year 2010-12 in the plant tissue culture laboratory, Department of Tree Physiology and Breeding, College of Forestry, Kerala Agricultural University (KAU), Vellanikkara, Thrissur district, Kerala (10° 31'N latitude and 76° 13'E longitude). The area is characterised by a warm humid tropical climate with distinct summer and rainy season. The location experiences a mean annual rain fall of 3000 mm most of which is received between June to September. The temperature varies from 22°C (December) to 34.8°C (March). The details of the materials used and techniques and the methodology employed in the experiment during the course of investigation are described in this chapter.

3.1. Propagule

Cocoa pods were collected from seven year old cocoa trees grown in poly clonal seed garden maintained by Cocoa Research Project, KAU (Plate 1a). Polyclonal seed garden consist of clones CCRP 1, CCRP 2, CCRP 4, CCRP 7, CCRP 9 and TISSA. The pods were collected based on the phenological observation mainly on colour of pod. The yellow ridged cocoa pods, aged between 100-120 days (Plate 1b) were collected for extraction of embryonic axes for the experiment. The site was visited at periodic intervals and pods were collected and brought immediately to the laboratory for conducting experiments.

Pods were washed in tap water to remove dust and pod surface cleaned and dried with a towel. The pods were cut open using a sharp knife and seeds with pulp were extracted. The pulp surrounding each seed was removed carefully without damaging the embryonic axis. Seeds devoid of pulp were washed with distilled water to clean the seeds. Seeds were soaked in 50% WP Carbendazim (Bavistin) fungicide for 30 minutes to reduce fungal contamination. After 30 minutes, seeds were washed thoroughly to remove the fungicide and brought to the

(a)

(b)

Plate 1. (a)Poly clonal seed garden of cocoa of Kerala agricultural University and (b) Yellow ridged cocoa pod collected for extraction of embryonic axis.

laminar air flow cabinet and excised with sterilized surgical blade and needle to extract embryonic axis.

For investigating the amount of cotyledon needed for the germination of seed, seeds were excised in different sizes (with half cotyledon, with ¼ cotyledon, without cotyledon) shown in Plate 2a. The excised embryo was inoculated in the media for storage. Observations were taken for rooting and shoot formation and suitable size of cotyledon attached to embryonic axis was found out. The excision of seed and inoculation were done in aseptic condition to avoid contamination during storage.

3.2. Media

3.2.1. *Encapsulation media*

In this study, alginate encapsulation was employed for the preparation of synthetic seed. The best combination of sodium alginate and calcium chloride standardised for cocoa by Sudhakara et al. (2000) was used for synthetic seed production.

Sodium alginate encapsulation method was used in synthetic seed. Forty grams of sodium alginate was weighed and added to one litre of distilled water taken in a beaker with constant stirring to avoid clumping of sodium alginate powder. The solution was heated slowly with constant stirring to obtain a gel form. Calcium chloride solution was used for making Calcium alginate which is a water-insoluble, gelatinous, cream coloured substance and it is also used for entrapment of enzymes and forming artificial seeds in plant tissue culture. The encapsulation media can be synthesised by adding of aqueous calcium chloride to aqueous sodium alginate. Eleven grams of aqueous calcium chloride ($CaCl_2$ $2H_2O$) was accurately weighed and dissolved in one litre of distilled water taken in a beaker. The above two solutions were then capped with aluminium foil and sterilized in an autoclave at 15 psi pressure for 30 minutes. Fresh solutions of sodium alginate and calcium chloride were used for encapsulation.

3.2.2. *Culture media*

MS tissue culture medium (Murashige and Skoog, 1962) was used for studying the viability and storage of embryonic axis as well as synthetic seed. The chemical

(a)

(b)

Plate 2. (a)Embryonic axis excised from seeds with different proportion of cotyledon and (b) Synthetic seeds made from cocoa embryonic axes.

composition of MS media is given in Table 1. The MS media in 50%, 25% and 10% levels were also used for storage studies of excised embryonic axis. 50%, 25% and 10% level denotes ½, ¼ and $^1/_{10}$ in amount of inorganic constituents per litre. Dry cotton and wet cotton were also used for the storage of both embryonic axis and synthetic seed.

Standard procedures (Gamborg and Shyluk, 1981) were followed for the preparation of the MS media. Stock solutions of major and minor nutrients were prepared by dissolving the required quantity of the chemicals in distilled water. Care was taken during the preparation of iron stock since it precipitates readily. To avoid this Na_2EDTA and $FeSO_4.7H_2O$ were dissolved in separate beakers with approximately 200 ml distilled water each. Both beakers were placed on hot plates and brought to the point of almost boiling. Then $FeSO_4.7H_2O$ solution was added slowly to Na_2EDTA over a 15 minute period with constant stirring. Then the volume was made up to one litre in a volumetric flask by adding distilled water. The mixture was allowed to cool in room temperature. The stock solutions were labelled indicating the stock number and date of preparation and stored in amber coloured bottles under refrigerated conditions. The stock solutions of nutrients were prepared fresh every four weeks and that of vitamins and amino acids every week.

Specific quantity of stock solution of the chemicals and growth regulators were pipetted out into 1000 ml beaker. Sucrose and inositol were added fresh and dissolved in the above solution. The volume was then made up to 1000 ml by adding distilled water. The pH of the solution was adjusted to the range of 5.7 - 5.8 using 1N NaOH or 1N HCl. Agar was added and the solution was boiled in microwave oven for melting agar completely. 10-15 ml of melted media was poured hot to the oven dried culture tubes. The containers with medium were then tightly plugged with absorbent cotton wool plugs. The media was autoclaved for 30 minutes at approximately 15psi pressure in a standard pressure cooker. After sterilization the culture vessels were immediately transferred to the culture room.

For standardizing media for storage, ½ MS, ¼ MS and $^1/_{10}$ MS were made from stock solutions. It is done by taking ½, ¼ and $^1/_{10}$ quantity of inorganic components required to prepare MS.

Table 1. Composition of the MS medium (Murashige and Skoog, 1969) used for the study on viability of synthetic seed and embryonic axis

Sl.No.	Components	Concentration (mgl^{-1})
	Inorganic :-	
1	$MgSO_4\ 7H_2O$	370
2	$CaCl_2\ 2H_2O$	440
3	KNO_3	1,900
4	NH_4NO_3	1,650
5	KH_2PO_4	170
6	$FeSO_4\ 7H_2O$	27.8
7	Na_2 EDTA	37.3
8	$MnSO_4\ 4H_2O$	22.3
9	$ZnSO_4\ 7H_2O$	8.6
10	$CuSO_4\ 5H_2O$	0.025
11	$CoC1_2\ 6H_2O$	0.025
12	KI	0.83
13	H_3BO_3	6.2
14	$Na_2MoO_4\ 2H_2O$	0.25
	Organic :-	
1	Myo-Inositol	100
2	Nicotinic Acid	0.5
3	Pyridoxine HCl	0.5
4	Thiamine HCl	0.1-1
5	Glycine	2
	Others :-	
1	Sucrose	30,000
2	Agar	7,000
3	pH	5.6 – 5.9

Synthetic seeds as well as embryonic axis were stored in wet cotton or dry cotton to investigate about their response to non-nutrient conditions. Sterilised absorbent cotton was used as dry cotton. A small amount of cotton was inserted into the glass bottle and sterilised under normal sterilization procedures. For wet cotton method, cotton was made wet with distilled water and sterilized by the above said method.

3.3. Desiccation treatments

Different relative humidities were maintained in glass desiccators of uniform size (160mm) by pouring 100 ml each of the solutions as shown in Table 2 (Agrawal, 1987). Distilled water (100 ml) was used to maintain 100 per cent relative humidity. The synthetic seeds were kept in sterilized petri plates inside the desiccators. Petroleum jelly was smeared in edges of desiccators before covering it with lid to maintain airtight condition.

Table 2. Chemicals and their concentration for achieving desired desiccation levels (Agrawal, 1987)

Chemical	Concentration	Relative humidity
Distilled water	100 ml	100%
KCl	100 ml Saturated solution	85.3%
NaCl	100 ml Saturated solution	75.6%
$Ca(NO_3)_2 \cdot 4H_2O$	100 ml Saturated solution	46.6%
KOH	423 g L^{-1} distilled water	30%
KOH	470 g L^{-1} distilled water	20%

3.4. Preparation of synthetic seed

The embryonic axis excised from the cocoa seed inside the laminar air flow were dropped into sodium alginate solution and stirred well. Each of the embryos was then carefully sucked, using a glass tube of 4mm diameter. The sucked gel coated embryos was then dropped into the $CaCl_2$ solution for complexation and allowed to form calcium alginate coating on embryonic axis. The encapsulated embryos with a translucent bead like appearance (Plate 2b) were allowed to remain in the calcium

chloride solution with intermittent stirring for 25-30 minutes to impart firmness. The synthetic seeds were transferred to a 100 ml beaker with sterilized distilled water to remove excess calcium ions. All the processes were done under aseptic condition.

The metal and glass instruments and other accessories used for tissue culture were wrapped in aluminium foil and sterilized in a standard pressure cooker at approximately 15psi pressure for 20 minutes. Forceps, scissors etc. were again dipped in alcohol and flamed on lamp at the time of use.

3.5. Inoculation and storage of the embryonic axis and synthetic seed

The inoculation operations were carried out under perfect aseptic conditions in a laminar air flow cabinet. To inoculate the embryonic axis and synthetic seed to the culture medium, the cotton wool plug of the culture vessel was removed and the vessel neck was first flamed over a gas burner kept in the chamber. The sterilised embryonic axis and synthetic seed were quickly transferred into the medium using sterile forceps. The neck of the culture vessels was once again flamed and the cotton wool plug replaced.

The culture vessels were then transferred to the culture racks where they were incubated at a temperature of 20 ± 2°C. Artificial illumination was provided using cool white fluorescent lamps. Photo period was fixed at 9 hours per day.

3.6. Storage of embryonic axis and synthetic seed in inhibiting medium

3.6.1. *Standardisation of medium with chemical inhibitor*

Studies were conducted to evaluate the effect of various plant growth regulators on rooting and shooting of embryonic axis and synthetic seed. For this study separate experiments were conducted using ½ MS as basal medium. Three chemical inhibitors (coumarin, ABA, Cycocel) either alone or in combination were added to the medium for studying storage response of embryonic axis and synthetic seed to these inhibitors. A total of 15 types of chemical inhibitor levels and combinations were used in the present study. Details regarding the various chemical inhibitors tried and concentrations used are furnished in Table 3. Observations on formation of root, shoot and growth pattern

was recorded at two days interval till the complete death of embryonic axis or synthetic seed.

Table 3. Germination inhibitors and their concentrations used for studying storage of embryonic axis and synthetic seed of cocoa (Basal Media ½ MS)

Germination inhibitor	Concentration
Coumarin	10^{-3} M
	10^{-4} M
	10^{-5} M
Abscisic Acid	10^{-4} M
	10^{-5} M
	10^{-6} M
Cycocel (CCC)	10^{-2} M
	10^{-3} M
	10^{-4}M
Coumarin and ABA	10^{-3} M coumarin + 10^{-5} M ABA
	10^{-4} M coumarin + 10^{-5} M ABA
	10^{-5} M coumarin + 10^{-5} M ABA
	10^{-3} M coumarin + 10^{-6} M ABA
	10^{-4} M coumarin + 10^{-6} M ABA
	10^{-5} M coumarin + 10^{-6} M ABA

Table 4. Osmoticum and their concentrations used for studying storage of embryonic axis and synthetic seed of cocoa (Basal Media ½ MS)

Osmoticum	Concentration
Sorbitol	100 mM
	250 mM
	500 mM
Mannitol	100 mM
	250 mM
	500 mM
Sodium Chloride	100 mM
	250 mM
	500 mM

3.6.2. *Standardisation of medium with osmoticum*

The experiment consisted of adding three osmotica (sorbitol, mannitol and sodium chloride) at three different levels (Table 4) to basal media. The embryonic axis and synthetic seeds were placed in the media and stored.

Plate 3. Desiccator with desiccation medium and synthetic seed

3.7. Effect of different desiccation treatments on storage of synthetic seeds

Synthetic seeds made from embryonic axis were subjected to desiccation in the desiccators set at different relative humidities. The synthetic seeds were kept in sterilized petri plates inside the desiccators and covered with the lid after coating the edges with petroleum jelly to keep the desiccators air tight (Plate 3). The synthetic seeds were pre-treated in desiccators set at relative humidities of 100%, 85.3%, 75.6%, 46.6%, 30% and 20% each for time duration of 3, 6, 9 and 12 hours. The entire work was done under sterile conditions inside a laminar flow cabinet. Germination characteristics of the synthetic seeds were studied before and after the treatment.

3.8. Observations

Each trial was conducted with minimum of 18 tubes or 18 seeds. The observations were recorded from 2nd day of inoculation and taken at regular intervals. The morphological changes during the storage period were also noted. The observations continued till the entire embryonic axis or synthetic seed were found dead.

The following observations were recorded from various experiments

a. Number of embryonic axis or synthetic seed germinated: Number of embryonic axis or synthetic seeds showed root growth were counted and expressed in percentage of total number of cultures in that replication
b. Number of embryonic axis or synthetic seeds showed shoot sprouting: Number of embryonic axis or synthetic seeds showed shoot sprouting were counted in each replication and expressed in percentage of total number of cultures.
c. Number of dead embryonic axis or synthetic seed : Embryonic axis or synthetic seeds which were found dead or contaminated during the storage were counted and expressed in percentage of total number of cultures in that replication.
d. Number of days for shoot formation: Number of days taken for shoot formation were recorded. Average number of days taken by each replication was noted.
e. Physical and morphological changes that occured to the embryonic axis or synthetic seed were observed and recorded with photographs.
f. Root and shoot growth pattern of embryonic axis or synthetic seed were observed and recorded with photographs.

g. Changes happened in the media were also observed and recorded.

3.9. Checking viability of embryonic axis or synthetic seed during storage

Viability of the embryonic axes or synthetic seeds were assessed by taking one or two embryonic axis or synthetic seed at random from the treatments which did not show shoot regeneration in the storing medium. They were then cultured into ½ MS medium and observed to confirm their viability up to that time. It was done only for the cultures which lasted more than 40 days.

3.10. Statistical analysis

The data recorded were Arc-sin transformed wherever necessary and statistically analysed using the ANOVA tests. Treatment means were compared using Duncan's multi range test. The analysis was done using statistical package SPSS 20.0. Microsoft excel was utilized for making graphs.

RESULTS

RESULTS

The results of various experiments on storage life of cocoa (*Theobroma cacao* L.) seeds through encapsulation and germination inhibition conducted at College of Forestry, Vellanikkara during 2010-2012 are presented in this chapter.

The standardization of enhancement in storage life of cocoa seed was done and results are presented in the following order

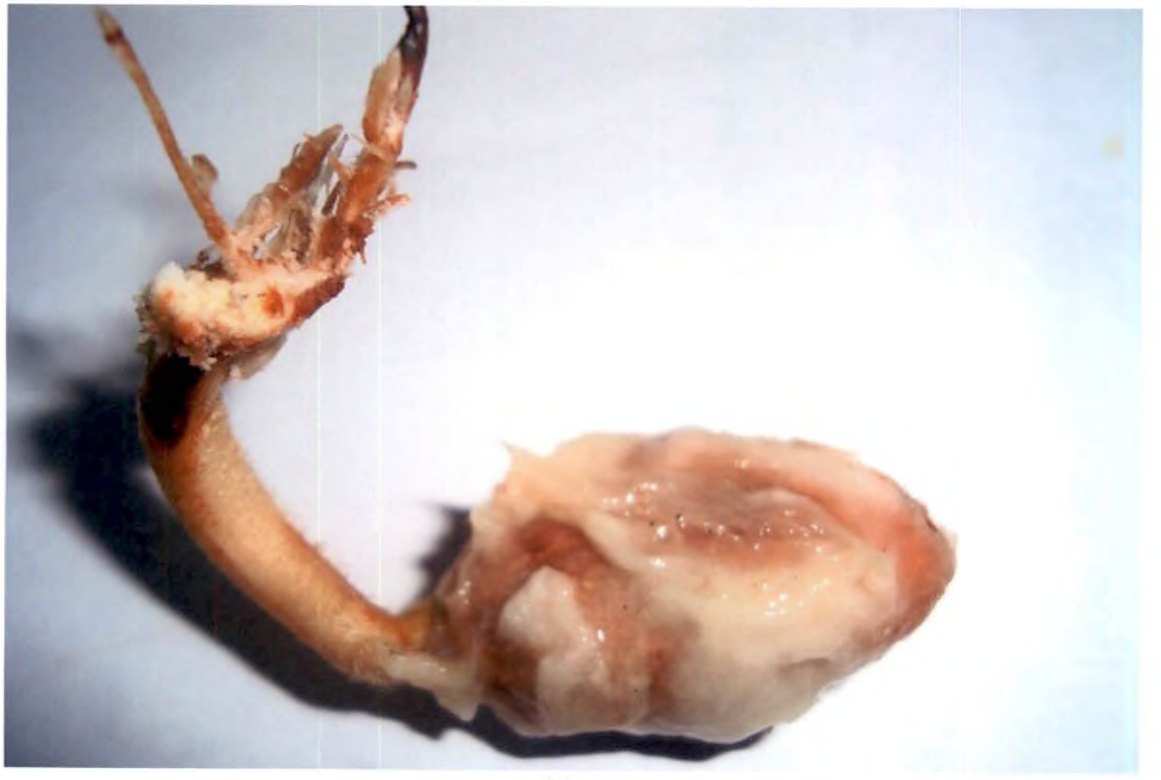
1. Standardization of embryonic axis with different proportion of cotyledon for *in vitro* storage and germination of embryonic axis
2. Standardization of storage media for *in vitro* germination of embryonic axis
3. Effect of germination inhibitors on *in vitro* germination and storage of embryonic axis
   3.1.Germination inhibitors used alone
   3.2. Germination inhibitors in combination
4. Effect of osmotica on *in vitro* germination and storage of embryonic axis
5. Effect of storage media on *in vitro* germination and storage of synthetic seed
6. Effect of germination inhibitor in encapsulation media on *in vitro* germination and storage of synthetic seed
7. Effect of storage media on longevity of embryonic axis and synthetic seed
8. Effect of desiccation on *in vitro* germination and storage of synthetic seed
9. Standardization of storage technique for synthetic seed

4.1. Standardization of microencapsulation techniques

4.1.1. *Standardization of embryonic axis with different proportion of cotyledon for in vitro germination of embryonic axis*

Cocoa pods for the study were collected from the polyclonal seed garden of Kerala Agricultural University. Embryos were collected from the seeds of 100-120 days old cocoa pods harvested based on the visual observation. It has been noted that when pods reach 100 to 120 days, the ridges of pods turn yellow and contain embryonic axis of 0.5 cm to 1 cm length. Immature cocoa seed does not have a distinguishable embryonic axis while over mature cocoa seed shows vivipary (Plate 4).

(a)



(b)

Plate 4. Vivipary seen in overmatured cocoa pod(a) and Cocoa seed from over matured pod(b).

The present study investigated influence of the presence of cotyledon on formation of root and shoot during the storage. Embryonic axes were extracted from seed with ½ cotyledon, ¼ cotyledon and without cotyledon (Plate 2a). The embryonic axes were cultured in ½ MS media to study the influence of presence of cotyledon on growth of embryonic axis. The results of the study are given in Table 5. There was an initial delay in root formation in embryonic axis without cotyledon (29.6%) compared to embryonic axis with cotyledon (95.8%, 84.7%). However, this difference disappeared from the 4th day onwards and all cultures had roots from 6th day of observation (Figure 1a). Cultures with embryonic axes devoid of cotyledon were able to produce root as in embryonic axis with cotyledon. Experiments having ¼ cotyledon and ½ cotyledon did not vary in root formation at any stage of experiment.

Shoot formation was absent (Figure 1b) in embryonic axis without cotyledon. In experiments of embryonic axis with ½ cotyledon and ¼ cotyledon, shoot regeneration started from 10th day and had shoot formation in 88.9% and 85.4% of cultures by 20 days respectively. There was no difference between these two treatments at any stage of the experiment. Shoot formation differed significantly between non-cotyledon embryonic axis and cotyledon attached embryonic axis.

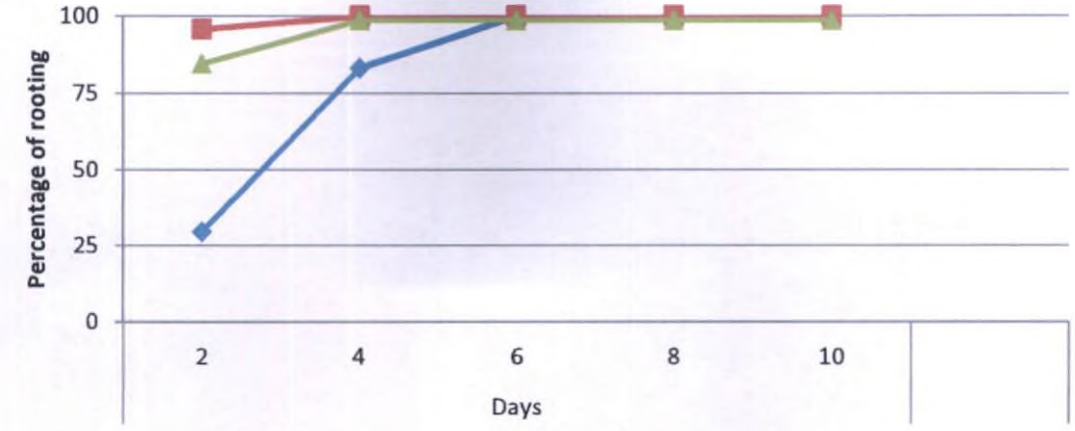
Embryonic axis without cotyledon showed higher mortality compared to experiments of embryonic axis with cotyledon. The embryonic axis without cotyledon died by 35 days (Figure 1c). Low mortality was observed in embryonic axis with cotyledon. There was no difference in the mortality rate between two treatments conducted with cotyledon. This shows that the necessity of cotyledon during the storage for maintaining viability and longevity.

Since embryonic axis with ½ cotyledon and ¼ cotyledon did not vary in level of shoot formation, the embryonic axis with ¼ cotyledon was selected for the synthetic seed preparation as well as for the further experiments. Further, it was also convenient to make synthetic seed in that size and reduced the area of storage without affecting the germination parameters.

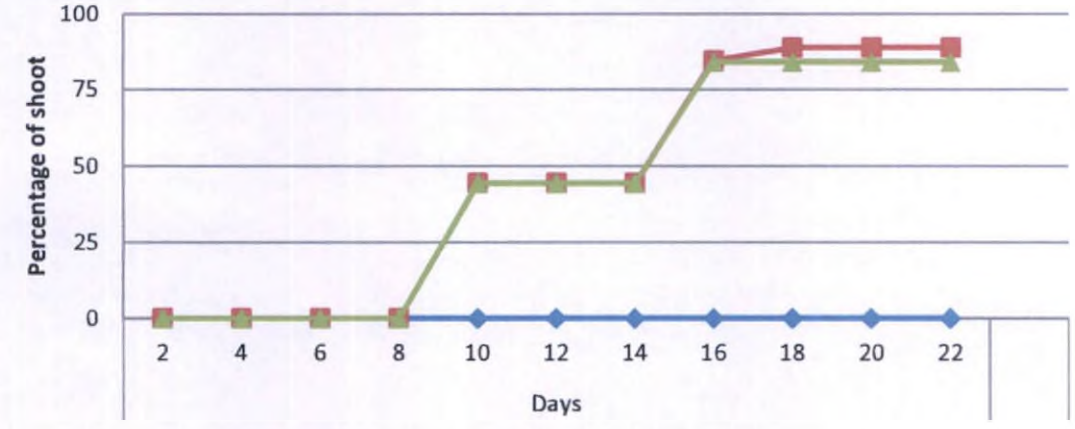
Table 5. Effect of different proportions of cotyledon on *in vitro* germination of embryonic axis of cocoa (Basal media- ½ MS)

Observation	Nature of embryonic axis	Days after culturing								
		05	10	15	20	25	30	35	40	45
Root (%)	without cotyledon	83.3 (56.3)	100 (89.9)	100 (89.9)	100 (89.9)	100 (89.9)	100 (89.9)	100 (89.9)	100 (89.9)	100 (89.9)
	with ¼ cotyledon	98.6 (80.3)	98.6 (80.3)	98.6 (80.3)	98.6 (80.3)	98.6 (80.3)	98.6 (80.3)	98.6 (80.3)	98.6 (80.3)	98.6 (80.3)
	with ½ cotyledon	100 (89.9)	100 (89.9)	100 (89.9)	100 (89.9)	100 (89.9)	100 (89.9)	100 (89.9)	100 (89.9)	100 (89.9)
	SEm±	2.3^{ns}	2.2^{ns}	2.2^{ns}	2.2^{ns}	2.2^{ns}	2.2^{ns}	2.2^{ns}	2.2^{ns}	2.2^{ns}
Shoot (%)	without cotyledon	0.0	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}
	with ¼ cotyledon	0.0	44.4^{a} (26.3)	85.4^{a} (58.6)	85.4^{a} (58.6)	85.4^{a} (58.6)	85.4^{a} (58.6)	85.4^{a} (58.6)	85.4^{a} (58.6)	85.4^{a} (58.6)
	with ½ cotyledon	0.0	44.4^{a} (26.3)	84.7^{a} (57.8)	88.9^{a} (62.7)	88.9^{a} (62.7)	88.9^{a} (62.7)	88.9^{a} (62.7)	88.9^{a} (62.7)	88.9^{a} (62.7)
	SEm±	0.0^{ns}	3.8*	7.6*	7.6*	7.6*	7.6*	7.6*	7.6*	7.6*
Dead (%)	without cotyledon	1.3 (0.7)	3.3 (1.8)	5.3 (3.3)	19.6 (11.3)	39.8^{a} (23.4)	58.1^{a} (35.5)	78.3^{a} (51.5)	78.3^{a} (51.5)	100^{a} (89.9)
	with ¼ cotyledon	1.4 (0.8)	1.4 (0.8)	14.6 (8.3)	14.6 (8.3)	14.6^{b} (8.3)	14.6^{b} (8.3)	14.6^{b} (8.3)	14.6^{b} (8.3)	14.6^{b} (8.3)
	with ½ cotyledon	0.0	0.0	8.3 (4.7)	11.1 (6.3)	11.1^{b} (6.3)	11.1^{b} (6.3)	11.1^{b} (6.3)	11.1^{b} (6.3)	11.1^{b} (6.3)
	SEm±	0.5^{ns}	0.7^{ns}	1.8^{ns}	2.2^{ns}	4.5*	6.2*	8.7*	8.7*	8.7*

* Significant at 0.05 levels; ns- non significant at 0.05 levels
(Figures with same superscript in a column do not differ significantly)
(Values in the parentheses are Arcsine transformed values)



(a)



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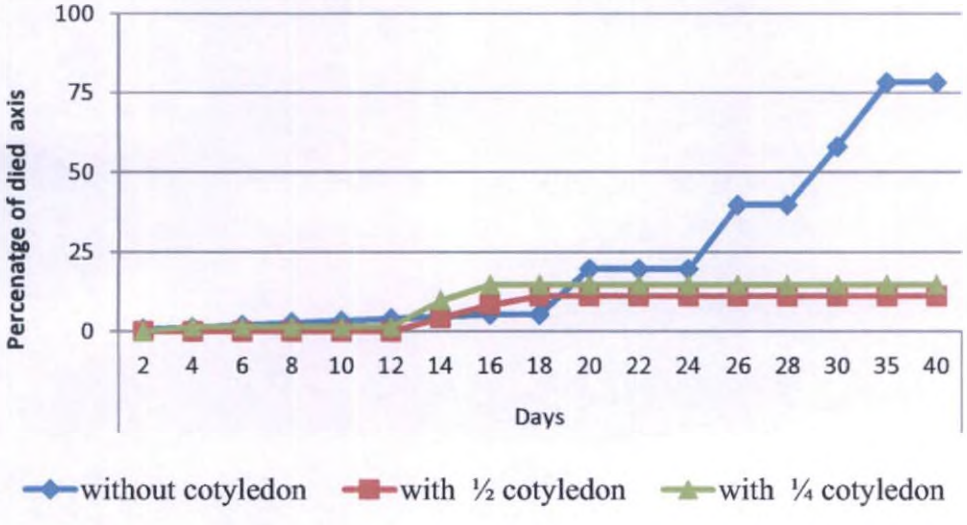


(c)

Figure. 1. Effect of different proportions of cotyledon on (a) root growth, (b) shoot growth and (c) death of cocoa embryonic axis (Basal media- ½ MS)

4.1.2. *Standardization of storage media for in vitro germination of embryonic axis*

In this study different culture media (½ MS, ¼ MS, $^1/_{10}$ MS, dry cotton and wet cotton) were used to study the storage condition suitable for embryonic axes. The formation of root, shoot and death rate of embryonic axis did not differ among any of the storage media (Table 6). However, all embryonic axes stored in dry cotton were found dead within 10 days of inoculation. The normal growth pattern of embryonic axis during storage is given in plate 5.

Rooting started from 2nd day and the cultures with MS basal media gave higher rooting in ½ MS (98.6%) ¼ MS (100%) and $^1/_{10}$ MS (100%) by the end of 5th day (Figure 2a). Rooting was not influenced by various levels of culture media used in the study. Shoot regeneration started from 10th day in ¼ MS and $^1/_{10}$ MS media. Higher percentage shoot regeneration (85.4%, 98.4% and 96.5%) was obtained in these media within 22 days (Figure 2b). In ½ MS media, shoot formation was delayed upto 15 days. The death rates of embryonic axes were found to be very less in ¼ MS (1.6%) and $^1/_{10}$ MS (3.5%) (Figure 2c). The formation of root and shoot and number of dead embryonic axes did not differ among the three MS media levels and wet cotton. Out of three MS media levels, ½ MS media was selected for further experiments due to delayed shoot formation.

4.2. Effect of germination inhibitors on *in vitro* germination and storage of embryonic axis

4.2.1. *Germination inhibitors used alone*

The chemicals, which are having growth inhibiting properties, were tried to reduce the rate of the growth of embryonic axis. The media was fortified with the chemical inhibitors that are widely used for germination inhibition. Coumarin (10^{-3}M, 10^{-4}M, 10^{-5}M) abscisic acid (10^{-4}M, 10^{-5}M, 10^{-6}M) and cycocel (10^{-2}M, 10^{-3}M, 10^{-4}M) were added in three different concentrations (Table 4). The results of the experiments are given below.

Root, shoot development and death of embryonic axis as influenced by chemical inhibitors of different levels present in the media is given in Table 7. Extent of rooting

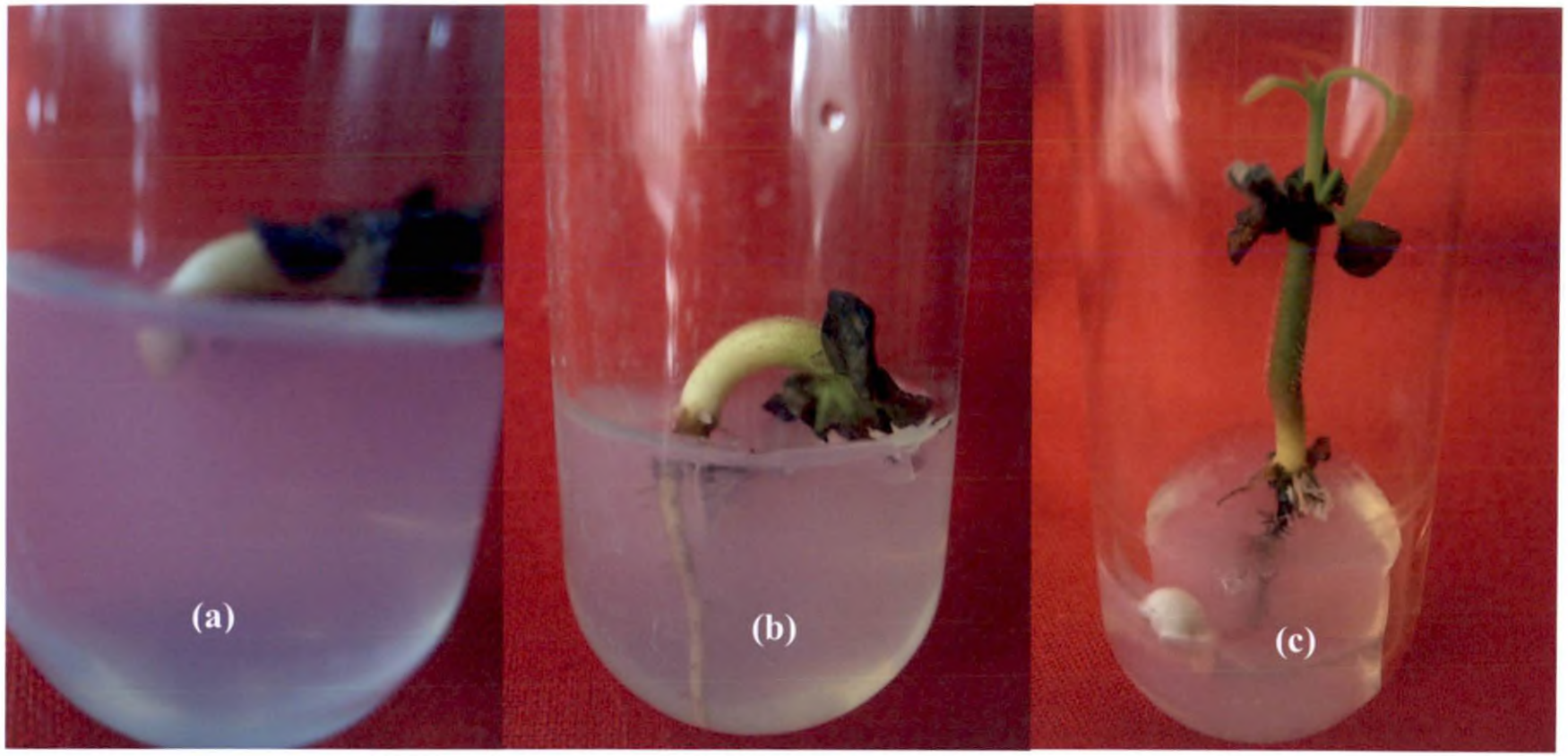
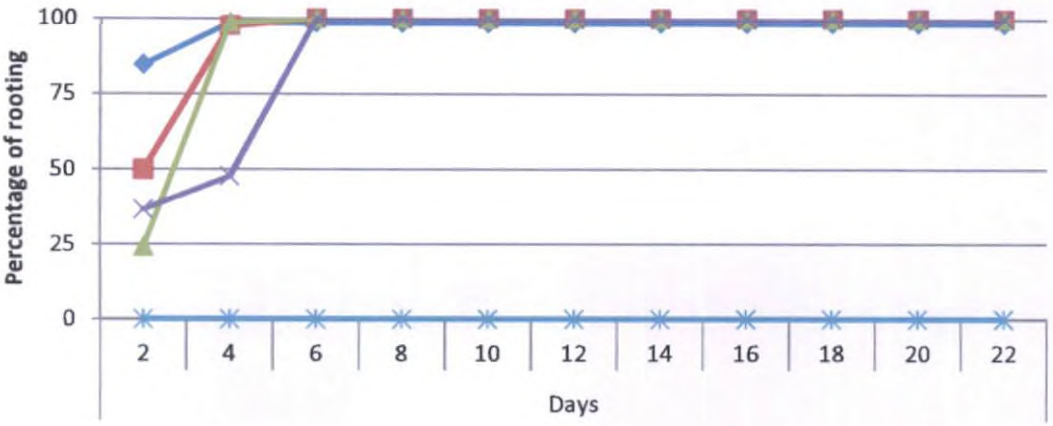
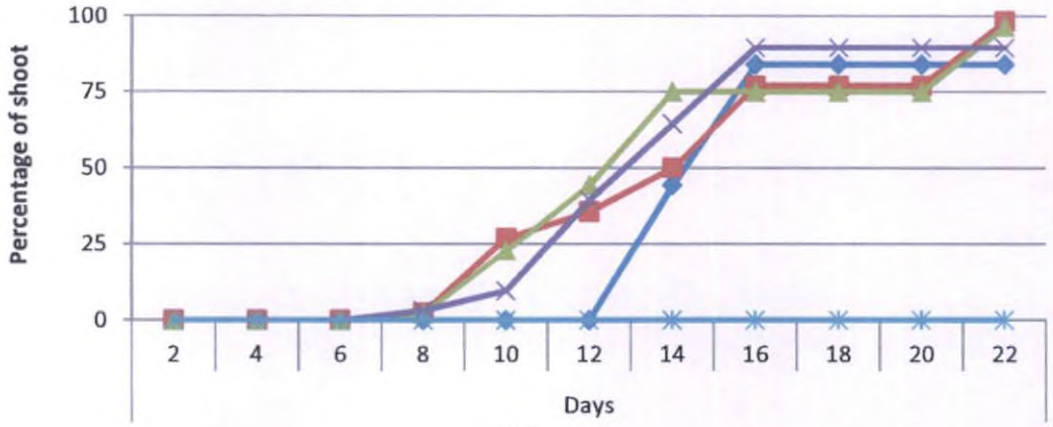


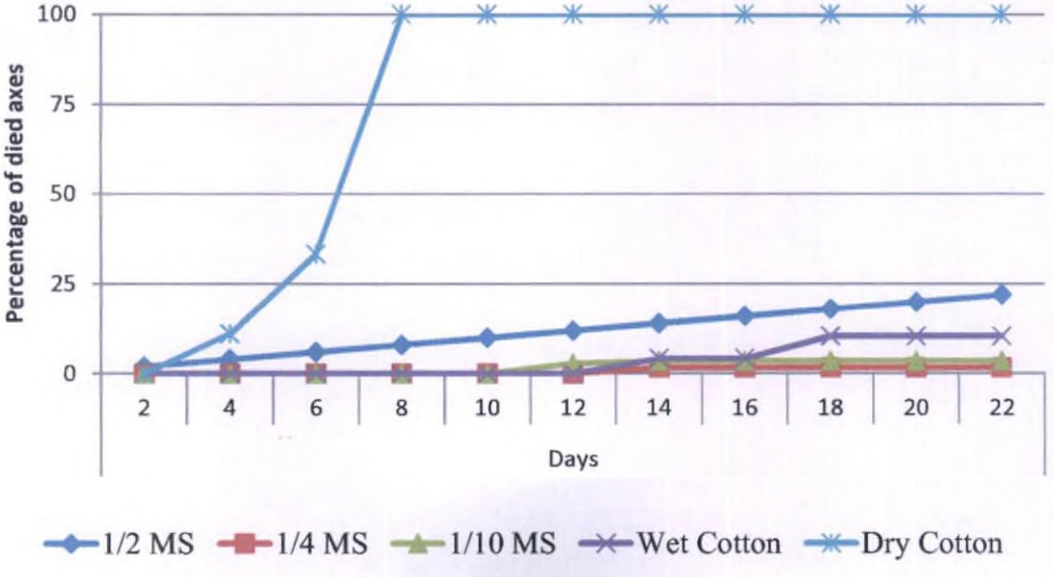
Plate 5. Germination of cocoa embryonic axes in basal media (a) 2nd day , (b) 8th day and (c) 20th day of storage.



(a)



(b)



(c)

Fig. 2. Effect of different storage media on (a) root growth, (b) shoot growth and (c) death of cocoa embryonic axis

Table 6. Effect of different storage media on *in vitro* germination of cocoa embryonic axis

Observation	Media	Days after culture				
		05	10	15	20	25
Root (%)	Dry Cotton	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]
	Wet Cotton	47.8[b] (28.5)	100.0[b] (89.9)	100.0[b] (89.9)	100.0[b] (89.9)	100.0[b] (89.9)
	1/2 MS	98.6[b] (80.3)	98.6[b] (80.3)	98.6[b] (80.3)	98.6[b] (80.3)	98.6[b] (80.3)
	1/4 MS	97.6[b] (77.3)	100.0[b] (89.9)	100.0[b] (89.9)	100.0[b] (89.9)	100.0[b] (89.9)
	1/10 MS	98.6[b] (80.3)	100.0[b] (89.9)	100.0[b] (89.9)	100.0[b] (89.9)	100.0[b] (89.9)
	SEm±	1.8*	1.3*	1.3*	1.3*	1.3*
Shoot (%)	Dry Cotton	0.0	0.0[a]	0.0[a]	0.0[a]	0.0[a]
	Wet Cotton	0.0	9.7[b] (5.5)	64.6[b] (40.2)	89.6[b] (63.6)	89.6[b] (63.6)
	1/2 MS	0.0	0.0[a]	44.4[b] (26.3)	84.0[b] (57.1)	84.0[b] (57.1)
	1/4 MS	0.0	27.0[b] (15.6)	50.0[b] (29.9)	77.0[b] (50.3)	98.4[b] (79.)
	1/10 MS	0.0	22.9[b] (13.2)	75.0[b] (48.5)	75.0[b] (48.5)	96.5[b] (74.7)
	SEm±	0.0[ns]	3.1*	5.8*	5.2*	5.5*
Dead (%)	Dry Cotton	11.1[b] (6.3)	100.0[b] (89.9)	100.0[b] (89.9)	100.0[c] (89.9)	100.0[c] (89.9)
	Wet Cotton	0.0[a]	0.0[a]	4.2[a] (2.4)	10.4[b] (5.9)	10.4[b] (5.9)
	1/2 MS	1.4[a] (0.8)	1.4[a] (0.8)	9.7[a] (5.5)	14.6[b] (8.3)	14.6[b] (8.3)
	1/4 MS	0.0[a]	0.0[a]	1.6[a] (0.9)	1.6[a] (0.9)	1.6[a] (0.9)
	1/10 MS	0.0[a]	0.0[a]	3.5[a] (2.0)	3.5[a] (2.0)	3.5[a] (2.0)
	SEm±	0.3*	0.3*	1.2*	2.7*	2.7*

* Significant at 0.05 levels; ns- non significant at 0.05 levels
(Figures with same superscript in a column do not differ significantly)
(Values in the parentheses are Arcsine transformed values)

was significantly different in 10^{-3}M coumarin, 10^{-4}M ABA and 10^{-6}M ABA for initial days compared to other concentrations. 10^{-4}M ABA had delayed root formation to 10 days and only 33.3% roots developed by 20 days. All inhibitors levels other than 10^{-4}M ABA had root emergence from 2nd day and more than 97% of culture had root within 10 days (Figure 3a). Cycocel (CCC) did not show any inhibitory influence on root regeneration in cocoa embryonic axes.

Coumarin initially delayed regeneration of roots in 10^{-3}M level but elongated embryonic axis produced abnormal swelling from 4th day. However, root growth was retarded by the medium containing 10^{-3}M coumarin. The elongated embryonic axis portion turned to dark brown colour when it came in contact with the medium instead of producing taproot. The presence of 10^{-3}M coumarin in the media lead to the production of fibrous roots from just above the tip of media contact. The darkening of tissue was observed on the emerged root tip along with bulging (Plate 6).

Chemical inhibitors significantly influenced shoot regeneration from the embryonic axis. Data on percentage of shoot regeneration is given in Table 7. No shoot regeneration was seen in cultures with 10^{-4}M ABA added media. The shoot regeneration started from 15th day in all cultures in the experiment, except cultures with 10^{-4}M ABA added media. Presence of 10^{-3}M coumarin in the media resulted in shoot regeneration only in 18.8% of cultures. At 10^{-4}M ABA, embryonic axes died without shoot regeneration. In chemical inhibitor levels, 10^{-4}M and 10^{-5}M coumarin shoot formation started from 15th day and maximum shoots are formed within 25 days of storage. Media with 10^{-5}M ABA and 10^{-6}M ABA and CCC concentrations (10^{-2}M, 10^{-3}M and10^{-4}M), did not influence the shoot formation. Shoot regeneration started from 10th day and maximum shooting obtained within 20 days (Figure 3b).

The death rate of embryonic axes was influenced by the presence of chemical inhibitor in the media. All embryonic axes stored in 10^{-4}M ABA were dead on 60th day. The death rates of embryonic axes were high in the two treatments (10^{-4}M ABA and 10^{-3}M coumarin) after 45 days of storage (Figure 3c). The presence of 10^{-4}M ABA in the basal media helped to extend the storage life of cocoa embryonic axis to a maximum of 55 days. The presence of cycocel in the media did not inhibit the regeneration of root and shoot in embryonic axes during storage. All embryonic axes found to have shoot

Table 7a. Effect of germination inhibitors on *in vitro* germination of cocoa embryonic axis (Basal media- ½ MS)

Inhibitors in media	Root development				Shoot development					
	Days after culture				Days after culture					
	05	10	15	20	05	10	15	20	25	30
10^{-3}M Coumarin	73.6^{b} (47.3)	97.2^{b} (76.3)	97.2^{b} (76.3)	97.2^{b} (76.3)	0.0	0.0	1.4^{a} (0.8)	18.8^{a} (10.8)	18.8^{a} (10.8)	18.8^{a} (10.8)
10^{-4}M Coumarin	100^{b} (89.9)	100^{b} (89.9)	100^{b} (89.9)	100^{b} (89.9)	0.0	0.0	1.4^{a} (0.8)	90.7^{bc} (65.0)	90.7^{bc} (65.0)	100^{c} (89.9)
10^{-5}M Coumarin	88.9^{b} (62.7)	100^{b} (89.9)	100^{b} (89.9)	100^{b} (89.9)	0.0	0.0	18.5^{a} (10.6)	70.4^{b} (44.7)	70.4^{b} (44.7)	87.0^{b} (60.4)
10^{-4}M ABA	0.0^{a}	11.1^{a} (6.3)	18.1^{a} (10.4)	33.3^{a} (19.4)	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
10^{-5}M ABA	100^{b} (89.9)	100^{b} (89.9)	100^{b} (89.9)	100^{b} (89.9)	0.0	0.0	37.0^{a} (21.7)	87.0^{bc} (60.4)	96.3^{c} (74.3)	96.3^{c} (74.3)
10^{-6}M ABA	87.0^{b} (60.4)	100^{b} (89.9)	100^{b} (89.9)	100^{b} (89.9)	0.0	0.0	75.9^{b} (49.3)	100^{c} (89.9)	100^{c} (89.9)	100^{c} (89.9)
10^{-2}M CCC	100^{b} (89.9)	100^{b} (89.9)	100^{b} (89.9)	100^{b} (89.9)	0.0	22.2 (12.82	70.4^{b} (44.7)	100^{c} (89.9)	100^{c} (89.9)	100^{c} (89.9)
10^{-3}M CCC	100^{b} (89.9)	100^{b} (89.9)	100^{b} (89.9)	100^{b} (89.9)	0.0	11.1 (6.3)	74.1^{b} (47.8)	100^{c} (89.9)	100^{c} (89.9)	100^{c} (89.9)
10^{-4}M CCC	100^{b} (89.9)	100^{b} (89.9)	100^{b} (89.9)	100^{b} (89.9)	0.0	14.8 (8.5)	81.5^{b} (54.5)	100.0^{c} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)
SEm±	5.6*	4.9*	4.7*	4.3*	0.0^{ns}	1.5^{ns}	6.7*	6.7*	6.8*	6.8*

* Significant at 0.05 levels; ns- non significant at 0.05 levels
(Figures with same superscript in a column do not differ significantly)
(Values in the parentheses are Arcsine transformed values)

Table 7b. Effect of germination inhibitors on death of cocoa embryonic axis (Basal media- ½ MS)

Inhibitors in media	Days after culture								
	05	10	15	20	30	45	50	55	60
10^{-3}M Coumarin	1.4 (0.8)	2.8 (1.6)	8.3 (4.7)	43.8^{b} (25.9)	73.6^{b} (47.3)	73.6^{b} (47.3)	73.6^{b} (47.3)	82.6^{b} (55.6)	82.6^{b} (55.6)
10^{-4}M Coumarin	0.0	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	0.0	0.0^{a}	0.0^{a}
10^{-5}M Coumarin	0.0	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	0.0	0.0^{a}	0.0^{a}
10^{-4}M ABA	0.0	0.0	0.0	0.0^{a}	0.0^{a}	43.1^{a} (25.5)	61.1^{b} (37.6)	61.1^{b} (37.6)	100^{c} (89.9)
10^{-5}M ABA	0.0	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
10^{-6}M ABA	0.0	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
10^{-2}M CCC	0.0	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
10^{-3}M CCC	0.0	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
10^{-4}M CCC	0.0	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
SEm±	0.2^{ns}	0.2^{ns}	0.8^{ns}	2.5*	4.5*	4.5*	4.7*	5.1*	6.5*

* Significant at 0.05 levels; ns- non significant at 0.05 levels
(Figures with same superscript in a column do not differ significantly)
(Values in the parentheses are transformed values)

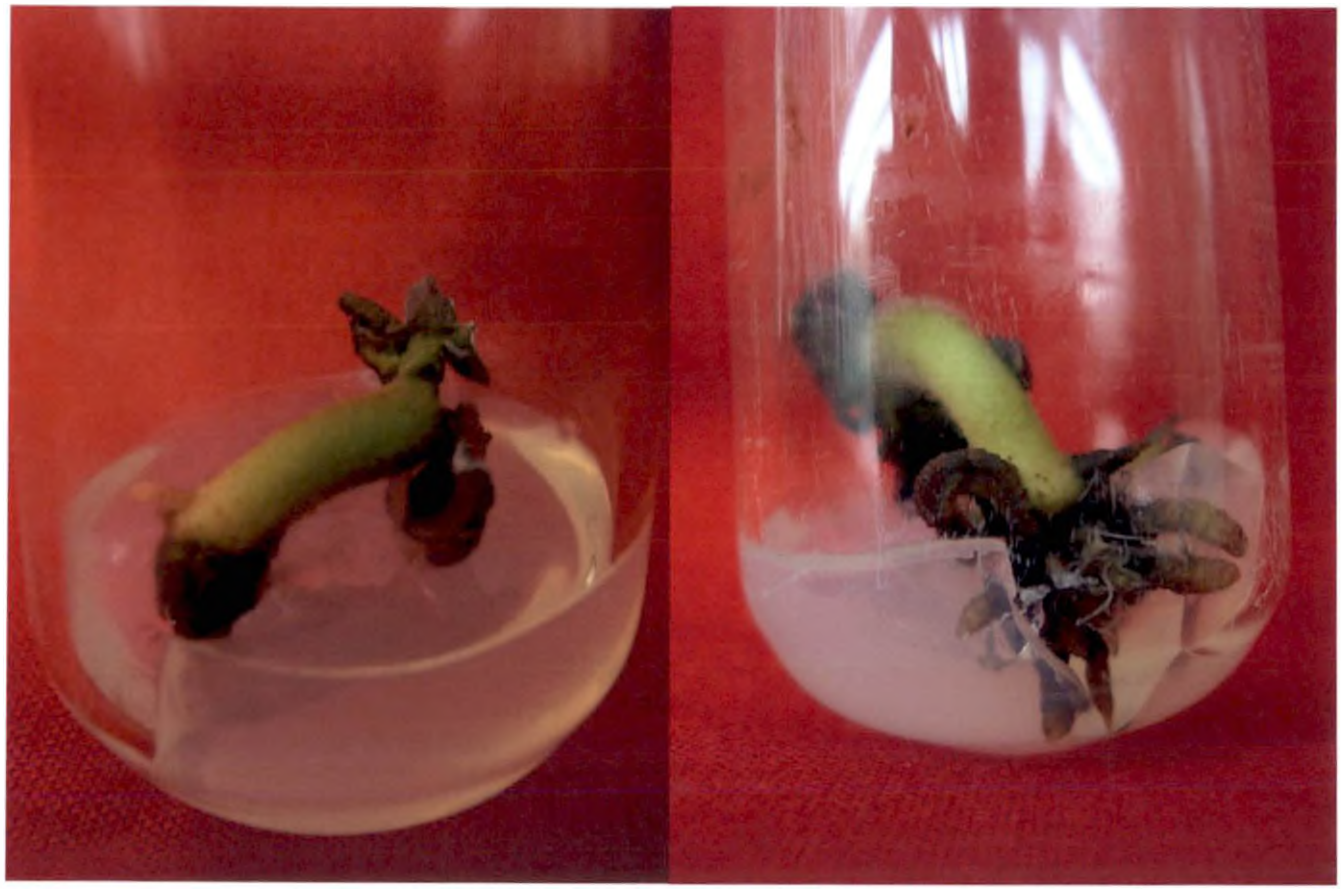
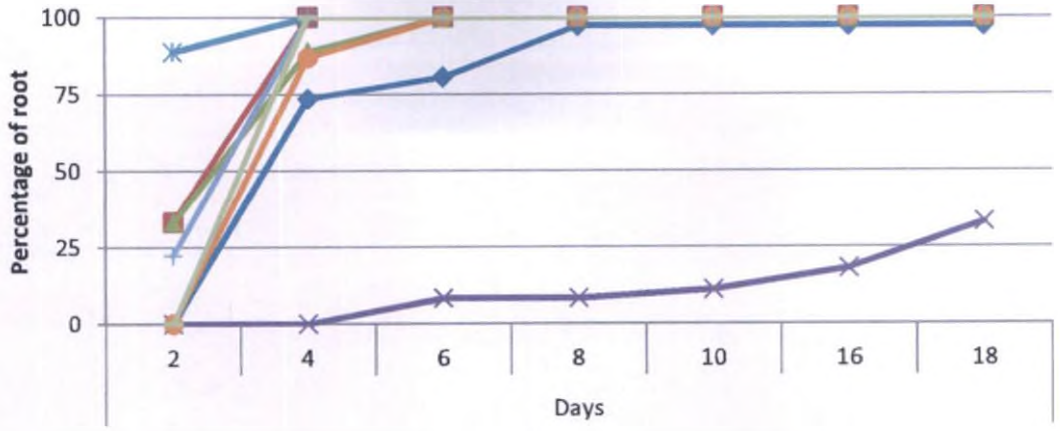


Plate 6. Bulging and abnormal root formation by cocoa embryonic axis stored in ½ MS $+10^{-3}$M coumarin



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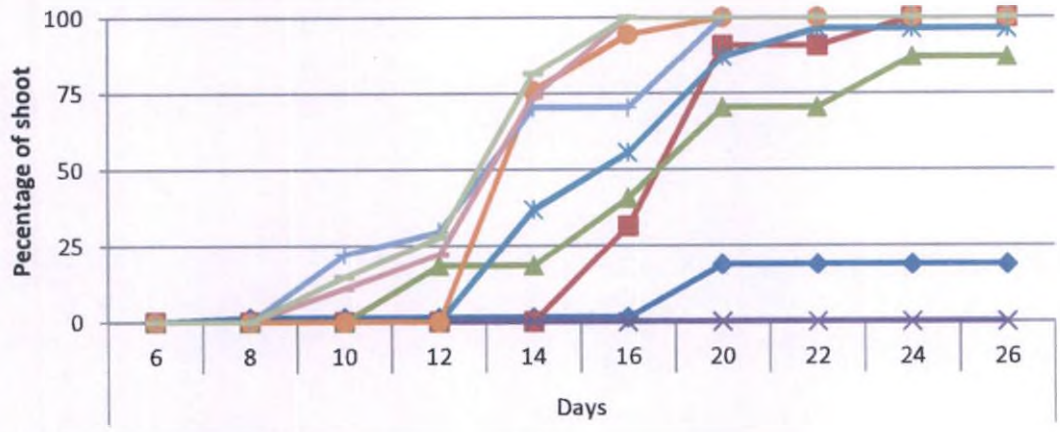

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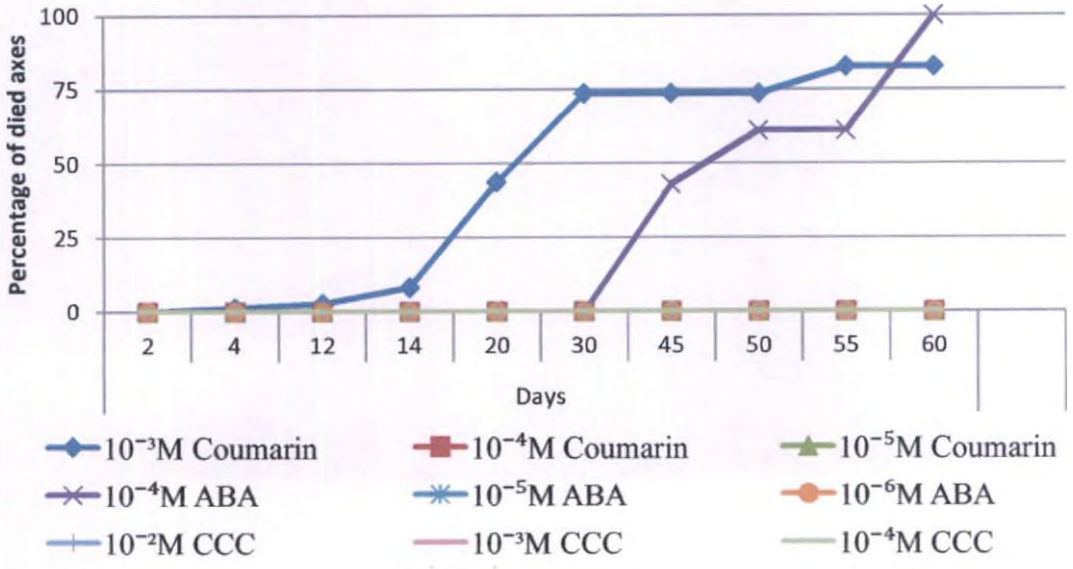


(c)

Fig. 3. Effect of germination inhibitors on (a) root growth, (b) shoot growth and (c) death of cocoa embryonic axis (Basal media- ½ MS)

by 20th day of storage. The lower concentration of ABA (10^{-5}M and 10^{-6}M) showed results similar to CCC.

The experiment had shown that higher concentration of ABA (10^{-4}M) have significant influence on longevity of embryonic axes. The seeds can be stored without germination up to a maximum extent of 56 days. Because of the abnormal growth patterns during storage, higher concentrations of coumarin were not selected for further experiments.

4.2.2. Germination *inhibitors in combination*

Combinations of inhibitors ABA and coumarin (Table 3) were tried to control the growth of embryos of cocoa. The results of the study are given in Table 8. The study indicated that root regeneration was significantly influenced by combination of 10^{-3}M coumarin + 10^{-5}M ABA and of 10^{-3}M coumarin + 10^{-6}M ABA. The embryonic axes stored in 10^{-3}M coumarin + 10^{-5}M ABA had only maximum root regeneration of 61.1% of cultures. Only 68.5% of 10^{-3}M coumarin + 10^{-6}M ABA culture had root regeneration. Other combinations of ABA and coumarin did not show any influence on root formation, as rooting was seen in all cultures within 10 days (Figure 4a).

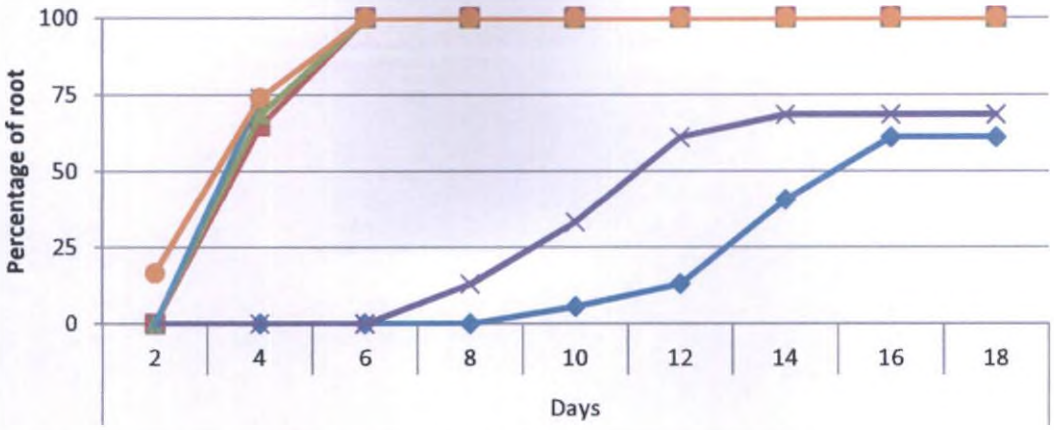
Shoot regeneration was also significantly influenced by having 10^{-3}M coumarin combination with 10^{-5}M and10^{-6}M ABA. Only 7.4% and 16.7 % of culture showed shoot regeneration in the above concentration of growth inhibitor. Other combinations of coumarin and ABA did not show significant influence on shoot formation (Figure 4b). The inhibitor combination of 10^{-4}M coumarin + 10^{-5}M ABA resulted within 57.4% of culture having shoots in 20 days. Embryonic axes cultured in 10^{-5}M coumarin + 10^{-5}M ABA added media, had shoot regeneration (77.8%) within 28 days (Table 8). The media with 10^{-4}M coumarin + 10^{-6}M ABA and 10^{-5}M coumarin + 10^{-6}M ABA resulted in shoot regeneration in 83.3% and 85.2% of the cultures respectively by the 25th day.

Extents of death of embryonic axes in cultures were significantly affected in 10^{-3}M coumarin combination with ABA. There was a significant death rate (92.6%) of embryonic axes in media having combination of 10^{-3}M coumarin +10^{-5}M ABA (Figure 4c) and all embryonic axes were found dead within 30 days (Table 8). In 10^{-6} M ABA and 10^{-3} M coumarin combination, 70.4% of culture were dead by 35 days. Higher death rate was also observed in 10^{-6} M ABA and 10^{-4} M coumarin (42.6%). Other

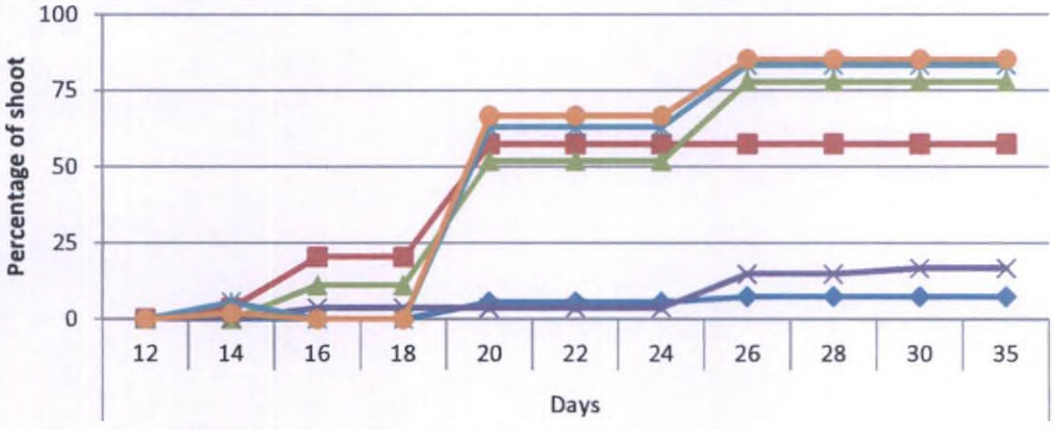
Table 8. Combined effect of germination inhibitors on *in vitro* germination of cocoa embryonic axis (Basal media- ½ MS)

Media	Days after culture																	
	05	10	15	20	10	15	20	25	30	05	10	15	20	25	30	35	40	45
	Root (%)				Shoot (%)					Dead (%)								
10^{-3} M Coumarin + 10^{-5} M ABA	0.0[a]	5.6[a] (3.2)	40.7[a] (24.0)	61.1[a] (37.6)	0.0	0.0[a]	5.6[a] (3.2)	7.4[a] (4.2)	7.4[a] (4.2)	0.0	5.6 (3.2)	16.7 (9.6)	33.3 (19.4)	70.4[b] (44.7)	92.6[b] (67.7)	92.6[b] (67.7)	92.6[b] (67.7)	92.6[b] (67.7)
10^{-4} M Coumarin + 10^{-5} M ABA	64.8[b] (40.3)	100[c] (89.9)	100[b] (89.9)	100[b] (89.9)	0.0	20.4[bc] (11.7)	57.4[b] (35.0)	57.4[b] (35.0)	57.4[b] (35.0)	0.0	0.0	13.0 (7.4)	31.5 (18.3)	33.3[a] (19.4)	42.6[ab] (25.2)	42.6[ab] (25.2)	42.6[ab] (25.2)	42.6[ab] (25.2)
10^{-5} M Coumarin + 10^{-5} M ABA	68.5[b] (43.2)	100[c] (89.9)	100[b] (89.9)	100[b] (89.9)	0.0	11.1[ab] (6.3)	51.9[b] (31.2)	77.8[bc] (51.0)	77.8[bc] (51.0)	0.0	0.0	5.6 (3.2)	13.0 (7.4)	14.8[a] (8.5)	14.8[a] (8.5)	22.2[a] (12.8)	22.2[a] (12.8)	22.2[a] (12.8)
10^{-3} M Coumarin + 10^{-6} M ABA	0.0[a]	33.3[b] (19.4)	68.5[a] (43.2)	68.5[a] (43.2)	0.0	3.7[a] (2.1)	3.7[a] (2.1)	14.8[a] (8.5)	16.7[a] (9.6)	3.7 (2.1)	3.7 (2.1)	20.4 (11.7)	25.9 (15.0)	42.6[ab] (25.2)	66.7[ab] (41.8)	70.4[ab] (44.7)	70.4[ab] (44.7)	83.3[b] (56.3)
10^{-4} M Coumarin + 10^{-6} M ABA	74.1[b] (47.8)	100[c] (89.9)	100[b] (89.9)	100[b] (89.9)	0.0	33.3[cd] (19.4)	63.0[b] (39.0)	83.3[c] (56.3)	83.3[c] (56.3)	0.0	0.0	13.0 (7.4)	13.0 (7.4)	16.7[a] (9.6)	16.7[a] (9.6)	16.7[a] (9.6)	16.7[a] (9.6)	16.7[a] (9.6)
10^{-5} M Coumarin + 10^{-6} M ABA	74.1[b] (47.8)	100[c] (89.9)	100[b] (89.9)	100[b] (89.9)	0.0	40.7[d] (24.0)	66.7[b] (41.8)	85.2[c] (58.4)	85.2[c] (58.4)	0.0	0.0	3.7 (2.1)	7.4 (4.2)	7.4[a] (4.2)	14.8[a]	14.8[a]	14.8[a]	14.8[a]
SEm±	10.2*	9.0*	6.8*	5.8*	0.0 ns	2.3*	4.2*	5.7*	5.7*	0.3[ns]	0.6[ns]	2.3[ns]	2.1[ns]	4.1*	8.2*	8.7*	8.7*	8.7*

* Significant at 0.05 levels; ns- non significant at 0.05 levels
(Figures with same superscript in a column do not differ significantly)
(Values in the parentheses are Arcsine transformed values)



(a)



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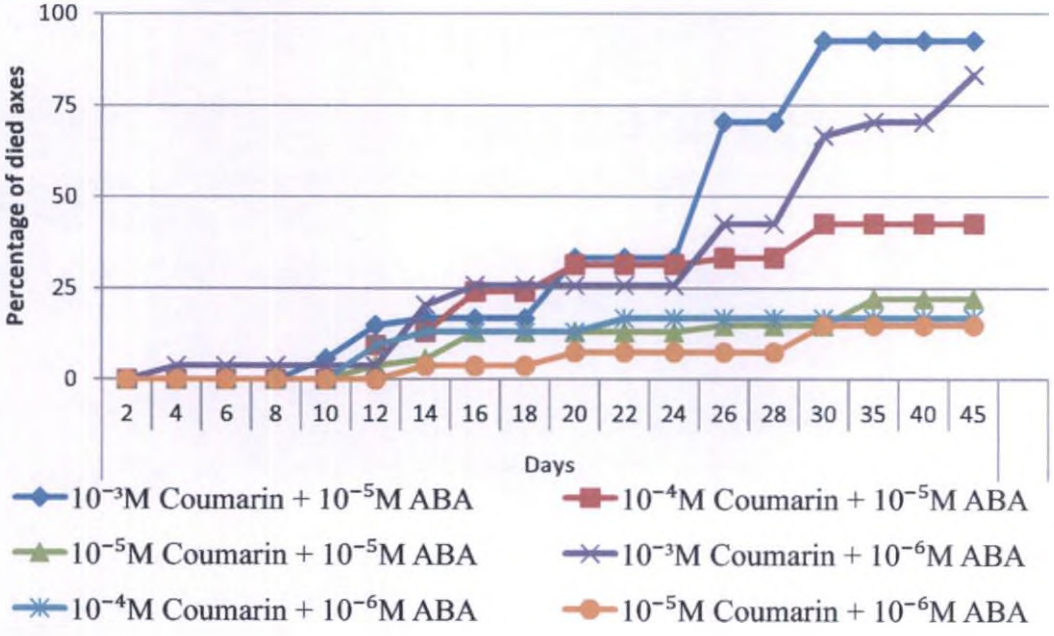


(c)

Fig. 4. Effect of germination inhibitor combinations on (a) root growth, (b) shoot growth and (c) death of cocoa embryonic axis (Basal media- ½ MS)

combinations of inhibitors in the media did not show influence on embryonic axes (Figure 4b).

The result suggests that 10^{-3}M coumarin was inhibiting the growth of the cocoa embryonic axes. The other concentrations were also responding similarly to the conditions when they are individually incorporated into the media. There was no combination effect of inhibitors found on the root formation of embryonic axes during the experiment. Higher death rate and lower germination of embryonic axes seen in 10^{-3} M coumarin added media. So the longevity of embryonic axes was adversely affected by the presence of 10^{-3} M coumarin in the media.

4.3. Effect of osmotica on *in vitro* germination and storage of embryonic axis

The present study investigated shoot and root regeneration of embryonic axis cultured in media added with different concentration of osmotica (Table 5). Sorbitol, mannitol and sodium chloride were added in 100 mM, 250 mM and 500 mM concentrations to the basal medium (½ MS) and shoot and root generation of embryonic axis was studied.

Rooting of embryonic axes in osmotica added condition varied significantly between the different osmotic levels (Table 9). In media with 100 mM of sorbitol and 100 mM mannitol no influence on rooting of embryonic axes was observed (Figure 5a). In both osmotic levels rooting was observed from 2^{nd} day of inoculation and maximum rooting was obtained by 10^{th} day (96.3% and 62.5% respectively). Root development was inhibited in media supplemented with 250 mM sorbitol, 500 mM sorbitol, 500 mM mannitol, 250 mM NaCl and 500 mM NaCl and the embryonic axes were found dead. Root development of embryonic axes was inhibited in these osmotic conditions.

In cocoa embryonic axes shoot regeneration were inhibited by all osmoticum except 100 mM sorbitol (Table 9). In cultures with 100 mM sorbitol shoot regeneration started from 14^{th} day and 85.2% of culture had shoot at the end of 40 days. However, in the case of 500 mM sorbitol added media shoot development was inhibited by the osmoticum up to 70 days of storage (Figure 5b). All other osmotica levels were found to be highly inhibiting the shoot formation. Embryonic axes cultured in the rest of the treatments did not have a significant shoot production even after 70 days of storage and was comparable to each other. All the embryonic axes were found dead in 500 mM

Table 9. Effect of osmotica on *in vitro* germination of cocoa embryonic axis (Basal media- ½ MS)

Media	Days after culture												
	Root (%)				Shoot (%)								
	05	10	15	20	10	15	20	25	30	40	60	65	70
100 mM Sorbitol	83.3^{b} (56.3)	96.3^{b} (74.3)	96.3^{b} (74.3)	96.3^{b} (74.3)	0.0	7.4 (4.2)	11.1 (6.3)	37.0^{b} (21.7)	79.6^{b} (52.7)	85.2^{b} (58.4)	85.2^{b} (58.4)	85.2^{c} (58.4)	85.2^{c} (58.4)
250 mM Sorbitol	4.4^{a} (2.5)	6.7^{a} (3.8)	6.7^{a} (3.8)	24.4^{a} (14.1)	0.0	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	17.8^{a} (10.2)	17.8^{a} (10.2)	17.8^{a} (10.2)
500 mM Sorbitol	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	2.2^{a} (1.2)
100 mM Mannitol	62.5^{b} (38.6)	73.6^{b} (47.3)	73.6^{b} (47.3)	73.6^{b} (47.3)	0.0	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
250 mM Mannitol	8.3^{a} (4.7)	16.7^{a} (9.6)	16.7^{a} (9.6)	16.7^{a} (9.6)	0.0	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
500 mM Mannitol	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
100 mM NaCl	34.7^{ab} (20.3)	66.7^{b} (41.8)	66.7^{b} (41.8)	66.7^{b} (41.8)	0.0	0.0	0.0	0.0^{a}	1.4^{a} (0.8)	1.4^{a} (0.8)	1.4^{a} (0.8)	1.4^{a} (0.8)	1.4^{a} (0.8)
250 mM NaCl	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
500 mM NaCl	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
SEm±	4.2*	5.6*	5.9*	5.9*	0.0^{ns}	0.3^{ns}	0.5^{ns}	1.8*	2.4*	2.7*	3.0*	3.1*	3.0*

* Significant at 0.05 levels; ns- non significant at 0.05 levels (Figures with same superscript in a column do not differ significantly)
(Values in the parentheses are Arcsine transformed values)

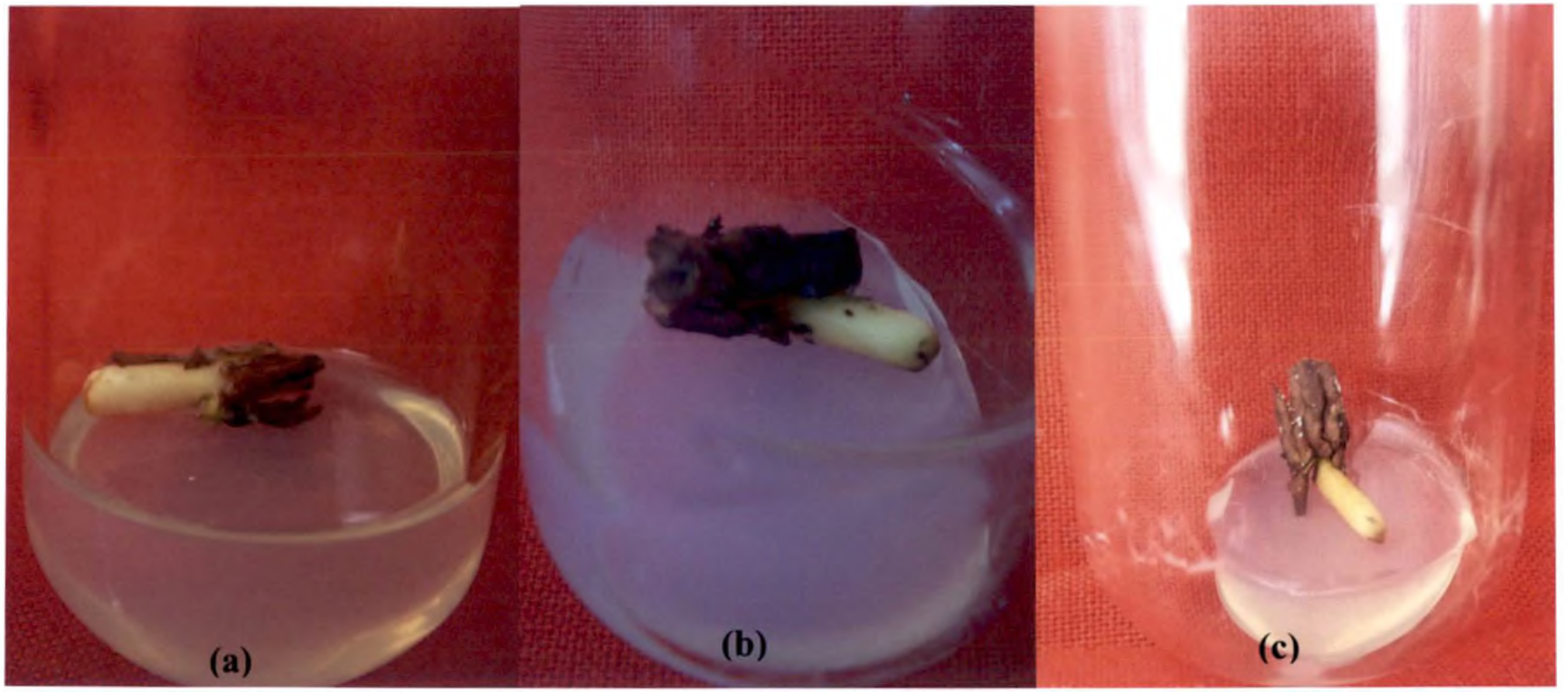


Plate 7. Embryonic axis of cocoa stored in ½ MS +250 mM sorbitol (a) 2nd day, (b) 25th day and (c) 60th day of storage.

NaCl media by 24th day of storage (Table 10). However, 100 mM NaCl showed less toxicity to embryonic axes.

The media added with different levels of osmotica significantly influenced the longevity of embryonic axes. Effect of sorbitol significantly differed from other osmotica tried. In sorbitol, cultures were surviving at 70 days after inoculation. Reduced shoot regeneration along with least death rate in sorbitol conditions showed possibility of storage of embryonic axes in that media up to 70 days. The embryonic axes were found viable up to 70 days of inoculation in media fortified with 250 mM sorbitol (Plate 7). mannitol and NaCl resulted in complete loss of cultures. The higher death rates of embryonic axes were found in the storage conditions where media fortified with mannitol and NaCl were used. The percentage of dead embryonic axes was not differed in mannitol and NaCl media (Figure 5c). By the end of 50 days of storage, all the embryonic axes were found dead in these media.

In mannitol supplemented media, crystallization was seen which lead to drying of media and death of embryonic axis (Plate 8). By 50 days of storage in mannitol added media all the embryonic axis were found dead. In 500 mM NaCl supplemented media dead embryonic axes were observed from 8th day and 100% death by 20th day (Figure 5c). In 250 mM NaCl added media all embryonic axes were found dead by the end of 40 days and at lower concentration (100 mM) embryos survived upto 50 days.

The study indicated that the osmotica influence the germination and longevity of embryonic axes. The embryonic axes survived upto 70 days in 250 mM sorbitol supplemented media and 65 days in 500 mM sorbitol added media. The germination of embryonic axes were delayed to 60 days in both media and it has shown potential of storing embryo in osmotic media upto 60 days. The growth of embryonic axes were highly influenced by these two storage condition but in 500 mM sorbitol supplemented media, embryonic axes had a higher death rate by the end of 70 days (97.8%). 250 mM Sorbitol supplemented media had a less death rate and germination of embryonic axes favored the storage .

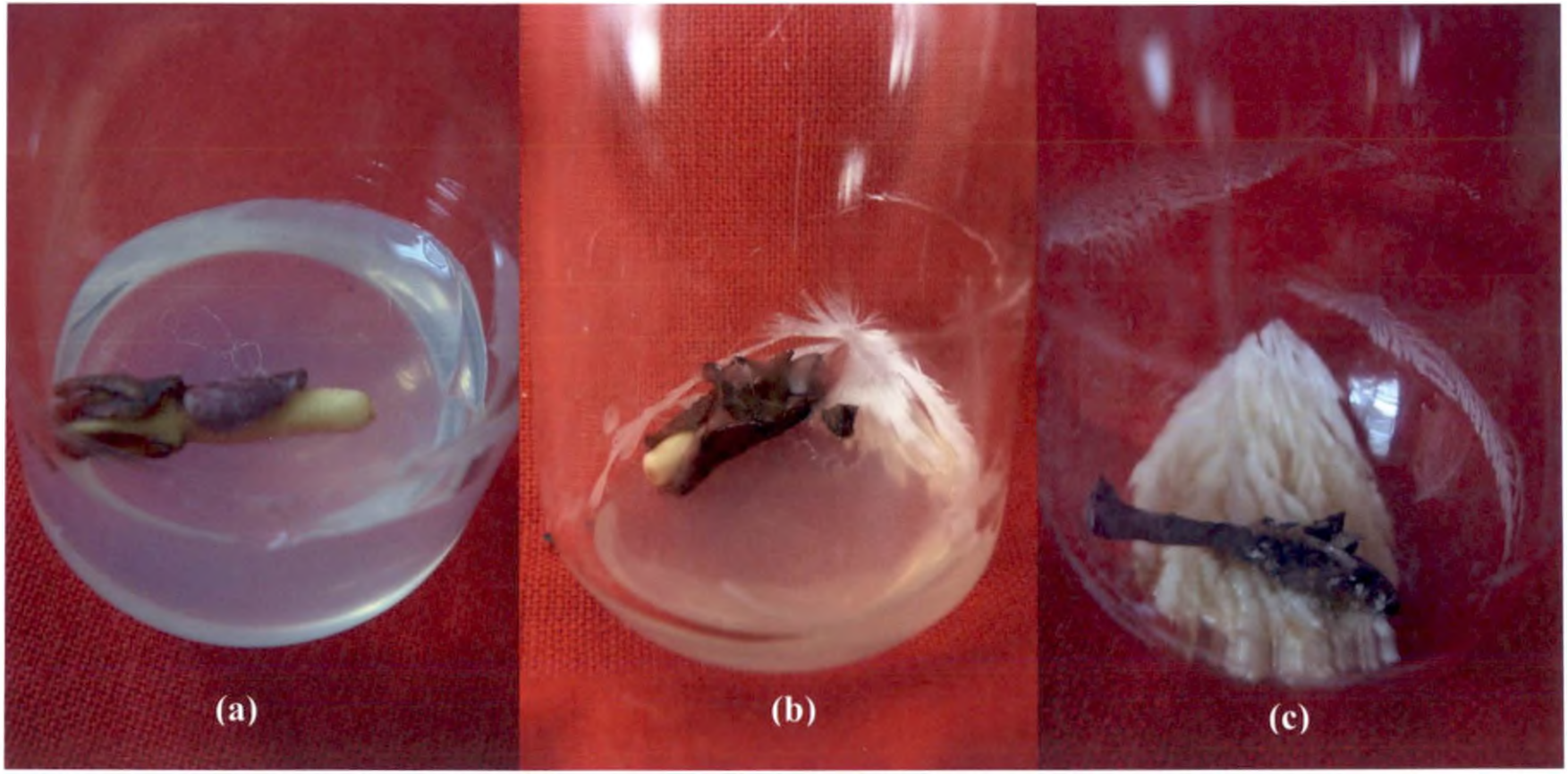
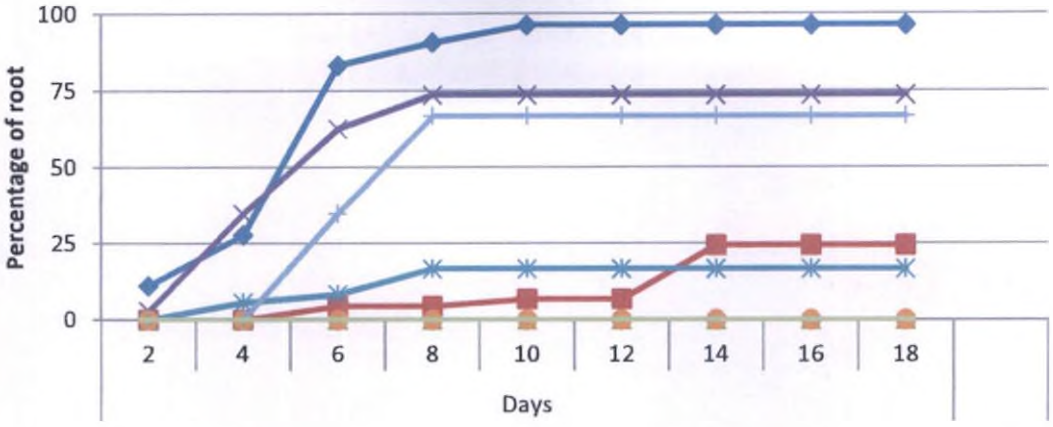
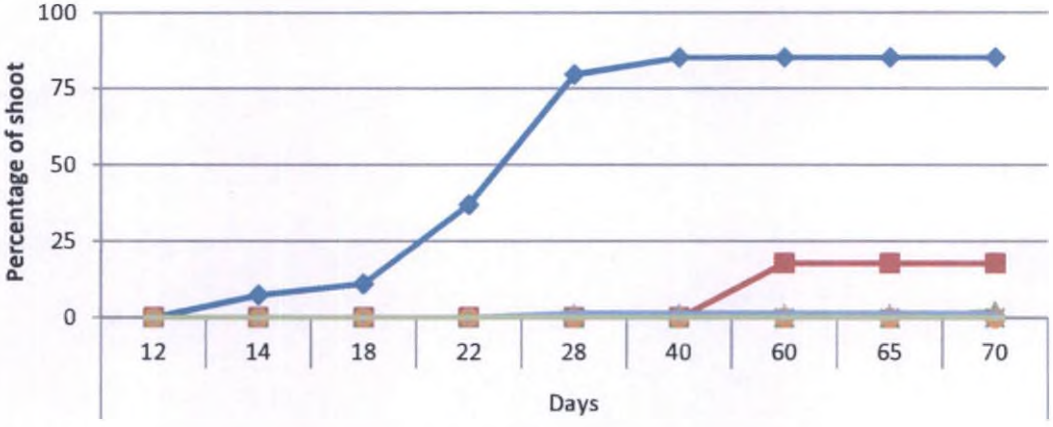


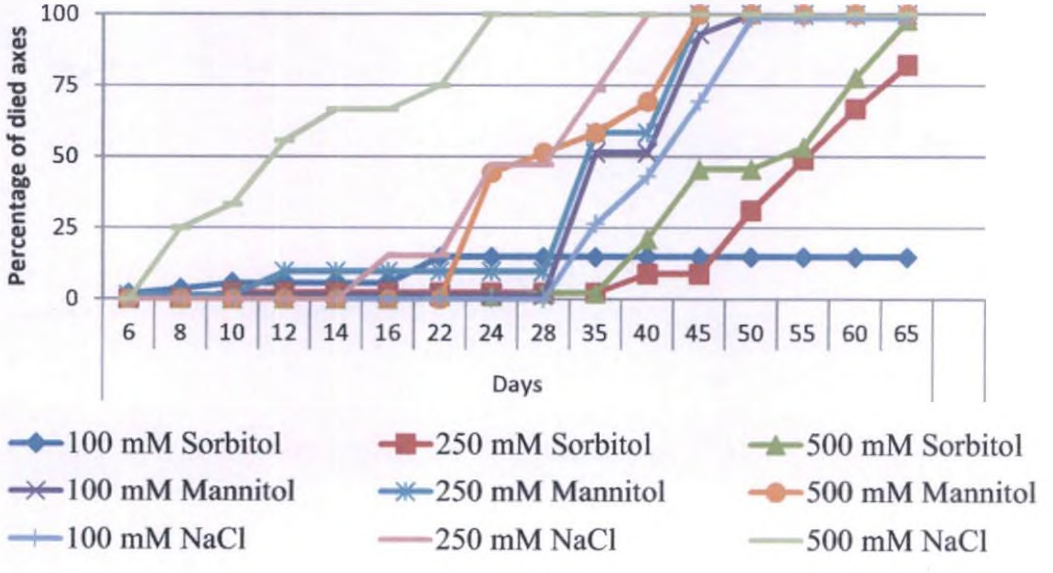
Plate 8. Crystallisation of media with 500 mM mannitol after (a) 2nd day, (b) 30th day and (c) 50th day of storage



(a)



(b)



(c)

Fig. 5. Effect of osmotica on (a) root growth, (b) shoot growth and (c) death of cocoa embryonic axis (Basal media- ½ MS)

Table 10. Effect of osmotica on death of cocoa embryonic axis (Basal media- ½ MS)

Media	Days after culture												
	Dead (%)												
	05	10	15	20	25	30	35	40	45	50	55	60	70
100 mM Sorbitol	1.9 (1.0)	5.6 (3.2)	5.6^{a} (3.2)	14.8^{a} (8.5)	14.8^{ab} (8.5)	14.8^{ab} (8.5)	14.8^{ab} (8.5)	14.8^{ab} (8.5)	14.8^{a} (8.5)	14.8^{a} (8.5)	14.8^{a} (8.5)	14.8^{a} (8.5)	14.8^{a} (8.5)
250 mM Sorbitol	0.0	2.2 (1.2)	2.2^{a} (1.2)	2.2^{a} (1.2)	2.2^{a} (1.2)	2.2^{a} (1.2)	2.2^{a} (1.2)	8.9^{a} (5.1)	8.9^{a} (5.1)	31.1^{ab} (18.1)	48.9^{ab} (29.2)	66.7^{ab} (41.8)	82.2^{ab} (55.2)
500 mM Sorbitol	0.0	0.0	0.0^{a}	1.1^{a} (0.6)	1.1^{a} (0.6)	2.2^{a} (1.2)	2.2^{a} (1.2)	21.1^{a} (12.1)	45.6^{ab} (27.1)	45.6^{ab} (27.1)	53.3^{ab} (32.2)	77.8^{ab} (51.0)	97.8^{c} (77.9)
100 mM Mannitol	0.0	1.4 (0.8)	1.4^{a} (0.8)	1.4^{a} (0.8)	1.4^{a} (0.8)	1.4^{a} (0.8)	51.4^{ab} (30.9)	51.4^{ab} (30.9)	93.1^{cd} (68.5)	100.0^{c} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)
250 mM Mannitol	1.4 (0.8)	1.4 (0.8)	9.7^{a} (5.5)	9.7^{a} (5.5)	9.7^{a} (5.5)	9.7^{a} (5.5)	58.3^{ab} (35.6)	58.3^{ab} (35.6)	100.0^{d} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)
500 mM Mannitol	0.0	0.0	0.0^{a}	0.0^{a}	44.4^{ab} (26.3)	51.4^{ab} (30.9)	58.3^{ab} (35.6)	69.4^{ab} (43.9)	100.0^{d} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)
100 mM NaCl	0.0	0.0	0.0^{a}	0.0^{a}	0.0	0.0^{a}	26.4^{a} (15.3)	43.1^{ab} (25.5)	69.4^{bc} (43.9)	98.6^{c} (80.3)	98.6^{c} (80.3)	98.6^{c} (80.3)	98.6^{c} (80.3)
250 mM NaCl	0.0	0.0	0.0^{a}	15.3^{a} (8.7)	47.2^{ab} (28.1)	47.2^{ab} (28.1)	73.6^{b} (47.3)	100.0^{b} (89.9)	100.0^{d} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)
500 mM NaCl	0.0	33.3 (19.4)	66.7^{b} (41.8)	75.0^{b} (48.5)	100.0^{c} (89.9)	100.0^{c} (89.9)	100.0^{b} (89.9)	100.0^{b} (89.9)	100.0^{d} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)
SEm±	0.1^{ns}	1.5^{ns}	3.3*	3.8*	4.0*	4.5*	5.6*	5.5*	5.7*	5.8*	5.8*	5.5*	5.3*

* Significant at 0.05 levels; ns- non significant at 0.05 levels (Figures with same superscript in a column do not differ significantly)
(Values in the parentheses are Arcsine transformed values)

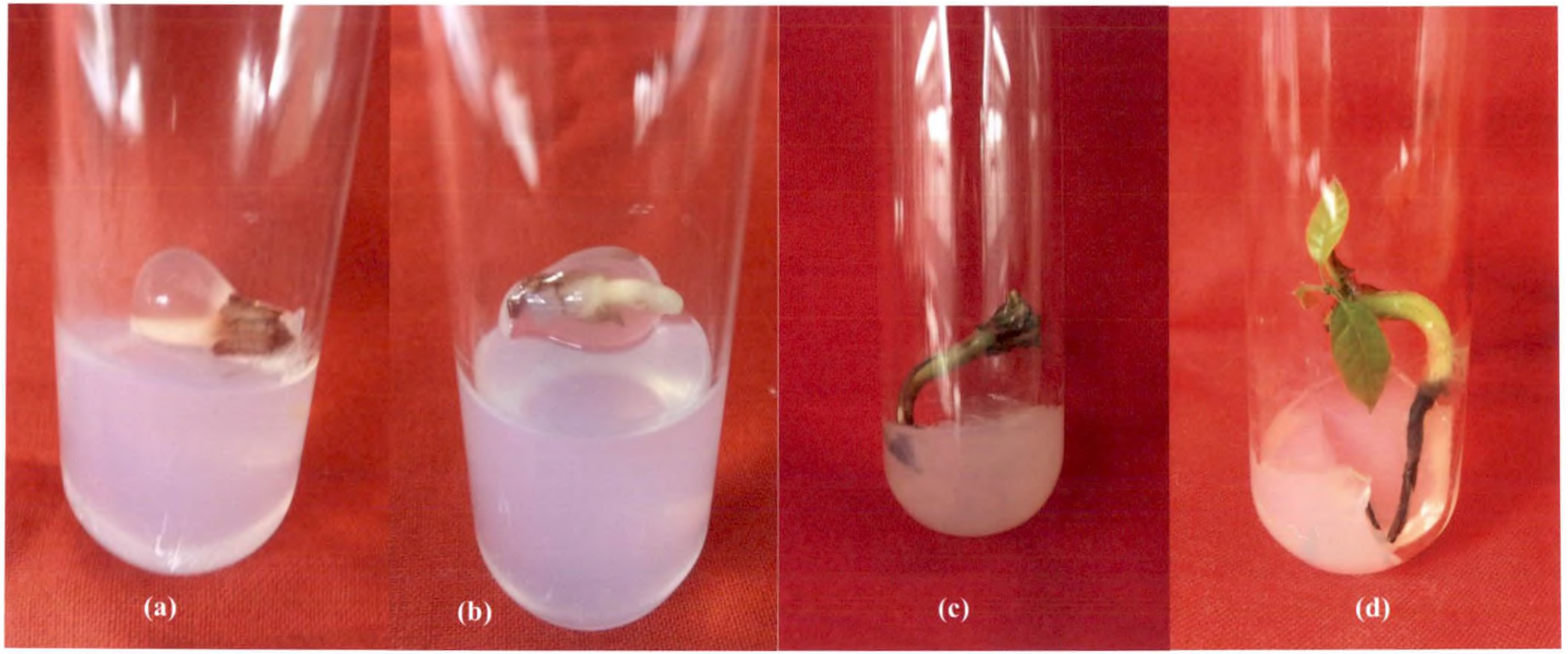


Plate 9. Germination of cocoa synthetic seed during storage of (a) 2 days (b) 5 days (c) 20 days (d) 35 days in ½ MS media

4.4. Effect of storage media on *in vitro* germination and storage of synthetic seed

The present study investigated the changes in "longevity" of encapsulated cocoa embryonic axes stored in different storage media. Encapsulated embryonic axes were stored in dry cotton and wet cotton to find out the survival during the storage condition. Synthetic seeds were also stored in the media added with most effective osmotic chemical concentration identified from the earlier described experiments. Synthetic seeds were prepared in aseptic conditions and stored in dry cotton, wet cotton, ½ MS, ¼ MS, ½ MS with 250 mM sorbitol and ½ MS with 500 mM sorbitol media to observe the survival of seeds in these conditions. The root formation and shoot development of synthetic seeds (Plate 9) were observed and the maximum longevity possible was recorded.

Synthetic seed stored in different media showed significant difference in root regeneration (Table 11) during the storage. In 500 mM sorbitol supplemented media, root regeneration of synthetic seeds was inhibited completely. In all other media, root development started from 5th day and maximum root development was obtained within 20 days of storage. More than 85% of synthetic seeds stored in wet cotton, ½ MS, ¼ MS and 250 mM sorbitol in ½ MS had root development within 20 days of storage (Figure 6a). Synthetic seeds stored in dry cotton and 500 mM sorbitol supplemented media did not have significant root development during the storage days. Highest root development was obtained in ¼ MS and wet cotton (96.3% and 92.4%). There was no significant difference in root development of synthetic seed at the end of 20 days in any of the different media tried except 500 mM sorbitol.

Synthetic seeds stored in different media exhibited significant difference in longevity. The shoot formation was found to be different among the various media of storage (Table 11). Dry cotton and 250 mM and 500 mM sorbitol containing media completely checked the shoot formation of synthetic seed. The shoot development of synthetic seeds stored in wet cotton started from 16th day and 70.8% of cultures produced shoot within 40 days (Figure 6b). By the end of 40 days of storage in ½ MS and ¼ MS media, 57.4% and 38.9% of cultures had shoot regeneration, respectively. It was seen that the normal shoot development could be delayed upto 40 days by encapsulation.

Table 11. Effect of different storage media on *in vitro* germination of cocoa synthetic seeds

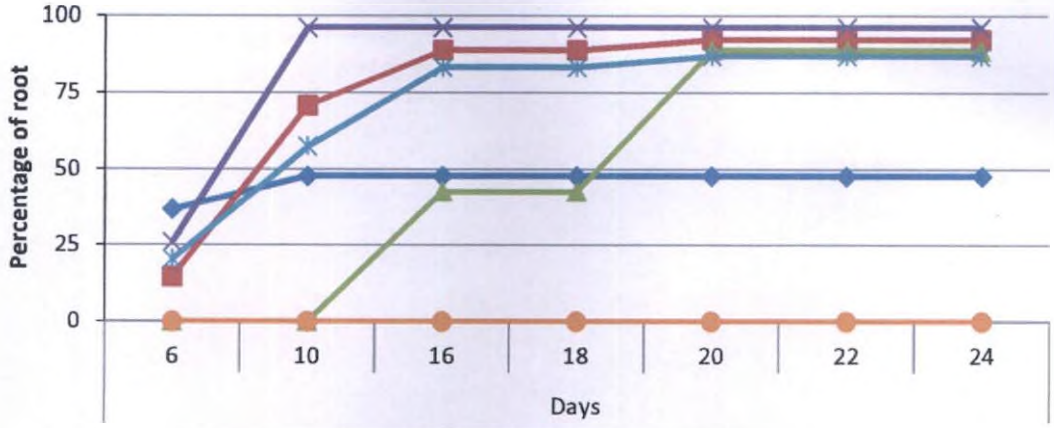
Synthetic Seed stored in	Days after culture										
	Root development (%)					Shoot development (%)					
	05	10	15	20	25	15	20	25	30	35	40
Dry Cotton	36.7^{b} (21.5)	47.8^{ab} (28.5)	47.8^{ab} (28.5)	47.8^{ab} (28.5)	47.8^{ab} (28.5)	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
Wet cotton	14.6^{ab} (8.3)	70.8^{bc} (45.0)	88.9^{b} (62.7)	92.4^{b} (67.4)	92.4^{b} (67.4)	0.0	9.7 (5.5)	39.6^{b} (23.3)	64.6^{b} (40.2)	70.1^{c} (44.4)	70.8^{c} (45.0)
½ MS	0.0^{a}	0.0^{a}	42.6^{ab} (25.2)	88.9^{b} (62.7)	88.9^{b} (62.7)	0.0	0.0	0.0^{a}	0.0^{a}	29.6^{ab} (17.2)	57.4^{b} (35.0)
¼ MS	25.9^{ab} (15.0)	96.3^{c} (74.3)	96.3^{b} (74.3)	96.3^{b} (74.3)	96.3^{b} (74.3)	0.0	0.0	18.5^{a} (10.6)	20.4^{a} (11.7)	38.9^{b} (22.8)	38.9^{b} (22.8)
½ MS + 250 mM Sorbitol	20.4^{ab} (11.7)	57.4^{ab} (35.0)	83.3^{b} (56.3)	87.0^{b} (60.4)	87.0^{b} (60.4)	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
½ MS+ 500 mM sorbitol	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
SEm±	2.9*	6.7*	7.2*	7.3*	7.3*	0.0^{ns}	0.9^{ns}	2.5*	4.1*	4.2*	4.3*

* Significant at 0.05 levels; ns- non significant at 0.05 levels
(Figures with same superscript in a column do not differ significantly)
(Values in the parentheses are Arcsine transformed values)

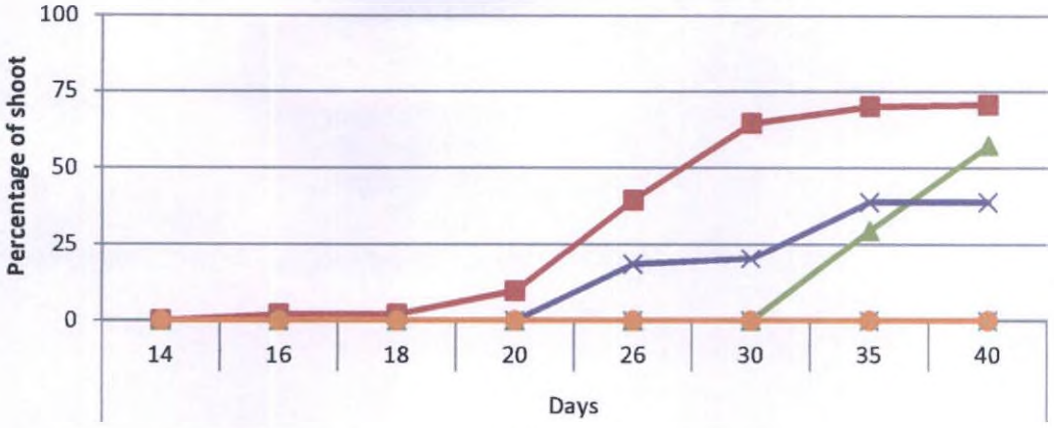
Table 12. Effect of different storage media on death of cocoa synthetic seeds

Synthetic Seed stored in	Percentage of dead synthetic seeds											
	Days after culture											
	10	15	20	25	30	35	40	45	50	55	60	70
Dry Cotton	11.1^{b} (6.3)	100.0^{c} (89.9)	100.0^{b} (89.9)	100.0^{b} (89.9)	100.0^{b} (89.9)	100.0^{b} (89.9)	100.0^{b} (89.9)	100.0^{b} (89.9)	100.0^{b} (89.9)	100.0^{b} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)
Wet Cotton	2.1^{ab} (1.2)	10.4^{b} (5.9)	17.4^{a} (10.0)	22.2^{a} (12.8)	28.5^{a} (16.5)	29.2^{a} (16.9)	29.2^{a} (16.9)	29.2^{a} (16.9)	29.2^{a} (16.9)	29.2^{a} (16.9)	29.2^{a} (16.9)	29.2^{a} (16.9)
½ MS	0.0^{a}	0.0^{a}	8.3^{a} (4.7)	19.4^{a} (11.1)	25.0^{a} (14.4)	30.6^{a} (17.8)	31.9^{a} (18.5)	42.6^{a} (25.5)	42.6^{a} (25.5)	42.6^{a} (25.5)	42.6^{a} (25.5)	42.6^{a} (25.5)
¼ MS	3.7^{ab} (2.1)	3.7^{ab} (2.1)	3.7^{a} (2.1)	22.2^{a} (12.8)	25.9^{a} (15.0)	33.3^{a} (19.4)	33.3^{a} (19.4)	33.3^{a} (19.4)	61.1^{ab} (37.6)	61.1^{ab} (37.6)	61.1^{ab} (37.6)	61.1^{ab} (37.6)
½ MS +250 mM Sorbitol	0.0^{a}	11.1^{b} (6.3)	11.1^{a} (6.3)	11.1^{a} (6.3)	22.2^{a} (12.8)	38.9^{a} (22.8)	38.9^{a} (22.8)	38.9^{a} (22.8)	50.0^{ab} (29.9)	50.0^{ab} (29.9)	77.8^{b} (51.0)	100.0^{c} (89.9)
½ MS+ 500 mM Sorbitol	0.0^{a}	5.6^{ab} (3.2)	11.1^{a} (6.3)	16.7^{a} (9.6)	16.7^{a} (9.6)	16.7^{a} (9.6)	33.3^{a} (19.4)	33.3^{a} (19.4)	50.0^{ab} (29.9)	55.6^{ab} (33.7)	100.0^{c} (89.9)	100.0^{c} (89.9)
SEm±	0.8*	6.8*	6.5*	6.2*	5.9*	5.8*	5.8*	5.8*	6.2*	6.4*	6.5*	6.5*

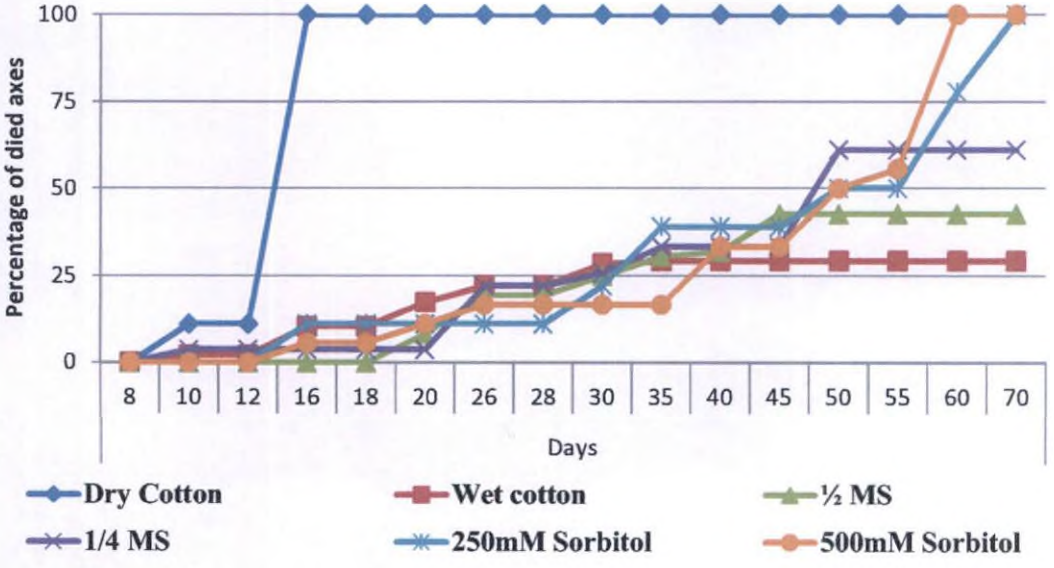
* Significant at 0.05 levels; ns- non significant at 0.05 levels
(Figures with same superscript in a column do not differ significantly)
(Values in the parentheses are Arcsine transformed values)



(a)



(b)



(c)

Fig. 6. Effect of different storage media on (a) root growth, (b) shoot growth and (c) death of cocoa synthetic seeds

The mortality of synthetic seeds varied significantly between the different media of storage. Synthetic seeds stored in dry cotton were found necrotic within 16 days of storage (Table 12). Low moisture content in dry cotton lead to drying of synthetic seed and death of embryonic axes. Synthetic seeds stored in 250 mM and 500 mM sorbitol containing media were viable up to 70 days and 60 days, respectively. In media having 250 mM and 500 mM sorbitol, growth was arrested upto 55 days and death rate was noticed after this period. In the above treatments after 70 days of storage all the synthetic seeds, which did not form shoot were found dead. The synthetic seeds stored in ½ MS, ¼ MS and wet cotton completed shoot regeneration within 35 days of storage.

The synthetic seeds stored in dry cotton lost their viability within 15 days. Dry cotton media had low moisture content which affected the longevity and resulted in complete death of synthetic seeds. Synthetic seeds stored in wet cotton condition had high germination. Maximum germination was obtained within 32 days and all synthetic seeds developed shoot in these period. Storage of synthetic seeds in ½ MS and ¼ MS was limited due to the complete germination of seeds. Germination capacity of synthetic seeds reached in 40 and 36 days in ½ MS and ¼ MS respectively. However, synthetic seeds stored in 250 mM sorbitol supplemented media were able to survive upto 70 days. In 500 mM sorbitol added media, synthetic seeds were able to maintain its viability upto 58 days.

4.5. Effect of germination inhibitor in encapsulation media on *in vitro* germination and storage of synthetic seed

To investigate the influence of inhibitor in the seed coat, the synthetic seeds were coated with culture media either alone or in combination with inhibitor/osmoticum. The embryonic axes were made into synthetic seeds by giving calcium alginate coat. The effective concentration of inhibitor or osmoticum, identified from the embryonic axes stored in inhibitor or osmoticum experiments was applied in the artificial seed coat. The synthetic seeds coated with basal media with inhibitors or osmoticum were stored in wet cotton to observe their regeneration potential during storage. The influence of artificial seed coat on growth of the synthetic seed were studied by observing the root and shoot development in storage. The number of cultures producing root and shoots are given in table 13.

Table 13. Effect of inhibitors in encapsulation on *in vitro* germination of cocoa synthetic seeds

Synthetic seed coat with	Days after culture															
	Root development				Shoot development											
	5	10	15	20	10	15	20	25	30	35	40	45	50	55	60	70
MS	64.8^{b} (40.3)	87.0^{b} (60.4)	92.6^{b} (67.7)	95.6^{b} (72.9)	0.0^{a}	40.7^{ab} (24.0)	75.9^{c} (49.3)	85.2^{d} (58.4)	85.2^{d} (58.4)	85.2^{c} (58.4)	85.2^{c} (58.4)	85.2^{d} (58.4)	85.2^{d} (58.4)	85.2^{d} (58.4)	85.2^{d} (58.4)	85.2^{c} (58.4)
½ MS	75.9^{b} (49.3)	79.6^{ab} (52.7)	79.6^{a} (52.7)	83.6^{a} (56.7)	14.8^{b} (8.5)	40.7^{b} (24.0)	64.8^{d} (40.37)	68.5^{bc} (43.2)	68.5^{c} (43.2)	68.5^{b} (43.2)	68.5^{b} (43.2)	68.5^{c} (43.2)	68.5^{c} (43.2)	68.5^{cd} (43.2)	68.5^{bcd} (43.2)	68.5^{bc} (43.2)
MS +250 mM Sorbitol	66.7^{b} (41.8)	100.0^{c} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)	0.0^{a}	0.0^{a}	48.1^{c} (28.74)	70.4^{bc} (44.7)	70.4^{c} (44.7)	70.4^{b} (44.7)	70.4^{b} (44.7)	70.4^{c} (44.7)	70.4^{c} (44.7)	70.4^{c} (44.7)	70.4^{bcd} (44.7)	70.4^{bc} (44.7)
½ MS +250 mM Sorbitol	77.8^{b} (51.0)	100.0^{c} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)	0.0^{a}	51.8^{b} (31.1)	79.6^{c} (52.7)	79.6^{cd} (52.7)	79.6^{cd} (52.7)	79.6^{c} (52.7)	79.6^{c} (52.7)	79.6^{d} (52.7)	79.6^{d} (52.7)	79.6^{d} (52.7)	79.6^{cd} (52.7)	79.6^{bc} (52.7)
MS +500 mM Sorbitol	27.8^{a} (16.1)	94.4^{c} (70.7)	94.4^{b} (70.7)	94.4^{b} (70.7)	0.0^{a}	0.0^{a}	35.2^{b} (20.6)	68.5^{bc} (43.2)	68.5^{c} (43.2)	68.5^{b} (43.2)	68.5^{b} (43.2)	68.5^{c} (43.2)	68.5^{c} (43.2)	68.5^{cd} (43.2)	68.5^{bcd} (43.2)	68.5^{bc} (43.2)
½ MS 500mM Sorbitol	88.9^{c} (62.7)	100.0^{c} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)	0.0^{a}	0.0^{a}	0.0^{a}	44.4^{b} (26.3)	63.0^{c} (39.0)	68.5^{b} (43.2)	68.5^{b} (43.2)	68.5^{c} (43.2)	68.5^{c} (43.2)	68.5^{cd} (43.2)	68.5^{bcd} (43.2)	68.5^{bc} (43.2)
250 mM Sorbitol	39.0^{a} (22.9)	64.0^{a} (39.7)	100.0^{c} (89.9)	100.0^{c} (89.9)	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	21.0^{ab} (12.1)	40.3^{b} (23.7)	54.3^{ab} (32.8)	63.8^{b} (39.6)
500 mM Sorbitol	31.8^{a} (18.5)	65.5^{a} (40.9)	100.0^{c} (89.9)	100.0^{c} (89.9)	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	2.8^{a} (1.6)	25.0^{b} (14.4)	33.3^{b} (19.4)	48.5^{bc} (29.0)	62.5^{bc} (38.6)	73.8^{bc} (47.5)
10^{-3}M ABA	34.5^{a} (20.1)	79.0^{ab} (52.1)	100.0^{c} (89.9)	100.0^{c} (89.9)	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	3.0^{a} (1.7)	4.5^{a} (2.5)	15.5^{a} (8.9)	33.0^{a} (19.4)	35.8^{a} (20.9)
SEm±	3.5*	3.9*	2.5*	2.5*	0.5*	2.4*	3.9*	4.4*	4.5*	4.5*	4.5*	4.1*	3.7*	3.1*	2.5*	2.3*

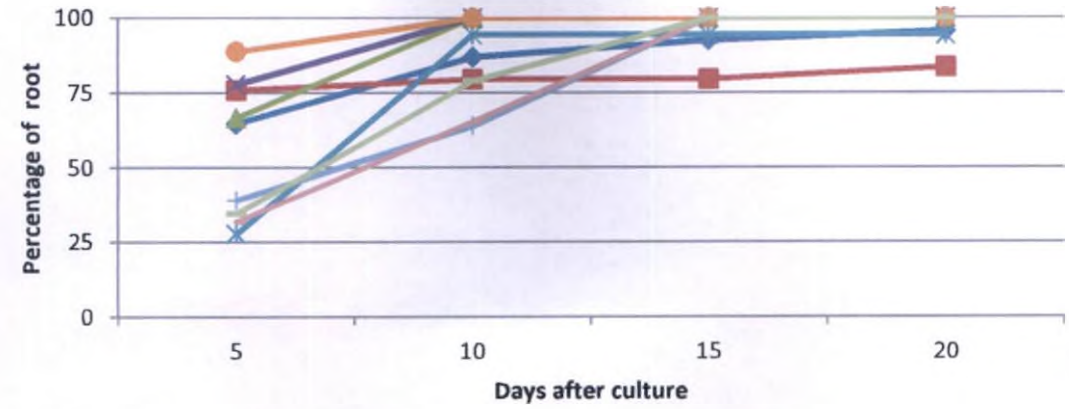
* Significant at 0.05 levels; ns- non significant at 0.05 levels (Figures with same superscript in a column do not differ significantly)
(Values in the parentheses are Arcsine transformed values)

The synthetic seeds coated with 500 mM sorbitol, 250 mM sorbitol and 10^{-3}M ABA had less rooting in the initial days. After 5 days of storage, only less than 40 percent of culture had root in synthetic seed coated with inhibitor or osmoticum. In the case of synthetic seeds coated with MS and ½ MS had root in more than 60 percent of culture (Figure 7a). However, the influence of artificial coat was only in initial phase and after 20 days, synthetic seeds in all treatments had more than 80 percent root formation.

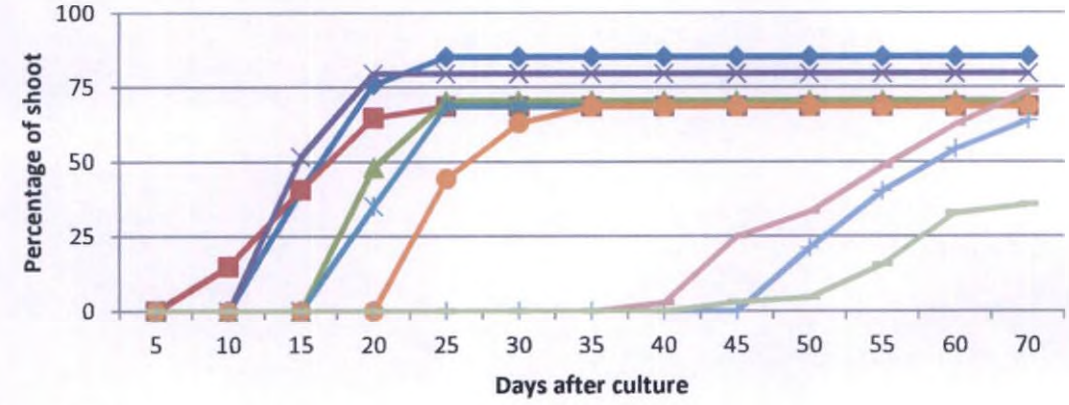
The presence of different chemicals in seed coat influenced shoot formation of synthetic seeds (Table 13). Synthetic seeds which had either MS or ½ MS alone in seed coat media showed early shoot formation during the storage. Shoot development was delayed by 40 days in synthetic seeds which had 250 mM sorbitol, 500 mM sorbitol and 10^{-3}M ABA in seed coat. The presence of basal media in the seed coat had accelerated the growth of synthetic seeds and produced high rates of shoot within short duration (25 to 30 days). Shoot formation was delayed in ½ MS+ 500 mM sorbitol coated synthetic seeds (Figure 7b).

The synthetic seeds coated with inhibitor or osmoticum had inhibiting influence on the shoot formation. In seed coat with 10^{-3}M ABA, the shoot formation was checked upto 45 days. A small percentage of seeds had formed shoot from 45 days and only 35 percent of seeds had shoot by the end of 70 days. Shoot formation in synthetic seed with 250 mM sorbitol in coat was delayed upto 50 days. After 50 days shoot formation started and 63.8 percent of culture had shoot by the end of 70 days. Similar shoot formation pattern was seen in 500 mM sorbitol coated seeds. The shoot formation started from 40 days and 73 percent of seeds had shoot after 70 days.

Synthetic seeds coated with MS and ½ MS alone did not prevent the growth of embryonic axes. In these seeds, shoot formation started from 10^{th} day of storage. The presence of MS media in artificial seed coat have influenced the growth positively. The shoot formation was completed within 30 days with a higher shoot regeneration percent(>65%), in synthetic seeds containing MS and ½ MS in arficial seed coat. In synthetic seed coated with MS levels (MS and ½ MS) and osmoticum (250 mM and 500 mM sorbitol), the osmotic effect was nullified by the MS media concentrations. The germination inhibition action obtained in embryonic axes were not seen in this condition.



(a)



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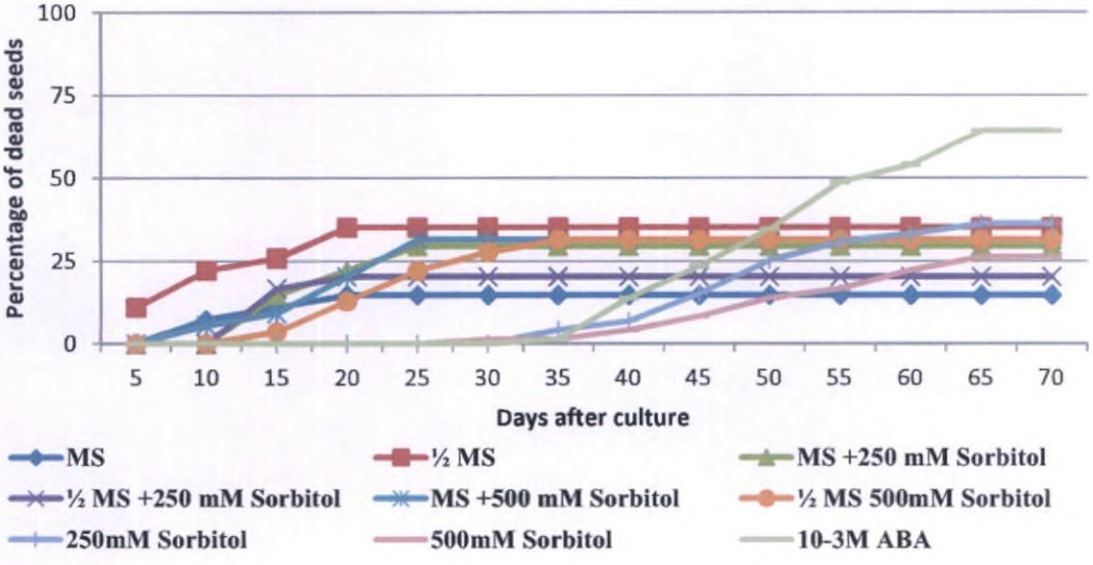

(c)

Fig. 7. Effect of inhibitors in encapsulation on (a) root growth, (b) shoot growth and (c) death of cocoa synthetic seeds

Table 14. Effect of inhibitor in encapsulation on death of cocoa synthetic seeds

Synthetic Seed Coat with	Days after culture													
	5	10	15	20	25	30	35	40	45	50	55	60	65	70
MS	0.0^{a}	7.4^{a} (4.2)	11.1^{ab} (6.3)	14.8^{b} (8.5)	14.8^{ab} (8.5)	14.8^{ab} (8.5)	14.8^{ab} (8.5)	14.8^{ab} (8.5)	14.8^{b} (8.5)	14.8^{a} (8.5)	14.8^{a} (8.5)	14.8^{a} (8.5)	14.8^{a} (8.5)	14.8^{a} (8.5)
½ MS	11.1^{b} (6.3)	22.2^{b} (12.8)	25.9^{c} (15.0)	35.2^{c} (20.6)	35.2^{c} (20.6)	35.2^{c} (20.6)	35.2^{c} (20.6)	35.2^{c} (20.6)	35.2^{b} (20.6)	35.2^{a} (20.6)	35.2^{ab} (20.6)	35.2^{ab} (20.6)	35.2^{a} (20.6)	35.2^{a} (20.6)
MS +250 mM Sorbitol	0.0^{a}	0.0^{a}	14.8^{abc} (8.5)	22.2^{b} (12.8)	29.6^{bc} (17.2)	29.6^{bc} (17.2)	29.6^{bc} (17.2)	29.6^{bc} (17.2)	29.6^{ab} (17.2)	29.6^{a} (17.2)	29.6^{ab} (17.2)	29.6^{a} (17.2)	29.6^{a} (17.21	29.6^{a} (17.2)
½ MS +250 mM Sorbitol	0.0^{a}	0.0^{a}	16.7^{bc} (9.6)	20.4^{b} (11.7)	20.4^{bc} (11.7)	20.4^{bc} (11.7)	20.4^{bc} (11.7)	20.4^{abc} (11.7)	20.4^{ab} (11.7)	20.4^{a} (11.7)	20.4^{a} (11.7)	20.4^{a} (11.7)	20.4^{a} (11.7)	20.4^{a} (11.7)
MS +500 mM Sorbitol	0.0^{a}	5.6^{a} (3.2)	9.3^{ab} (5.3)	20.4^{b} (11.7)	31.5^{c} (18.3)	31.5^{c} (18.3)	31.5^{c} (18.3)	31.5^{bc} (18.3)	31.5^{b} (18.3)	31.5^{a} (18.3)	31.5^{ab} (18.3)	31.5^{ab} (18.3)	31.5^{a} (18.3)	31.5^{a} (18.3)
½ MS 500mM Sorbitol	0.0^{a}	0.0^{a}	3.7^{ab} (2.1)	13.0^{b} (7.4)	22.2^{bc} (12.8)	27.8^{bc} (16.1)	31.5^{bc} (18.3)	31.5^{bc} (18.3)	31.5^{b} (18.3)	31.5^{a} (18.3)	31.5^{ab} (18.3)	31.5^{ab} (18.3)	31.5^{a} (18.3)	31.5^{a} (18.3)
250 mM Sorbitol	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	4.3^{a} (2.4)	7.0^{a} (4.0)	15.3^{ab} (8.7)	25.3^{a} (14.6)	30.8^{ab} (17.9)	33.3^{ab} (19.4)	36.3^{a} (21.2)	36.3^{a} (21.2)
500 mM Sorbitol	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	1.5^{a} (0.8)	1.5^{a} (0.8)	4.3^{a} (2.4)	8.5^{a} (4.8)	14.0^{a} (8.0)	16.8^{a} (9.6)	22.3^{a} (12.8)	26.3^{a} (15.2)	26.3^{a} (15.2)
10^{-3}M ABA	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	1.5^{a} (0.8)	14.0^{ab} (8.0)	23.5^{ab} (13.5)	34.8^{a} (20.3)	48.8^{b} (29.2)	54.0^{b} (32.6)	64.3^{b} (40.0)	64.3^{b} (40.0)
SEm±	0.5*	0.8*	1.1*	1.4*	1.6*	1.7*	1.6*	1.6*	1.4*	1.5*	1.7*	1.8*	2.0*	2.0*

* Significant at 0.05 levels; ns- non significant at 0.05 levels (Figures with same superscript in a column do not differ significantly)
(Values in the parentheses are Arcsine transformed values)

The different seed coating with different media and inhibitor combinations did not influence the mortality of synthetic seeds (Table 14). The longevity of synthetic seeds coated with MS and ½ MS alone lasted only upto 20 days. Synthetic seeds coated with MS media + 250 mM sorbitol, had slightly higher longevity than MS media coated alone. Enhanced longevity was observed in synthetic seeds, which are coated with inhibitor alone. Growth of synthetic seeds coated with 250 mM sorbitol, 500 mM sorbitol and 10^{-3}M ABA was inhibited. The inhibitor action increased the longevity upto 70 days (Figure 7c).

The synthetic seeds coated with different media and inhibitors had influence on the growth of the synthetic seed. The time taken for maximum germination was less in seed coated with MS and ½ MS. There was significant delay in germination due to presence of sorbitol and ABA alone in the seed coat. The maximum germination was delayed upto 60 days in these cultures (Table 13). In addition, longevity of seeds was higher in 250 mM sorbitol, 500 mM sorbitol and 10^{-3}M ABA coated seeds. They were viable upto 65 days. It was seen that seeds could be stored upto 70 days through coating the seeds with above chemical concentrations. The germination was also arrested in these seeds upto 60 days.

4.6. Effect of storage media on longevity of embryonic axis and synthetic seed

To investigate about the storage response of embryonic axes and synthetic seed in different storage condition an experiment was done with propagule (embryonic axis and synthetic seed) and different media as two factors. Data on embryonic axis and synthetic seed stored in different media were analyzed for their longevity during storage. The days upto which propagules were viable without shoot formation were counted and analyzed in this experiment. The difference in longevity of the embryonic axis and synthetic seed and influence of medium on longevity of propagule were analyzed and the results are given in table 15.

It was found that, there was a significant difference between embryonic axis and synthetic seed in longevity. Synthetic seeds were viable for longer time than excised embryonic axes. The embryonic axes had maximum longevity of 29 days while

synthetic seeds survived upto 41 days. The conversion of embryonic axes to synthetic seeds increased longevity of embryo.

Table 15. Effect of different storage media on longevity of embryonic axis and synthetic seeds of cocoa

Media	Longevity (days)			
	Embryonic axes	Synthetic seed	Mean	SEm±
Dry cotton	8.0^{h}	15.0^{g}	12.3^{o}	1.4*
Wet cotton	16.0^{fg}	32.5^{e}	28.0^{m}	1.3*
½ MS	15.2^{g}	40.0^{d}	22.0^{n}	3.4*
¼ MS	18.2^{fg}	36.6^{d}	23.8^{n}	3.0*
$^{1}/_{10}$ MS	20.0^{f}	31.6^{e}	23.1^{n}	2.2*
½ MS + 250 mM sorbitol	65.4^{b}	70.0^{a}	67.7^{l}	1.7*
½ MS + 500 mM sorbitol	60.8^{c}	64.3^{b}	62.5^{l}	1.4*
Mean	29.1^{y}	41.5^{x}		
SEm±	3.3*	3.1*		

(Figures with same superscript do not differ significantly)
* Significant at 0.05 levels; ns-non significant at 0.05 levels
Superscripts *x,y* are used for comparison of means of longevity of propagule , *l,m,n,o* are used for comparison of means of longevity in media. *a,b,c,d,e,f,g,h* are used for comparison of interaction. SEm± for interaction = 2.4

Longevity of propagule was also influenced by the media used for storage. Maximum longevity was observed in media supplemented with 250 mM sorbitol and 500 mM sorbitol. The propagules had longevity of 62 to 67 days in both media. The least longevity of 12 days observed for propagules stored in dry cotton.

Interaction of media and propagule had significant influence in the controlling longevity of seeds during storage. The lowest longevity of 8 days was seen in embryonic axis stored in dry cotton. The maximum longevity of 70 days was observed for synthetic seeds stored in media supplemented with 250 mM sorbitol (Figure 8).

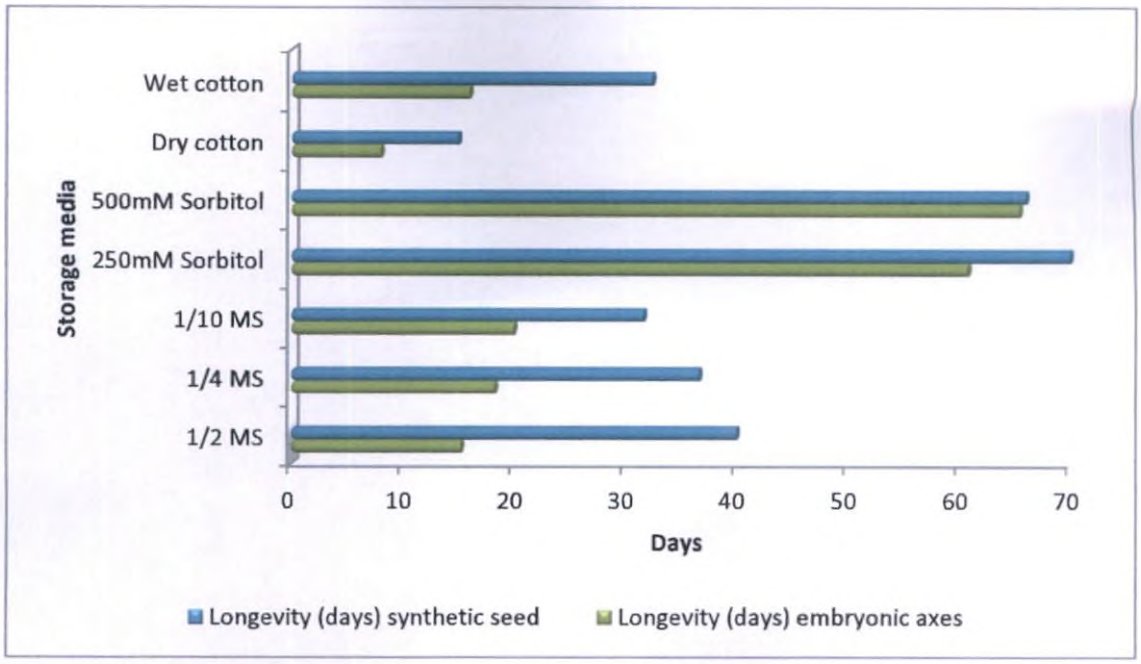

Fig. 8. Effect of different storage media on longevity of embryonic axis and synthetic seeds of cocoa

4.7. Effect of desiccation on *in vitro* germination and storage of synthetic seed

To study the influence of desiccation on synthetic seed, the seeds were subjected to different level of relative humidities (20%, 30% ,46.6%, 78.6%, 85.3% and 100%) for 3, 6, 9 and 12 hours and stored in wet cotton. The time taken for root regeneration and longevity were observed and given in Table 16.

Root regeneration was influenced by the level of desiccation and duration of desiccation treatment. Desiccation levels of 20, 30 and 46.6 percent RH, influenced root regeneration. On an average synthetic seeds took 10 days for completing root regeneration. However, under normal in vitro condition (100% RH) root formation was completed within 5 days of storage (Table 16). The duration of desiccation also influenced the root regeneration. Exposing the synthetic seeds for longer duration of desiccation (12 hours) influenced root emergence. Desiccation for 3 hours had the low influence on root growth of the synthetic seeds (Figure 9). Root regeneration was not influenced by the interaction of desiccation and duration. Longevity of synthetic seeds were influenced by desiccation treatments. Desiccation of 20% RH had more influence

on the synthetic seeds. Longevity of 54 days observed in synthetic seeds exposed to 20% RH. Desiccation levels of 46.6% RH, and 30% RH

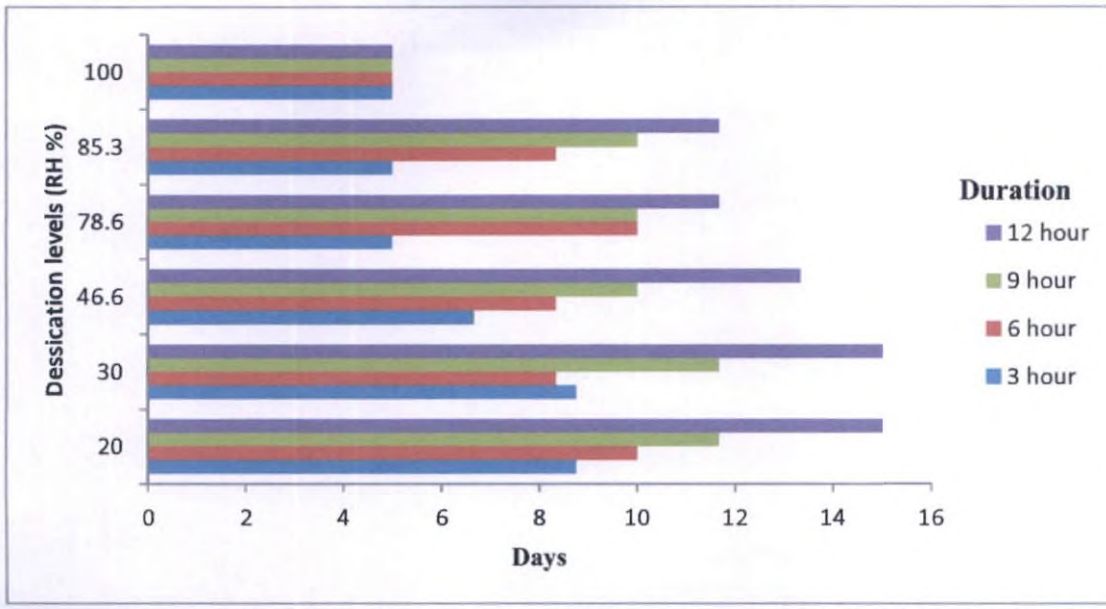

Fig.9. Effect of different levels and duration of desiccation on root emergence of cocoa synthetic seed

Table 16. Effect of different levels and duration of desiccation on days taken for root emergence of cocoa synthetic seeds

Desiccation RH (%)	Duration of desiccation (hour)				Mean	SEm±
	3	6	9	12		
20	8.75	10.00	11.67	15.00	11.35^{o}	0.83*
30	8.75	8.33	11.67	15.00	10.94^{no}	0.95*
46.6	6.67	8.33	10.00	13.33	9.58^{mno}	0.96*
78.6	5.00	10.00	10.00	11.67	9.17^{mn}	0.83*
85.3	5.00	8.33	10.00	11.67	8.75^{m}	0.89*
100	5.00	5.00	5.00	5.00	5.00^{l}	0.0^{ns}
Mean	6.53^{a}	8.33^{b}	9.72^{c}	11.94^{d}	9.20	
SEm±	0.54*	0.57*	0.63*	0.91*		

* Significant at 0.05 levels; ns-non significant at 0.05 levels.
Figures with same superscript do not differ significantly
Superscripts *a,b,c,d* are used for comparison of means of duration of desiccation treatments, *l,m,n,o* are used for comparison of means of desiccation levels. SEm± for interaction = 0.4

Table 17. Effect of different levels and duration of desiccation on longevity of cocoa synthetic seeds

Desiccation RH (%)	Duration of desiccation (hour)				Mean	SEm±
	3	6	9	12		
20	51.2^{ab}	55.0^{a}	55.0^{a}	55.0^{a}	54.1^{o}	0.60^{ns}
30	45.0^{de}	51.6^{ab}	51.6^{ab}	53.3^{ab}	50.4^{n}	1.13*
46.6	45.0^{de}	50.0^{bc}	50.0^{bc}	55.0^{a}	50.0^{n}	1.06*
78.6	45.0^{de}	46.6^{cd}	50.0^{bc}	51.6^{ab}	48.3^{n}	1.12*
85.3	45.0^{de}	45.0^{de}	45.0^{de}	50.0^{bc}	46.2^{m}	0.65*
100	41.6^{e}	41.6^{e}	41.6^{e}	43.3^{de}	42.0^{l}	0.74*
Mean	45.5^{x}	48.3^{y}	48.9^{y}	51.3^{z}	48.5	
SEm±	0.75*	1.14*	1.18*	1.05*		

(Figures with same superscript do not differ significantly) * Significant at 0.05 levels; ns-non significant at 0.05 levels. Superscripts *x,y,z* are used for comparison of means of duration of desiccation treatments, *l,m,n,o* are used for comparison of means of desiccation levels. *a,b,c,d,e* are used for comparison of interaction. SEm± for interaction = 0.55

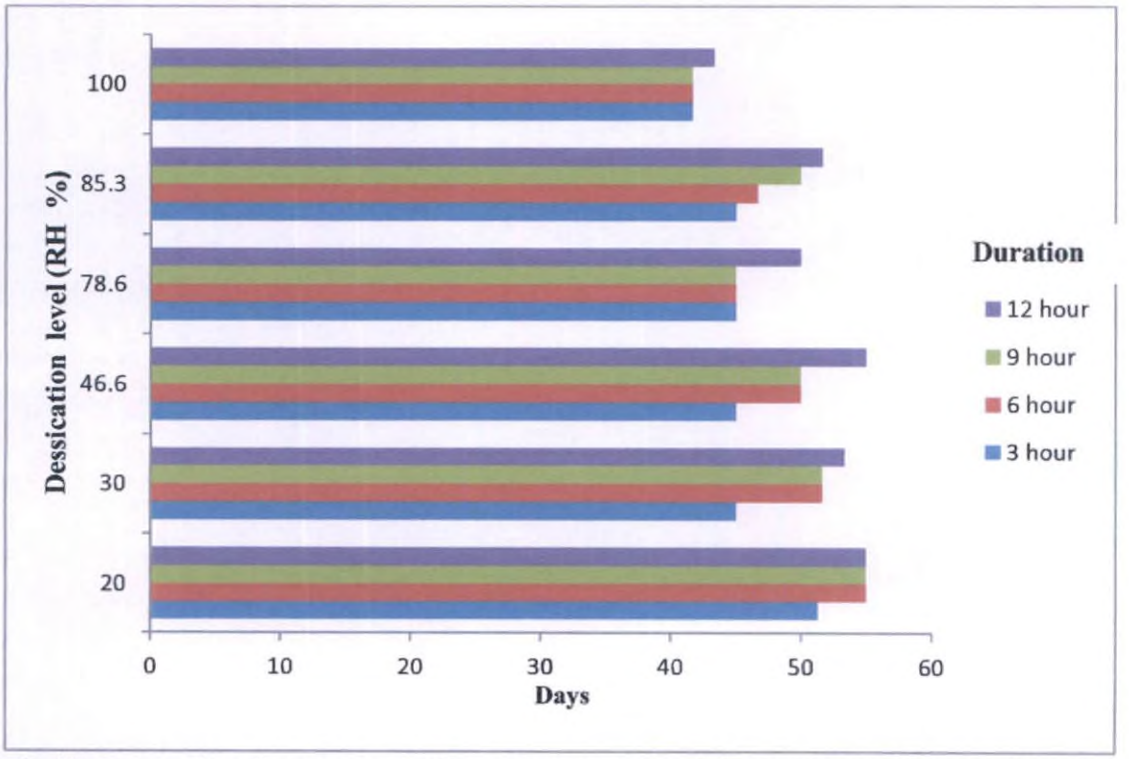

Fig.10. Longevity of cocoa synthetic seeds exposed to different levels of desiccation for different duration

also had enhanced longevity to more than 50 days (Table 17). Duration of desiccation treatment influenced the longevity of synthetic seeds. Longevity of synthetic seed was higher (51 days) in seeds exposed to 12 hour desiccation (Table 17). Desiccation and duration together influenced the synthetic seed longevity in certain cases. Synthetic seed exposed to desiccation of 20 percent for 6, 9 and 12 hours had shown higher longevity. Seeds stored in 100% RH had lower longevity (Figure 10).

4.8. Standardization of storage technique for synthetic seed

Based on all the above experimental results, it was observed that synthetic seeds had a better longevity during storage. Longevity of synthetic seed was more in media supplemented 250 mM sorbitol. The synthetic seeds stored in 250 mM sorbitol had longevity upto 70 days. The maximum possible storage time for synthetic seeds was assessed by storing in 250 mM sorbitol supplemented media. The seeds were transferred to wet cotton during 55, 60 and 65 days after culture. The days taken for shoot regeneration and percentage of seeds forming shoot were counted (Table 18).

Synthetic seeds stored in media supplemented with 250 mM sorbitol for 55 days before transferring to wet cotton showed 80 percent germination after 34 days of transfer. However, synthetic seed stored for longer duration of 60 and 65 days in 250 mM sorbitol supplemented media showed reduced and early germination on 27 and 25 days, respectively. So the maximum storage time possible for encapsulated embryo was with the media supplemented with 250 mM sorbitol for 55 days before transferring to wet cotton (89 days).

Table 18. Potential storage possible with synthetic seeds stored in ½ MS+250 mM sorbitol

Days of storage in ½ MS+250 mM sorbitol (A)	Days taken for germination in wet Cotton (B)	Percentage of culture which had shoot	Total storage days possible (A+B)
55	34	80	89
60	27	60	87
65	25	60	80

DISCUSSION

DISCUSSION

Theobroma cacao is one of the well-known cash crops grown throughout the humid tropics with about 9.5 million hectares spread over 57 countries (FAOSTAT, 2012). It is a major agroforestry crop in humid regions. Propagation of cocoa tree is difficult due to its recalcitrant seeds. The conventional nursery works are difficult for mass propagation of good quality seeds from the available polyclonal plantations. Moreover, lack of dormancy and enhanced vivipary create difficulties in spreading the cultivation of the crop to non-conventional areas. Attempts were made to increase the storage time through different methods. The synthetic seeds in cocoa has shown a higher longevity when compared to normal seeds (Sudhakara et al., 2000).

The present study investigates the possibility to further elongate the storage potential of synthetic seeds helped from zygotic embryo of cocoa seeds. The synthetic seeds were treated with the common germination inhibiting conditions during the storage. The effect of inhibiting conditions were first observed on the naked embryonic axes and the information were used for selecting appropriate storage conditions of synthetic seeds. The study was carried out in cocoa pods collected from cocoa polyclonal garden in the Kerala Agricultural University and the experiments were conducted at Plant Tissue Culture Laboratory, Department of Tree Physiology and Breeding, College of Forestry. The results of the study are discussed below, in the light of available literature and presented below.

5.1. Standardization of microencapsulation techniques

The present study revealed the influence of cotyledon in the shoot regeneration of cocoa embryo. Embryonic axes stored without cotyledon had root growth only. Shoot regeneration was not observed due to mortality in cultures. The embryonic axis was in the state of active division, so the root emergence happened within five days of storage. Root regeneration was not influenced by the cotyledon. The embryonic axes excised without cotyledon did not show shoot regeneration during the course of study. Embryonic axes with ½ cotyledon and ¼ cotyledon had higher percentage of shoot formation in culture media (Figure 11). The treatments had comparable death rate in cultures. The embryonic axes without cotyledon had a

higher death rate during the storage. The presence of cotyledon helped to increase the longevity and viability for long time.

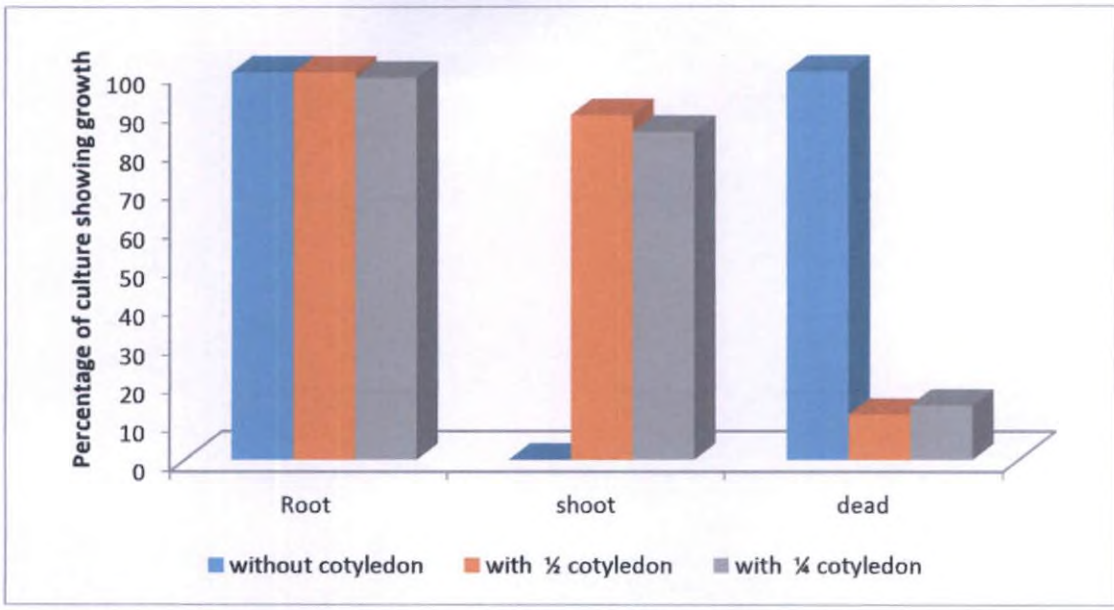

Fig. 11. Effect of different proportions of cotyledon on *in vitro* germination of cocoa embryonic axis (Basal media- ½ MS)

The shoot regeneration started two weeks after the root regeneration. The embryonic axes without cotyledon dried during this period. It was seen that the site of the attachment of cotyledon with the embryonic axis is the site of regeneration of shoot (Plate 10). The absence of cotyledon in that region influences the shoot regeneration. The attachment of cotyledon enabled embryonic axis to produce the first pair of cotyledonary leaves. The disturbance in that position of embryo adversely affected the shoot formation. Another reason could be that the removal of cotyledon physically damaged the meristem. The attachment of cotyledon influenced the viability of embryonic axes. The death rate of embryonic axes were found less in the case of embryonic axes attached with cotyledon.

In all storage conditions except low moisture condition (dry cotton), the embryonic axis showed a normal growth pattern. The investigation for the media for storage had shown that dry cotton storage was least efficient. The dry cotton lead to excessive drying of embryonic axis, which resulted in the death of embryonic axes (Sudhakara et al., 2000). Embryonic axes stored in other media like wet cotton, ½

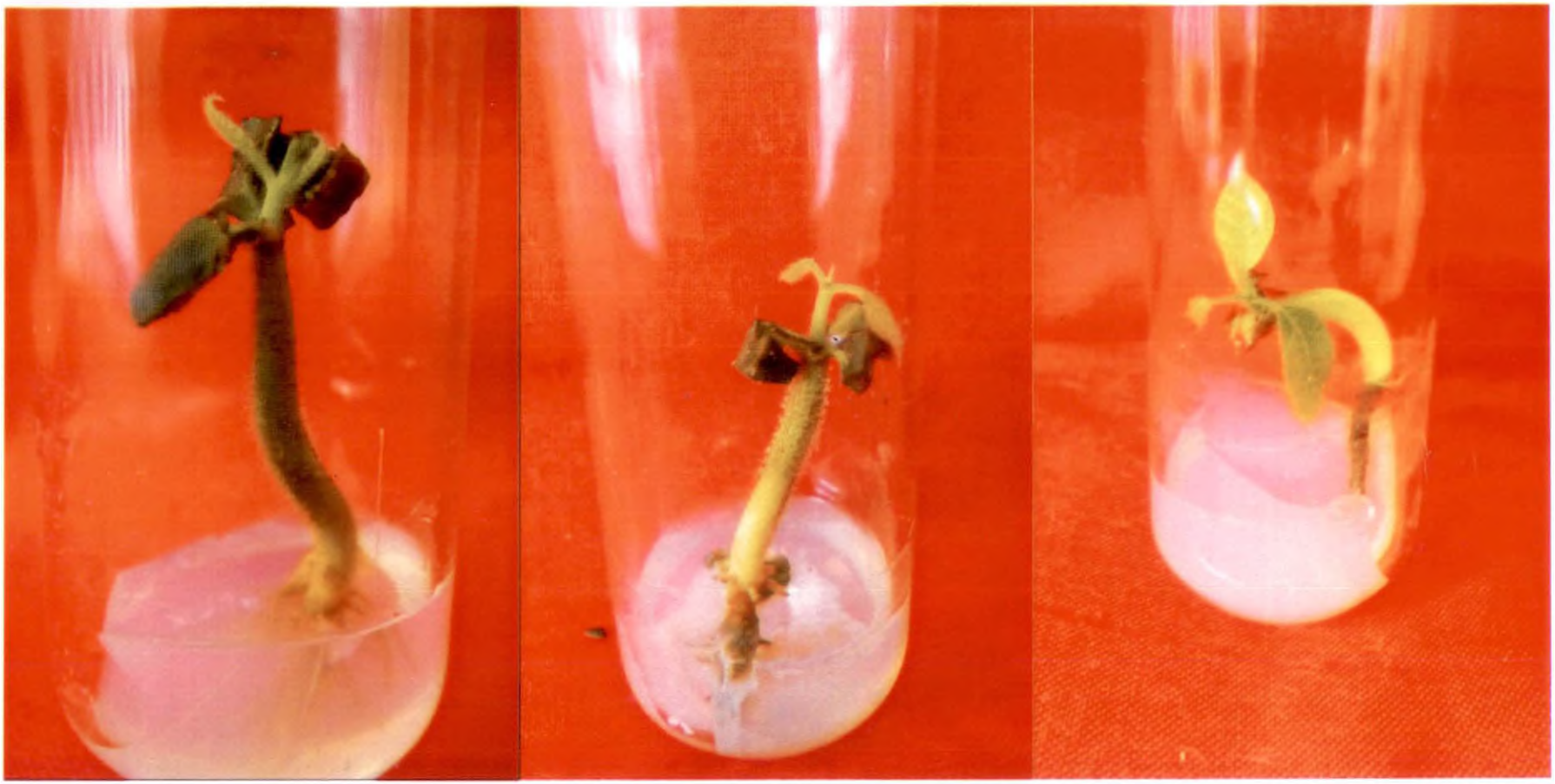

Plate 10. Cotyledon opening and leaf emergence of embryonic axes

MS, ¼ Ms and $^{1}/_{10}$ MS had high percentage of germination within 25 days of storage (Figure 12). It is already known that viability of recalcitrant seeds depend on the moisture content of the surrounding environment (Roberts, 1973; Baskin and Baskin, 1998). The presence of nutrient medium does not influence the viability and germination. Reduction of moisture in the surrounding environment adversely affected the longevity of recalcitrant seeds.

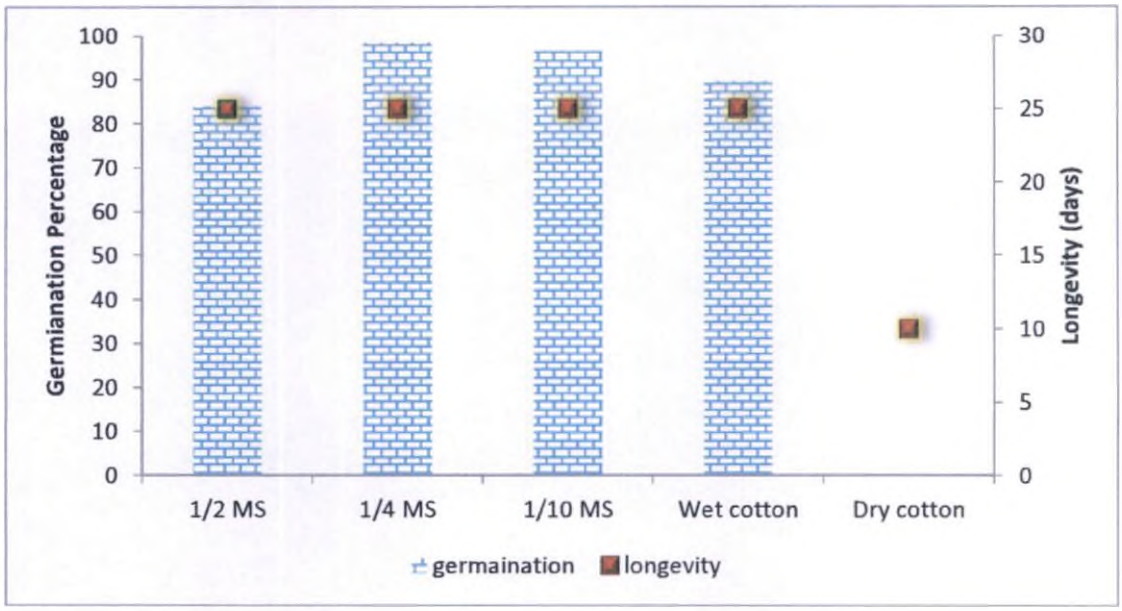

Fig. 12. Germination percentage and longevity of cocoa embryonic axes stored in different media

The embryonic axis with ¼ cotyledon was selected as the propagule and ½ MS medium as the basal media for the rest of the experiments. The minimum attachment of cotyledon ensured high germination. It also helped in easy making of synthetic seeds. Half MS medium added as a supporting medium for the growth of embryonic axes. Half MS had been identified as a basal medium for the storage of embryonic axes by Sudhakara et al. (2000) and Sunilkumar et al. (2000).

5.2 Effect of germination inhibitors in the media on regeneration of embryonic axis

To increase the longevity of embryonic axes, different germination inhibitors were added to the media. The ½ MS medium with the germination inhibitors had

influenced the growth of the embryonic axis. The presence of ABA in the media delayed root formation (Figure 13). ABA inhibited the germination by preventing embryonic growth. Da Silva et al. (2004) reported a transient rise in endogenous ABA content during germination in the embryonic cells, suggesting that ABA inhibits cell wall extensibility by not permitting an increase in cell turgor. ABA has a similar effect when applied directly on zygotic embryos. Present study indicated that root regeneration was influenced by the presence of exogenous ABA in higher (10^{-4}M) concentrations (Table 7a). Only 33 percent of cultures had root regeneration during storage. But embryonic axes stored in lower concentrations (10^{-5}M and 10^{-6}M) of ABA had high root regeneration (100%) and had similar growth pattern seen in embryonic axes stored in media without inhibitor.

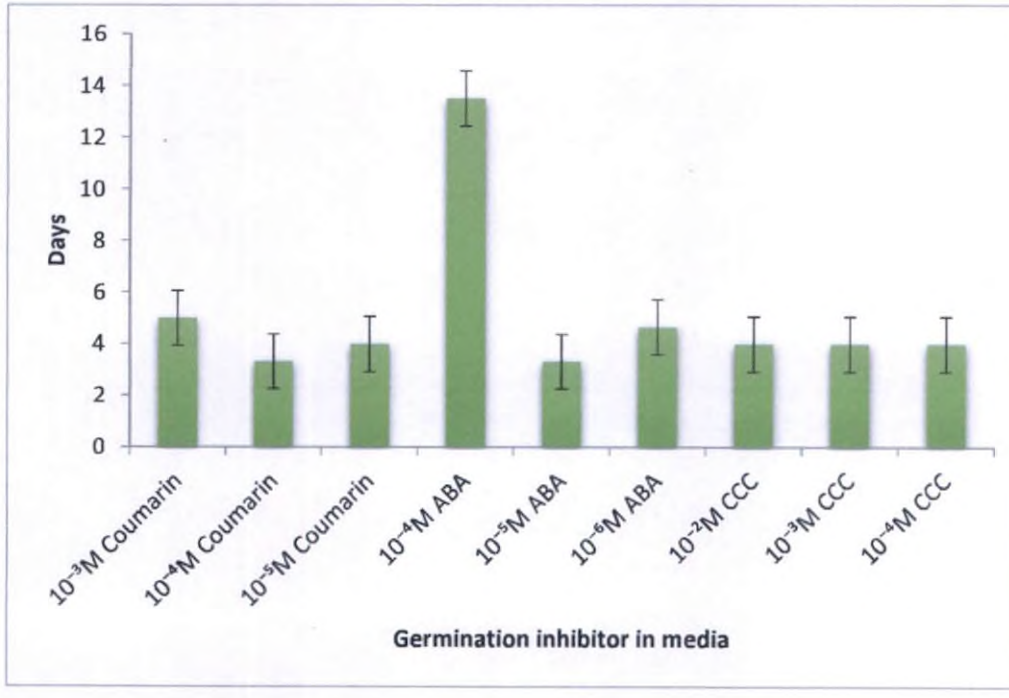

Fig. 13. Days taken for complete root regeneration of cocoa embryonic axes stored in different inhibitor added media (Basal media – ½ MS)

Longevity of embryonic axes showed variation with respect to different media. Media fortified with 10^{-4}M ABA completely inhibited the embryonic axes germination. The embryonic axes were viable up to 56 days (Table 19). The ABA in exogenous condition as in dormant seeds effects the growth of seeds. Embryonic ABA played a central role in induction and maintenance of seed dormancy and inhibited the transition from embryonic to germination growth (Rodríguez-Gacio et al., 2009). The presence of ABA in the medium had the same effect observed when it was present in the seed coat. The studies of ABA presence showed that during germination, ABA declined while GA_4 increased and higher ABA was found in un-germinated seeds when compared to germinated seeds (Zhang, 2008).

Table 19. Effect of germination inhibitors on longevity of cocoa embryonic axes (Basal media- ½ MS)

Inhibitors in Media	Longevity of seed (days)
10^{-3}M Coumarin	31.7^{b}
10^{-4}M Coumarin	22.7^{cb}
10^{-5}M Coumarin	22.7^{cb}
10^{-4}M ABA	56.0^{a}
10^{-5}M ABA	20.0^{c}
10^{-6}M ABA	18.7^{c}
10^{-2}M CCC	18.0^{c}
10^{-3}M CCC	16.0^{c}
10^{-4}M CCC	15.3^{c}
½ MS	16.0^{c}
SEm±	2.35*

(Figures with same superscript in a column do not differ significantly)
* Significant at 0.05 levels; ns- non significant at 0.05 levels

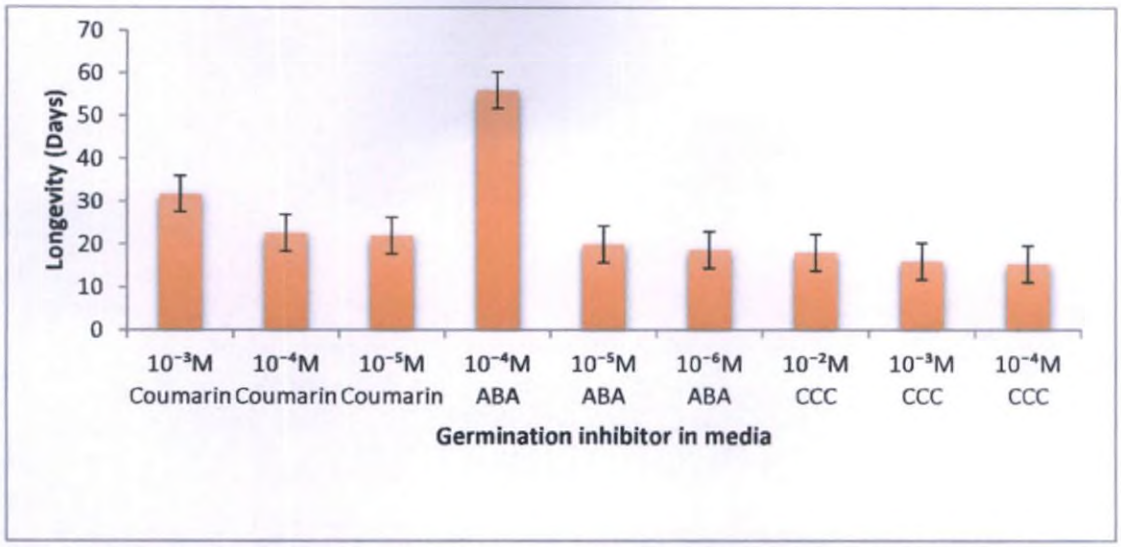

Fig. 14. Longevity of cocoa embryonic axes stored in germination inhibitor added media (Basal media – ½ MS)

ABA played an important role in maintaining longevity of embryonic axes and inducing dormancy (Figure 14). When the embryonic axes in ABA containing media were transferred to ½ MS media they germinated within a short time. It is known that increased ABA in plant cells inhibit DNA replication and cell division, which results in retarded plant growth (Finkelstein et al., 2002; Swiatek et al., 2002). In the present study, ABA in more than 10^{-4}M concentration only had influence on the growth of embryonic axes. ABA concentrations 10^{-5}M and 10^{-6}M were found to be ineffective to inhibit the growth of embryonic axes. So for the effective inhibiting action of ABA, concentrations more than 10^{-4}M should be added to the media. The presence of other inhibitors did not enhance longevity of embryonic axis in the experiment.

Higher concentration of coumarin showed an influence on the growth of the embryonic axis. The emerged root was malformed with bulging at the tip. The colour of bulged tip changed to brown during the storage (Plate 6). At higher concentration of coumarin the rate of germination was poor in the initial period of 25-30 days. Higher concentration of coumarin inhibiting seed germination was also observed in different species (Aliotta et al., 1992, 1994: Abenavoli et al., 2006). The

greatest inhibitory effect of 10^{-3} M coumarin was observed in seed germination and radicle elongation (Peal and Williams, 2002; Williams and Bartholomew, 2011). Coumarin is an inducer of coat-imposed dormancy through the inhibition of water uptake during seed imbibition (Aliotta et al. 1992, 1993). The inhibition of water uptake from the media probably resulted in the colour change at the tip of growth. The water intake inhibition could have resulted in the production of fibrous roots to the media. The fibrous root formation could have helped the embryonic axes to survive the inhibiting condition.

Even being a growth inhibitor, CCC did not influence the embryonic growth during storage. The higher concentration of CCC (10^{-2}M) did not show any inhibiting action on the embryonic axis. The regular growth pattern of embryonic axes seen in media without inhibitor was obtained in CCC supplemented media. Kumaran et al. (1994) reported that seedling growth in terms of root length, shoot length and number of leaves was enhanced by CCC at lower concentration for a shorter soaking duration and at higher concentration for a longer soaking period. Probably the concentration, attempted during the study was not sufficient to inhibit germination.

The combinations of inhibitors had shown the same effect on embryonic axes as when they were applied individually (Figure 15). Coumarin (10^{-3}M) influenced the growth of embryonic axes as in the condition when it was applied alone in the media. The result indicated that the inhibition of coumarin and ABA are independent of each other and there is no synergic effect to inhibit the growth in lower concentrations of both inhibitors (Table 20).

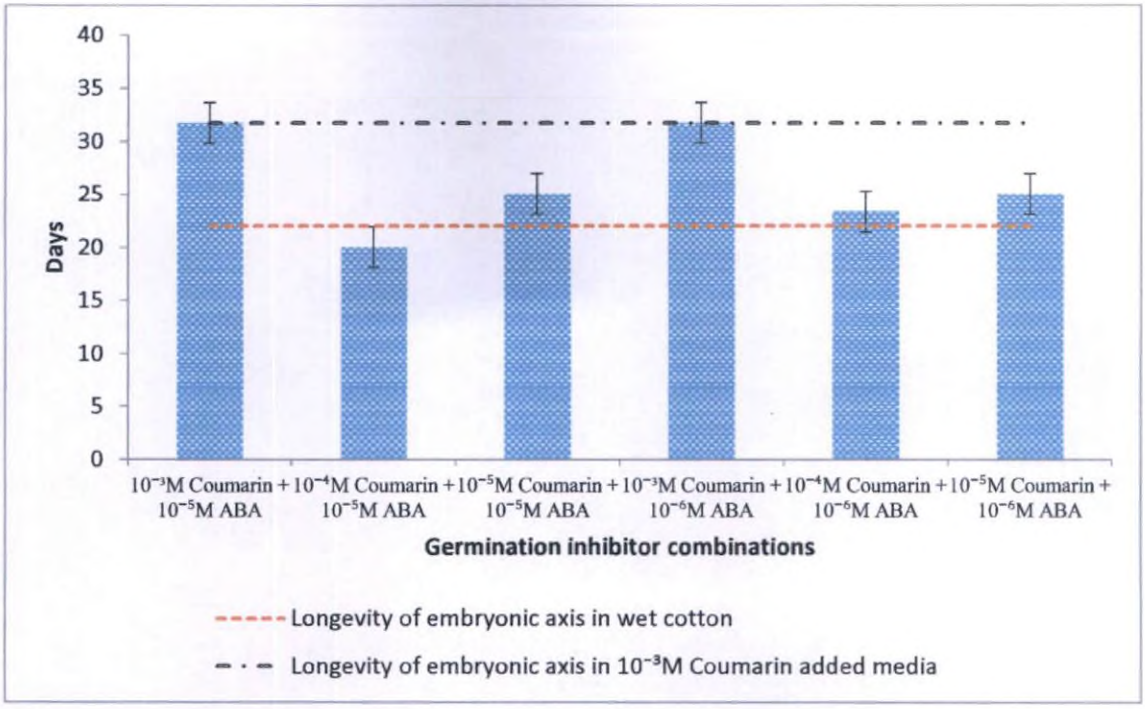

Fig. 15. Combined effect of germination inhibitors on longevity of cocoa embryonic axes (Basal media – ½ MS)

Table 20. Combined effect of germination inhibitors on longevity of cocoa embryonic axes (Basal media- ½ MS)

Inhibitor combination in Media	Longevity of seed (days)
10^{-3}M Coumarin+ 10^{-5}M ABA	31.7^{a}
10^{-4}M Coumarin+ 10^{-5}M ABA	20.0^{c}
10^{-5}M Coumarin + 10^{-5}M ABA	25.0^{bc}
10^{-3}M Coumarin+ 10^{-6}M ABA	31.7^{a}
10^{-4}M Coumarin+ 10^{-6}M ABA	23.3^{bc}
10^{-5}M Coumarin+ 10^{-6}M ABA	25.0^{bc}
10^{-3}M Coumarin	31.7^{a}
SEm±	1.16*

(Figures with same superscript in a column do not differ significantly)
* Significant at 0.05 levels; ns- non significant at 0.05 levels

5.3. Effect of osmotica in the media on the growth of embryonic axis

The germination of seeds starts with the imbibition of water. The osmoticum in the media changes the water potential in the media and it reduces the intake of water by the seed for its germination (Mehra et al., 2003). The osmotica added media had influenced the growth of embryonic axes stored in it in different ways.

Enhanced longevity was observed in sorbitol added media. The sorbitol in the media delayed the shoot regeneration. Death rates of embryonic axes were also less upto 70 days (Table 21). The sorbitol added media maintained its water potential without damaging the embryonic axes. The 250 mM sorbitol inhibited germination in an effective way. The embryonic axes were able to survive upto 70 days without affecting its viability.

Shoot regeneration in the 250 mM sorbitol supplemented media delayed to 60 days and it may be due to an effective control over the water imbibition of embryonic axes. The water potential of the media may be near threshold value for imbibition which effectively controlled the growth. The water availability was effectively checked in 250 mM sorbitol condition. In NaCl and mannitol added media the availability of water was completely checked by the osmotic concentration. But in 250 mM sorbitol condition the water potential may be in equilibrium with embryonic axes water potential, resulting in a reduced uptake of water without loss of viability of embryonic axes.

NaCl had the most deleterious effect on the embryonic axes. High concentration of NaCl (250 mM and 500 mM) only had longevity of 34 and 15 days respectively (Table 21). The concentration of NaCl had a negative relationship on longevity. The increasing NaCl concentration decreased longevity of embryonic axes. Root and shoot regeneration was negligible at all concentrations of NaCl. The NaCl presence in the media reduced the availability of water for absorption. Also it increased the salt concentrations, and resulted in salt stress on embryonic axes and prevented the growth and started dying. The higher mortality rate in higher concentrations (250 mM and 500 mM) of NaCl showed the non-availability of water

to the embryonic axes. The recalcitrant embryonic axes cannot survive without the presence of water content is evident from dry cotton condition. The 500 mM NaCl condition also influenced embryonic axes as in the case of dry cotton. The embryonic axes was found dead due to excessive drying in both cases. The embryonic axes colour changed from white to dark brown during the storage. Another reason could be the salt stress by the NaCl. Salt stress causes decline in seed germination, shoot and root lengths, fresh mass and seedling vigor (Misra et al., 1996; Promila and Kumar, 2000; Misra and Dwivedi, 2004). The higher concentration of NaCl caused severe salt stress condition to the embryonic axes and it limited further growth.

Table 21. Effect of osmotica on longevity of cocoa embryonic axes (Basal media- ½ MS)

Osmoticum added in Media	Longevity of seed (days)
100 mM Sorbitol	36.0^{b}
250 mM Sorbitol	65.4^{d}
500 mM Sorbitol	60.8^{d}
100 mM Mannitol	42.5^{bc}
250 mM Mannitol	43.0^{bc}
500 mM Mannitol	47.0^{c}
100 mM NaCl	48.0^{c}
250 mM NaCl	34.0^{b}
500 mM NaCl	15.3^{a}
½ MS	16.0^{a}
SEm±	2.69*

(Figures with same superscript in a column do not differ significantly)
* Significant at 0.05 levels; ns- non significant at 0.05 levels

Mannitol added media enhanced the longevity upto 42 days. But crystallization made the media dry and affected embryonic axes viability. The viability was lost rapidly after 30 days due to crystalisation in the media (Figure 16).

Mannitol in 250 mM and 500 mM concentrations had more deleterious effect. The death of embryonic axes was lower than dry cotton and NaCl condition.

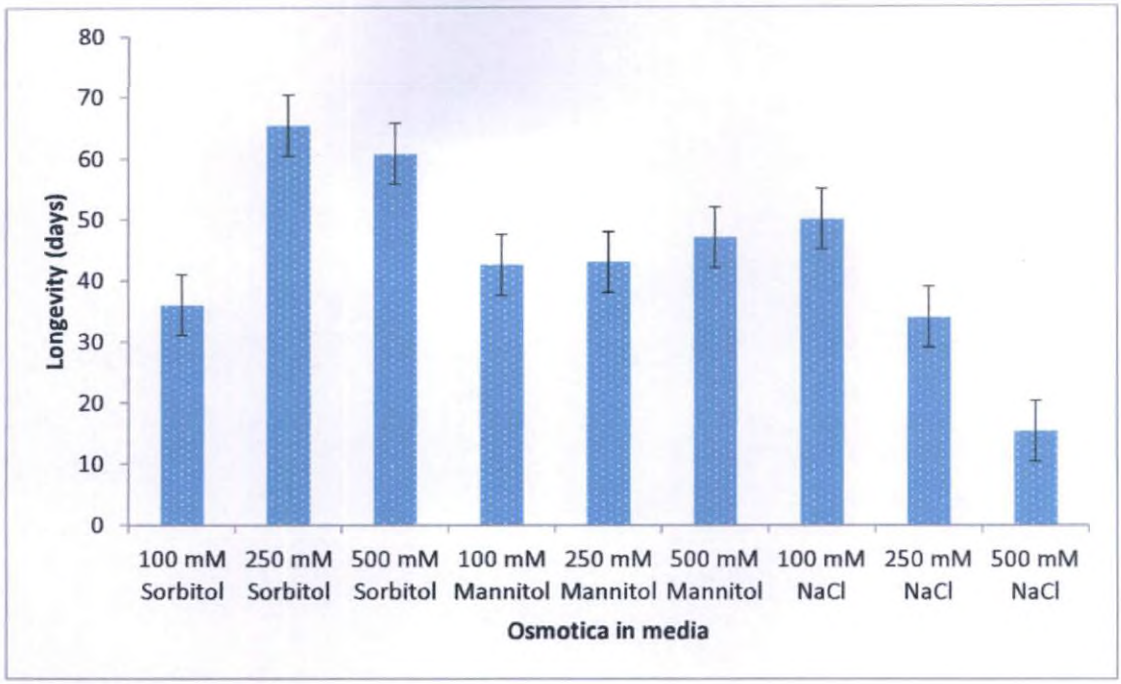

Fig. 16. Longevity of cocoa embryonic axes stored in osmotica added media (Basal media – ½ MS)

5.4. Synthetic seed storage

Recalcitrant seed storage becomes difficult due to two reasons mainly, one is, it loses viability in low moisture conditions, and other is absence of inhibiting conditions. The nature has provided rich cotyledon condition near the embryonic axes for continuous growth. It helps the seed to have continuous growth from the beginning. The recalcitrant nature is supposed to be due to seed coat and storage tissue (King and Robert, 1980). For effective storage the continuous growth of the seed embryo should be checked by artificial methods.

In the present study, longevity of synthetic seeds were found higher than embryonic axes stored in the same medium. A substantial increase in longevity from 29 days to 40 days was observed after encapsulation of embryonic axes (Table 15).

The continuous growth of embryonic axes was inhibited by the encapsulation procedure. From the present investigation, the conversion of embryo to synthetic seed is found to be one of the best methods to check the continuous growth. The reduced cotyledon presence and encapsulation had affected the growth of embryo. Embryonic axes were converted to synthetic seeds by removing major part of the cotyledon. The non-availability of required amount of nutrients from the cotyledon may have inhibited the fast growth of embryonic axes. Similarly, the root emergence and finding new sources for nutrients may have delayed the shoot formation.

The embryonic axes stored in dry cotton had longevity of eight days while synthetic seeds had 15 days. In wet cotton embryonic axes had 16 days and synthetic seeds had 32 days of longevity (Table 15). This increased longevity may be due to the high moisture holding capacity of encapsulation. The synthetic seeds stored in dry cotton had longevity equal to embryonic axes stored in wet cotton. Encapsulation had provided a higher moisture environment to the embryonic axes. So it is evident that moisture had an important role in longevity. The encapsulation provided a better moisture level near the embryonic axes as in wet cotton. So even if stored in dry cotton, the synthetic seeds were able to survive upto 16 days. The encapsulation also provides higher moisture content around the embryonic axes compared to naked embryonic axes and it was evident from the storage of embryonic axes and synthetic seed in dry cotton. The higher longevity was observed in the synthetic seed (Table 15). This was due to less drying rate near the embryonic axes provided by the encapsulation.

The higher percentage of root formation in synthetic seeds stored in dry cotton was due to the moisture in the artificial coat compared to embryonic axis (Figure 17). Synthetic seeds were able to produce roots due to this moisture availability. The lack of availability of moisture had increased mortality in embryonic axes stored in dry cotton. But in later stages of storage of synthetic seeds in dry cotton, the availability of moisture content was almost nil. This lead to increased mortality in the synthetic seeds after 10 days of storage in dry cotton condition. Generally the axes of the recalcitrant seeds have considerably high water content than the cotyledons (Berjak etal., 1989; Maithani etal., 1989; Fu et al., 1993).

It was well known in recalcitrant seeds that, if drying continues and reaches less than "critical moisture content" (King and Roberts, 1979, 1980) or "lowest safe moisture content" (Tompsett, 1984)., viability is eventually reduced to zero.

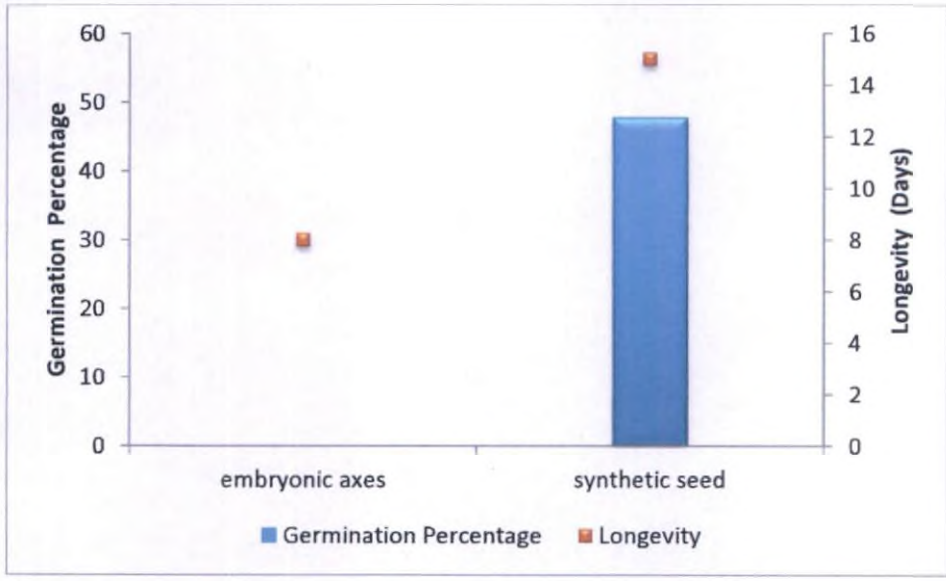

Fig. 17. Germination percentage and longevity of embryonic axes and synthetic seed of cocoa in dry cotton

Longevity of embryonic axes were increased by its conversion to synthetic seeds. The artificial coat on embryonic axes helped to maintain viability through increased level of moisture content. Also it helped to delay shoot formation. The root formation and shoot emergence were affected by the encapsulation of calcium alginate. The embryonic axes have to overcome the external covering during the growth. This extra coating delayed the root formation to certain extent. The emerged root elongated and entered into the media lead to the accumulation of the entire coat on the other end of the embryonic axes i.e., at the site of shoot regeneration. The presence of gel seed coat at this area delayed the emergence of leaves. The gel played as a mechanical barrier to open up. As the embryonic axis grows the gel coat breaked and leaf emerged. The delaying of germination by the encapsulation increased the longevity of synthetic seeds when compared to embryonic axes. The calcium alginate coat also helped embryonic axes from rapid drying. It also helped in maintaining the moisture content near the embryonic axes.

When synthetic seeds were stored in inhibitor added media, they showed increased longevity than embryonic axes stored in the same media. The synthetic seed stored in 250 mM sorbitol added media showed an increased longevity of 70 days (Table 22). Increased longevity was also observed in other inhibitor concentrations. The synthetic seeds were able to be stored upto 70 days without germination. Seeds were unable to grow in the inhibitor added media.

Table 22. Effect of different storage media on longevity of cocoa synthetic seed

Synthetic seed stored in	Longevity of seed (days)
Dry cotton	15.0^{e}
Wet cotton	32.5^{d}
½ MS	40.0^{c}
¼ MS	36.6^{cd}
250 mM sorbitol	70.0^{a}
500 mM sorbitol	60.8^{b}
SEm±	3.51*

(Figures with same superscript in a column do not differ significantly)
* Significant at 0.05 levels; ns- non significant at 0.05 levels

5.5. Effect of germination inhibitor in encapsulation media on *in vitro* storage and germination of synthetic seed

Seed coat plays an important role in dormancy of seeds. The presence of inhibitors in the seed coat helps in inducing dormancy as in natural conditions. The observations from the present study showed that, MS concentrations in the encapsulation negatively influenced the storage of synthetic seeds. The presence of MS media in the encapsulation made embryonic axes to a higher growth rate and reduced longevity. It resulted in the early germination of seeds (25 days). MS and ½ MS in the encapsulation accelerated the germination of synthetic seeds and reduced the storage potential. MS media components in the encapsulation favoured the growth of seeds in the storage condition. Even higher concentrations of inhibitor along with MS media concentrations were not able to stop the growth of the

embryonic axes. This had shown the cotyledon influence on the growth. The MS media concentrations in the coat gave a similar environment as cotyledon. The availability of nutrient near the embryonic axes in available form with sufficient moisture made them grow fast. The presence of MS media (MS and ½ MS) in the artificial coat reduced longevity to 20-35 days (Table 23) which is similar to cotyledonal condition. MS media in encapsulation gave a suitable environment for growth of embryonic axes.

Table 23. Effect of different inhibitors in encapsulation on longevity of cocoa synthetic seeds

Synthetic Seed Coat with	Longevity of seed (days)
MS	23.3^{cd}
½ MS	21.6^{de}
MS +250 mM Sorbitol	25.0^{c}
½ MS +250 mM Sorbitol	20.0^{e}
MS +500 mM Sorbitol	25.0^{c}
½ MS+ 500 mM Sorbitol	35.0^{b}
250 mM Sorbitol	65.0^{a}
500 mM Sorbitol	65.0^{a}
10^{-3} M ABA	65.0^{a}
SEm±	3.38

(Figures with same superscript in a column do not differ significantly)
* Significant at 0.05 levels; ns- non significant at 0.05 levels

To induce dormancy in synthetic seeds, effective chemical inhibitor combinations were added to the encapsulation. It had a significant increase in longevity. The presence of osmotic condition (250 mM and 500 mM sorbitol) had played important role in increasing longevity (Figure 18). Regulating water potential

near the embryonic axes is the most effective technique to keep embryo live and without germination. The presence of osmoticum in the encapsulation controlled the water transport between the axes and media of storage. The coated embryonic axes were stored in wet cotton condition. Even in the presence of high moisture content in the media the synthetic seeds were unable to continue its normal growth due to the presence of osmoticum in the artificial coat. Encapsulation played as a barrier to absorb water from the media. This checked the growth of the embryonic axes and helped in delaying germination. This had shown that manipulation of encapsulation with chemical inhibitors can improve duration of storage without germination.

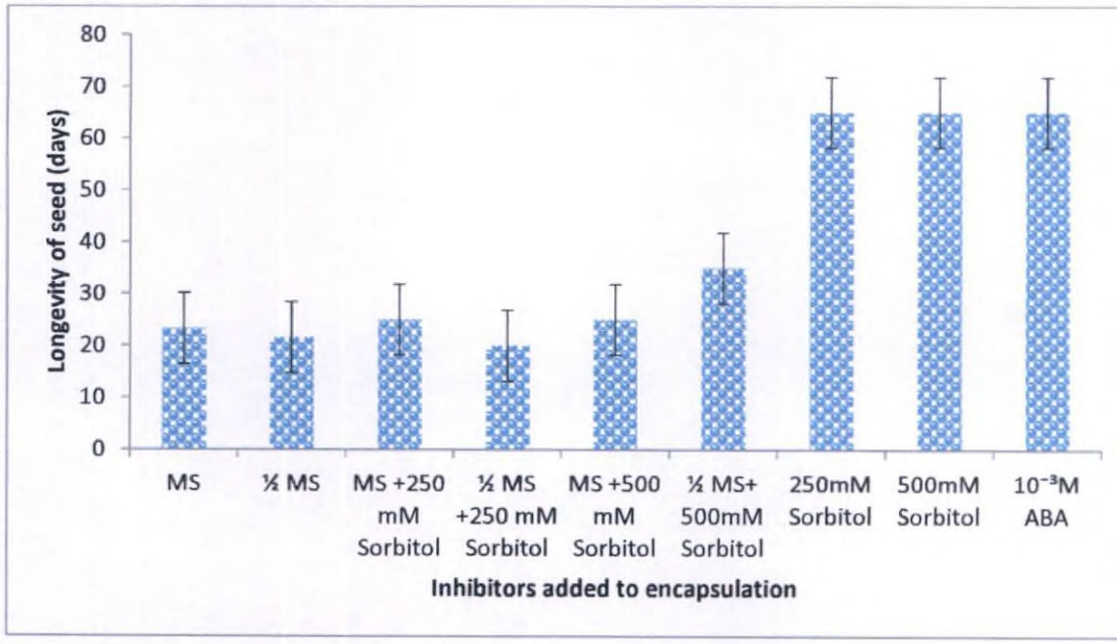

Fig. 18. Longevity of cocoa synthetic seeds having different chemicals in encapsulation

Due to continuous growth nature of embryonic axes, root formation was not affected by the presence of inhibitor in the artificial coat. The root formation was normal in all the conditions. The presence of encapsulation also provided necessary moisture condition for the regeneration of root. But in the case of shoot regeneration the inhibitors action played a good role. Inhibitors in the encapsulation delayed shoot formation. The hormonal as well as osmotic effect on the embryonic axes delayed emergence of leaves.

Presence of 10^{-3}M ABA in the encapsulation also checked the germination of synthetic seeds. Presence of ABA in seed coat induced dormancy was proved in different studies (Kawakami et al., 1997; Gonai et al., 2004; Benech-Arnold et al., 2006; Miller et al., 2006; Gianinetti and Vernieri, 2007; Goggin et al., 2009). Abscisic acid (ABA) regulates many processes during the plant life cycle, including key events during seed formation, such as the deposition of storage reserves and prevention of precocious germination. ABA is known to regulate phase III water uptake in the metabolically active embryo (Bewley, 1997; Kucera et al., 2005) through its influence on water relations (Schopfer et al., 1979; Schopfer and Plachy, 1984; Ni and Bradford, 1992). ABA is involved in regulating the onset of dormancy and in maintaining the dormant state.

5.6. Desiccation treatments on synthetic seed

Recalcitrant seed survival is influenced by the level of desiccation (Pammenter and Berjak, 1999). In the present study, synthetic seeds exposed to higher relative humidity had earlier root emergence. It was observed that longevity of synthetic seed increased by application of desiccation treatments. The level of desiccation and duration influenced the root emergence of synthetic seed. Initiation of root growth was delayed up to 11 days in 20 percent relative humidity compared to five days in 100 percent relative humidity. This shows the effectiveness of desiccation on delaying the root growth in synthetic seed. The increase in duration of desiccation also had significant influence in root emergence. The time taken for the root emergence was increased with increasing dessication time. In synthetic seeds exposed to 12 hour desiccation, root emergence was seen after 11 days. The maximum delay in root emergence was seen in synthetic seeds desiccated to 20 percent relative humidity for 12 hours (Table 16). Longevity of synthetic seeds was increased with lower desiccation. When synthetic seeds was exposed to 20 percent relative humidity, longevity increased to 54 days. The duration of desiccation also influenced the longevity of synthetic seed. Synthetic seeds exposed to 12 hour desiccation had an increased longevity of 51 days (Table 17). The synthetic seeds desiccated in 20 percent relative humidity for more than 6 hour had maximum longevity of 55 days.

Previous studies with embryonic axes of cocoa (Sudhakara et al., 2000) showed that, embryonic axes are desiccation sensitive. Loss of viability within 10 days was seen in embryonic axes exposed to 20 percent relative humidity. From the present study, it is found that synthetic seed can withstand higher desiccation rates. It seems that the alginate encapsulation enabled recalcitrant cocoa embryonic axis to withstand the desiccation. Encapsulation may be prevening the deleterious effects of desiccation on the embryo. The protective action of encapsulation on *in vitro* storage studies of synthetic seeds reported earlier by Ikhlaq et al. (2010). An alginate gelled matrix surrounding embryonic axes slowing the process of desiccation and providing the mechanical support to protect the tissue within encapsulation medium during storage (Sujatha and Kumari, 2007). The encapsulation on embryonic axes enabled it to maintain viability even after high desiccation. The ability to withstand desiccation is a characteristic of orthodox seeds. The conversion of recalcitrant seed to synthetic seed may be inducing orthodox nature to recalcitrant seeds. The encapsulation played an important role in giving desiccation tolerance. It may be checking the deleterious effect of desiccation on the embryonic axes. Effectiveness of the protective coating and possibility to store the propagules was also confirmed by Ballester et al. (1997) who reported that survival percentage of synthetic seeds was better than those of non-encapsulated ones.

5.7. Standardization of storage technique

In previous studies *Theobroma cocoa* storage was lasted upto 6-12 days in harvested pods (Nair, 1987) and encapsulation of zygotic embryo had enhanced the longevity upto 3 weeks (Sudhakara et al., 2000). In the present experiments further enhancement in storage was tried by controlling germination through hormonal and osmotic effect.

From all the experiments in the present study, it was evident that synthetic seed have more advantageous response for storage. The 250 mM sorbitol or 10^{-3} M ABA in the encapsulation only delayed the germination to 70 days. So for the storage upto 70days method of incoperating inhibitor in the encapsulation can be adopted. The synthetic seeds with inhibitor coat can be stored in the wet cotton for

70 days effectively. But the seeds will start germination from 60 days of storage and leaf emergence got completed with 70 days

Synthetic seeds stored in ½ MS +250 mM sorbitol media had checked the germination upto 70 days effectively. The synthetic seeds were transferred to wet cotton between the experiment germinated after 25-30days (Table 18). The synthetic seeds stored in ½ MS +250 mM sorbitol added media for 55 days and transferred to wet cotton had maximum storage time upto 89 days. The increased storage time in the sorbitol added media reduced the germination percentage of seeds in wet cotton. This was due to the exposure of synthetic seeds in inhibiting condition for longer time. It reduces the viability of seeds. The number of viable seeds becomes less if we exposed the seeds to inhibiting condition above a limit. The mortality in all experiment with inhibitors was due to the over exposure of seeds to inhibiting condition. The mortality starts in 250 mM sorbitol added media from 55 days. So it the most appropriate time to get maximum viable seeds.

The best method for storing cocoa through synthetic seed as found in the present study is as follows

1. Collect the yellow ridged pod i.e., 100-120 days old cocoa pod.
2. Extract embryonic axes with ¼ cotyledon, without damaging embryo
3. Encapsulate it with calcium alginate
4. Store synthetic seeds in ½ MS + 250 mM sorbitol media for 55 days.
5. Transfer synthetic seeds to wet cotton after 55 days
   The germination will start 89 days after collection of cocoa pod.

SUMMARY

SUMMARY

The present study on "Enhancement of storage life of cocoa (*Theobroma cacao* L.) seeds through encapsulation and germination inhibition." was carried out at plant tissue culture laboratory, Department of Tree Physiology and Breeding, College of Forestry, Vellanikkara, during 2010-2012.

The programme envisaged standardisation of embryonic axes with different proportion of cotyledon and response of the embryo towards different media, enhancement of storage life of recalcitrant seeds by encapsulation and inducing dormancy using different concentrations of germination inhibitors or osmotic conditions. Salient findings of the study are summarized here under,

1. Embryonic axes without cotyledon failed to regeneration. Retaining ½ or ¼th cotyledon resulted in shoot regeneration of embryonic axes.

2. Embryonic axes having ¼ cotyledon were selected for the experiments because of their good germination and size.

3. Storage of embryonic axes in different media showed significant difference in longevity.

4. Embryonic axes stored in dry cotton had least longevity and germination.

5. Embryonic axes stored in ½ MS media had higher germination and longevity.

6. Embryonic axes stored in germination inhibitors had significant difference in root, shoot regeneration, and longevity.

7. The media with 10^{-3}M coumarin influenced the growth of embryonic axes.

8. Bulging and fibrous roots were observed in embryonic axes stored in 10^{-3}M coumarin. This may be due to the inhibitory action of coumarin on embryo growth.

9. Dormancy inducing action of ABA controlled the root and shoot regeneration of embryonic axes. Media with 10^{-4}M ABA checked the germination of embryonic axes and had a longevity of 56 days.

10. Cycocel did not showed significant influence on the growth of the embryonic axes.
11. The combinations of germination inhibitor added in the media had similar influence as when added individually. Interaction effects of inhibitors were not observed.

12. Presence of osmoticum in the media influenced growth of the embryonic axes. Higher osmotic levels controlled the water uptake and thus had an influence on the growth of embryonic axes.

13. Embryonic axes stored in ½ MS+250 mM sorbitol showed higher longevity of 65 days.

14. Media supplemented with higher concentrations of mannitol (250 mM and 500 mM) resulted in crystallization of the media during the storage and reduced the longevity of embryonic axes.

15. Presence of higher NaCl concentration (250 mM and 500 mM) reduced the longevity of embryonic axes.

16. Embryonic axes given encapsulation enhanced the longevity of embryo. Encapsulation enhanced the longevity of embryonic axes from 29 days to 40 days.

17. Maximum longevity observed in synthetic seed stored in 250 mM sorbitol added media and least seen in seeds stored in dry cotton.

18. Presence of MS media concentrations in the encapsulation reduced the time taken for germination.

19. MS media concentrations and inhibitor in the encapsulation showed no influence on the growth of synthetic seed.

20. Significant enhancement in longevity upto 65 days, was observed in seeds having inhibitor in the encapsulation.

21. Desiccation treatments on synthetic seed had effect on longevity (48 days). It was due to the presence of artificial seed coat which limits the desiccation to reach embryonic axes.

22. Desiccation of 20 percent RH had higher longevity of 54 days. Longevity decreased with increasing desiccation RH level.

23. The maximum storage time of 89 days was obtaining with synthetics seeds stored in 250 mM sorbitol added media for 55days, and transferred to wet cotton for germination.

REFERENCES

REFERENCES

Abenavoli, M.R., Cacco, G., Sorgonà, A., Marabottini, R., Paolacci, A.R., Ciaffi, M., and Badiani, M. 2006. The inhibitory effects of coumarin on the germination of durum wheat (*Triticum turgidum* ssp. durum, cv. Simeto) seeds. J. Chem. Ecol., 32: 489-506.

Abenavoli, M.R., De Santis, C., Sidari, M., Sorgona, A., Badiani, M., and Cacco, G. 2001. Influence of coumarin on the net nitrate uptake in durum wheat. New Phytol., 150: 619-627.

Abenavoli, M.R., Nicolò, A., Lupini, A., Oliva, S., and Sorgonà, A. 2008. Effects of different allelochemicals on root morphology of *Arabidopsis thaliana*. Allelopathy J., 22: 245-252.

Abenavoli, M.R., Sorgonà, A., Albano, S., and Cacco, G. 2004. Coumarin differentially affects the morphology of different root types of maize seedlings. J. Chem. Ecol., 30: 1871-1883.

Abenavoli, M.R., Sorgonà, A., Sidari, M., Badiani, M., and Fuggi, A. 2003. Coumarin inhibits the growth of carrot (*Daucus carota* L. cv. Saint Valery) cells in suspension culture. J. Plant Physiol., 160: 227-237.

Agarwal, P.K. 1987. Seed storage and packing techniques. In: Agrawal, P.K., and Dadlani, M. (eds), Seed Science and Technology, South Asian Publishers, New Delhi, 191p.

Agboola, D.A. 1998. Effect of saline solutions and salt stress on seed germination of some tropical forest tree species. Trop. Biol., 45(2): 324-331.

Aliotta, G., Cafiero, G., Fiorentino, A., and Strumia, S. 1993. Inhibition of radish germination and root growth by coumarin and phenylpropanoids. J. Chem. Ecol., 19: 175-183.

Aliotta, G., Fuggi, A., and Strumia, S. 1992. Coat-imposed dormancy by coumarin in radish seeds: the influence of light. G. Bot. Ital., 126: 631-637.

Ali-Rachedi, S., Bouinot, D., Wagner, M.H., Bonnet, M., Sotta, B., Grappin, P., and Jullien, M. 2004. Changes in endogenous abscisic acid levels during dormancy release and maintenance of mature seeds: studies with the Cape Verde Islands ecotype, the dormant model of *Arabidopsis thaliana*. Planta., 219: 479-488.

Al-Khayri, J.M. and Al-Bahrany, A.M. 2002. Callus growth and proline accumulation in response to sorbitol and sucrose induced osmotic stressing rice. Biol. Plant., 45: 609-611.

Anandarajah, K. and McKersie, B.D. 1990. Manipulating the desiccation tolerance and vigor of dry somatic embryos of *Medicago sativa* L. with sucrose, heat shock and abscisic acid. Plant Cell Rep., 9(8): 451-455.

Arunkumar, M.B., Vakeswaran, V., and Krishnasamy, V. 2005. Enhancement of synthetic seed conversion to seedlings in hybrid rice. Plant Cell Tissue. Organ Cult., 81: 97-100.

Bais, H.P., Park, S.W., Weir, T.L., Callaway, R.M., and Vivanco, J.M. 2004. How plants communicate using the underground information superhighway. Trends Plant Sci., 9: 26-32.

Bajji, M., Kinet, J.M., and Lutts, S. 2002. Osmotic and ionic effects of NaCl on germination, early seedling growth, and ion content of *Atriplex halimus* (Chenopodiaceae). Can. J. Bot., 80: 297-304.

Ballester, A. Janeiro, L.V., and Vieitez, A.M. 1997. Cold storage of shoot cultures and alginate encapsulation of shoot tips of *Camellia japonica* L. and *Camellia reticulata* L. Sci. Hortic., 71: 67-78.

Bapat, V. A. 1993. Studies on synthetic seeds of sandalwood (*Santalum album*) and mulberry (*Morus indica*). In : Redenbaugh K. (Ed.) Synseeds : Application of Synthetic Seeds to Crop Improvement, CRC Press Inc., Boca, Raton, Ca, USA, 381-408.

Bapat, V. A. and Rao, P. S.1988. Sandalwood plantlets from synthetic seeds. Plant Cell Rep., 7: 434-436.

Bapat, V.A. and Rao, P.S. 1992. Plantlet regeneration from encapsulated and non encapsulated somatic embryos of a forest tree - sandalwood. J. Plant Biochem. Biotech., 1: 109-113.

Bapat, V.A., Mhatre, M., and Rao, P.S. 1987. Propagation of *Morus indica* (mulberry) by encapsulated shoot buds. Plant Cell Rep., 6: 393-395.

Baskin, J.M. and Baskin, C.C. 1998. Seeds: Ecology, biogeography and evolution of dormancy and germination. Academic Press, San Diego, 666p.

Baumert, A., Mock, H.P., Schmidt, J., Herbers, K., Sonnewald, U., and Strack, D. 2001. Patterns of phenylpropanoids in non inoculated and potato virus Y-inoculation leaves of transgenic tobacco plants expressing yeast-derived invertase. Phytochemistry, 56: 535-541.

Bekheet, S.A., Taha, H.S., Saker, M.M., and Moursya, H.A. 2002. A synthetic seed system of Date Palm through somatic embryos encapsulation. An. Agric. Sci., 47(1): 325-337.

Benech-Arnold, R.L., Gualanol, N., Leymarie, J., Come, D., and Corbineau, F. 2006. *Hypoxia interferes* with ABA metabolism and increases ABA sensitivity in embryos of dormant barley grains. J. Exp. Bot., 57(6): 1423-1430.

Berjak, P. 1996. The Role of micro-organisms in deterioration during storage of recalcitrant and intermediate seeds. In: Quédraogo, A.S., Poulsen, K., and Stubsgaard, I. (eds), Intermediate/recalcitrant tropical forest seeds, International Plant Genetic Resource Institute, Rome. pp 121-126.

Berjak, P. and Pammenter, N.W. 1997. Progress in the understanding and manipulation of desiccation-sensitive (recalcitrant) seeds. In: Ellis, R.H. Black, M., Murdoch, A.J., and Hong, T.D. (eds), Basic and applied aspects of seed biology, Kluwer Academic Publishers, Dordrecht, Netherlands. pp 689-703.

Berjak, P., Farrant, J.M., and Pammenter, N.W. 1990. The basis of recalcitrant seed behaviour. In: Taylorson, R.B. (ed.), Recent advances in the development and germination of seeds. Plenum Press, New York. pp 89-108.

Berjak, P., Farrant, J.M., Mycock, D.J., and Pammenter, N.W. 1989. Homoiohydrous (recalcitrant) seeds: The enigma of their desiccation sensitivity and state of water in axes of *Landolphia kirkii* Dyer. Planta., 186: 249–261.

Berjak, P., Vertucci, C.W., and Pammenter, N.W. 1993. Effects of developmental status and dehydration rate on characteristics of water and desiccation-sensitivity in recalcitrant seeds of *Camellia sinensis*. Seed Sci. Res., 3: 155-166.

Bertin, C, Yang X., and Weston, L.A. 2003. The role of root exudates and allelochemicals in the rhizosphere. Plant Soil., 256: 67-83.

Bewley, J.D. 1997. Seed germination and dormancy. Plant Cell, 9: 1055-1066.

Bewley, J.D. and Black, M. 1994. Seeds physiology of development and germination. Plenum Press, New York. 445p.

Bhattacharjee, A. and Gupta, K. 1981. Effect of CCC on retardation of seedling growth and metabolism of sunflower. Sci. Cult., 47: 266-267.

Bonner, F.T. 1990. Storage of seeds: Potential and limitations for germplasm conservation. For. Ecol. Mgmt., 35: 35-43.

Bourgaud, F., Hehn, A., Larbat, R., Doerper, S., Gontier, E., Kellner, S., and Matern, U. 2006. Biosynthesis of coumarins in plants: A major pathway still to be unrevelled for cytochrome P450 enzymes. Phytochem. Rev., 5: 293-308.

Bradford, K.J. and Nonogaki, H. 2007. Seed development, dormancy and germination. Blackwell. Publishing Plant Science, Oxford, 392p.

Brooker, N. L., Kuzimichev, Y., Laas, J., and Pavlis, R. 2007. Evaluation of coumarin derivatives as anti-fungal agents against soil-borne fungal pathogens. Commun. Agric. Appl. Biol. Sci., 72: 785-793.

Carman, J.G. 1988. Improved somatic embryogenesis in wheat by partial stimulation of the *in vitro* oxygen growth regulator and desiccation environments. Planta, 175 : 417-424.

Carpinella, M.C., Ferrayoli, C.G., and Palacios, S.M. 2005. Antifungal synergistic effect of scopoletin, a hydroxycoumarin isolated from *Melia azedarach* L. fruits. J. Agric. Food Chem., 53: 2922-2927.

Chen, Z. H., Yao, Y. G., Guan, Y. C., Qian, W. J., Lin, D. T., and Liu, G. Z. 1992. Studies on the propagules used for artificial seeds. In : Biotechnology in Agriculture Proceedings of the First Asia - Pacific Conference on Agricultural Biotechnology, Kluwer Academic Publishers, Netherlands, pp 279-282.

Chetia, S. and Devi, J. 1996. Regeneration of three orchid species from artificial seeds. In : Souvenir Nation. Symp. Hort. Biotechnol., Horticultural Society of India and IIHR, Bangalore, p.6.

Chin, H.F. 1988. Recalcitrant Seeds - A Status Report. International Board for Plant Genetic Resources, Rome, 28p.

Chin, H.F. and Roberts, E.H. 1980. Recalcitrant crop seeds. Tropical Press Sdn. Bhd., Kuala Lumpur, Malaysia, 427p.

Chin, H.F., Aziz, M., Ang, B.B., and Hamzah, G. 1981. The effect of moisture content and temperature on the ultra structure and viability of seeds of *Hevea brasiliensis*. Seed Sci. Technol., 9: 411-422.

Come, D. and Corbineau, F. 1996. Metabolic damage related to desiccation sensitivity. In: Quédraogo, A.S., Poulsen, K., Stubsgaard, F. (eds), Intermediate/recalcitrant tropical forest tree seeds, International Plant Genetic Resources Institute, Rome. pp 83-97.

Da Silva, E.E.A., Toorop, P.E., Van Aelst, A.C., and Hilhorst, H.W.M. 2004. Abscisic acid controls embryo growth potential and endosperm cap weakening during coffee (*Coffea arabica* cv, Rubi) seed germination. Planta., 220: 251-261.

Datta, K.B., Kanjilal, B., and Sarkar, D. 1999. Artificial seed technology : Development of a protocol in *Geodoxum densiflorum* : An endangered orchid. Curr. Sci., 76: 1142-1145.

Datta, S.K. and Potrykus, I. 1989. Artificial seeds in barley : encapsulation of microspore derived embryos. Theor. Appl. Genet., 77: 820-824.

Debolt, S., Gutierrez, R., Ehrhardt, D. W., Melo, C.V., Ross, L., Cutler, S.R., Somerville, C., and Bonetta, D. 2007. Morlin, an inhibitor of cortical microtubule dynamics and cellulose synthase movement. Proc. Natl. Acad. Sci. USA, 104: 5854-5859.

Dorell, D.G. 1973. Controlling plant height in sunflower with growth retardants. Can. J. Pl. Sci., 33: 417-428.

Duan, D., Liu, X., Khan, M.A., and Gul, B. 2004. Effects of salt and water stress on the seed germination of *Chenopodium glaucum* L. Pak. J. Bot., 36(4): 793-800.

Egan, T.P., Ungar, I.A., and Meekins, J.F. 1997. The effect of different salts of sodium and potassium on the germination of *Atriplex prostrata* (Chenopodiaceae). J. Plant Nutr. 20(12): 1723-1730.

Ellis, R.H. 1991. The longevity of seeds. Hort. Sci., 26:1119-1125.

Ellis, R.H., and Roberts, E.H. 1980. Improved equations for the prediction of seed longevity. Ann. of Bot., 45: 13-30.

Ellis, R.H., Hong, T.D., Roberts, E.H., and Tao, K. 1990. Low moisture-content limits to relations between seed longevity and moisture. Ann. of Bot., 65: 493-504.

Engels, J. and Engelmann, F. 1998. Introductory statement. Seed Sci. Res., 8: 1-2.

Ewart, A.J. 1908. On the longevity of seeds. Proc. of the Royal Society, Victoria, 21: 1-210.

FAOSTAT(Food and Agriculture Organization Statistics Division). 2012. Available : http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor [1 October 2012].

Farrant, J.M., Pammenter N.W., and Berjak, P. 1986. The increasing desiccation sensitivity of recalcitrant *Avicennia marina* seeds with storage time. Physiologia Plant., 67: 291-298.

Farrant, J.M., Pammenter, N.W., and Berjak, P. 1988. Recalcitrance - a current assessment. Seed Sci. Tech., 16: 155-166.

Farrant, J.M., Pammenter, N.W., and Berjak, P. 1989. Germination-associated events and the desiccation sensitivity of recalcitrant seeds - a case study in three unrelated species. Planta., 178: 189-198.

Farrant, J.M., Pammenter, N.W., and Berjak, P. 1993. Seed development in relation to desiccation tolerance: A comparison between desiccation-sensitive (recalcitrant) seeds of *Avicennia marina* and desiccation-tolerant types. Seed Sci. Res., 3: 1-13.

Feurtado, J.A., Yang, J., Ambrose, S.J., Cutler, A.J. Abrams, S.R., and Kermode, A.R. 2007. Disrupting abscisic acid homeostasis in western white pine (*Pinus monticola*) seeds induces dormancy termination and changes in abscisic acid catabolites. J. Plant Growth Regul., 26: 46-54.

Finch-Savage, W.E. 1992. Seed development in the recalcitrant species *Quercus robur* L.: Development of germinability and desiccation tolerance. Seed Sci. Res., 2: 17-22.

Finch-Savage, W.E. and Blake, P.S. 1994. Indeterminate development in desiccation-sensitive seeds of *Quercus robur* L. Seed Sci. Res., 4: 127-133.

Finch-Savage, W.E., Pramanik, S.K., and Bewley, J.D. 1994. The expression of dehydrin proteins in desiccation-sensitive (recalcitrant) seeds of temperate trees. Planta., 193: 478-485.

Finkelstein, R.R. and Crouch, M.L. 1986. Rapeseed embryo development in culture on high osmoticum is similar to that in seeds. Plant Physiol., 81: 907-912.

Finkelstein, R.R., Gampala, S.S., and Rock, C.D. 2002. Abscisic acid signaling in seeds and seedlings. Plant Cell., 14: 15-45.

Frankel, O.H. 1990. Germplasm conservation and utilization in horticulture. Horticultural Biotechnology. Wiley-Liss Inc., New York. pp 5-17.

Fu, J., Zhang, B.Z., Wang, X.P., Qiao, Y.Z., and Huang, X.L. 1990. Physiological studies on desiccation, wet storage and cryopreservation of recalcitrant seeds of three fruit species and their excised embryonic axes. Seed Sci. Tech., 18: 743-754.

Fu, J.R., Xia, Q.H., and Tang, L.F. 1993. Effects of desiccation on excised embryonic axes of three recalcitrant seeds and studies on cryopreservation. Seed Sci. Tech., 21: 85-95.

Fujii, J. A., Slade, D., and Redenbaugh, K. 1988. Improving plantlet growth and vigour from alfalfa artificial seed. In-vitro Cell Dev. Biol. Plant, 24(3): 70.

Gamborg, O.L. and Shyluk J.P. 1981. Nutrition, media and characteristics of plant cell and tissue cultures. In: Thorpe,T.A. (ed.) Plant Tissue Culture Methods and Application in Agriculture. Academic Press, New York, pp 21-44.

Ganapathi, T.R., Higgs, N.S., Balint-Kurti, P.J., Arntzen, C.J., May, G.D., and Van Eck, J.M. 2001. *Agrobacterium*-mediated transformation of the embryogenic cell suspensions of the banana cultivars Rasthali (AAB). Plant Cell Rep., 20: 157-162.

Garciarrubio, A., Legaria, J.P., and Covarrubias, A.A. 1997. Abscisic acid inhibits germination of mature Arabidopsis seeds by limiting the availability of energy and nutrients. Planta, 203: 182-187.

George, L. and Eapen, S. 1995. Encapsulation of somatic embryos of finger millet, *Eleusine coracana* Gaertn. Indian J. Exp. Biol., 33: 291-293.

Gianinetti, A. and Vernieri, P. 2007. On the role of abscisic acid in seed dormancy of red rice. J. Exp. Bot., 58: 3449-3462.

Gill, N.K., Gill, R., and Gosal, S.S. 2002. Somatic embryogenesis and plant regeneration in some commercial cultivars of sugarcane. Crop Improv., 29(1): 28-34.

Goggin, D.E., Steadman, K.J., Emery, R.J.N., Farrow, S.C., Benech-Arnold, R.L., and Powles, S.B. 2009. ABA inhibits germination but not dormancy release in mature imbibed seeds of *Lolium rigidum* Gaud. J. Exp. Bot., 60: 3387-3396.

Gonai, T., Kawahara, S., Tougou, M., Satoh, S., Hashiba, T., Hirai, N., Kawaide, H., Kamiya, Y., and Yoshioka, T. 2004. Abscisic acid in the thermoinhibition of lettuce seed germination and enhancement of its catabolism by gibberellin. J. Exp. Bot., 55: 111-118.

Gray, D.J. 1987. Synthetic seed technology for the mass cloning of crop plants: problems and prospective. Horti. Sci., 22: 795-814.

Gubler, F., Hughes, T., Waterhouse, P., and Jacobsen, J. 2008. Regulation of dormancy in barley by blue light and after-ripening: effects on abscisic acid and gibberellin metabolism. Plant Physiol., 147: 886-896.

Guo, W., Tiwari, G., Tang, J., and Wang, S. 2008. Frequency, moisture and temperature dependent dielectric properties of chickpea flour. Biosystems Eng., 101: 217-224.

Gupta, P.K. and Durzan, D.J. 1987. Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. Biotechnol., 5: 147-151.

Hamilton, D.F. 1976. Physiology and biochemistry of seeds. Can. J. Bot., 54: 1068-1073.

Hanson, J. 1985. Procedures for Handling Seeds in Genebanks. International Board for Plant Genetic Resources, Rome. 115p.

Harrington, J.F. 1963. Practical instructions and advice on seed storage. Proc. Int. Seed Test. Assoc., 28: 989-994.

Hong, T.D. and Ellis, R.H. 1990. A comparision of maturation drying, germination, and desiccation tolerance between developing seeds of *Acer pseudoplatanus* L. and *Acer platanoides* L. New Phytologist. 116: 589-596.

Hong, T.D. and Ellis, R.H. 1996. A protocol to determine seed storage behaviour, Technical Bulletin 1. International Plant Genetic Resources Institute, Rome. 62p

Huarte, H.R. and Benech-Arnold, R.L. 2010. Hormonal nature of seed responses to fluctuating temperatures in *Cynara cardunculus* (L.). Seed Sci. Res., 20: 39-45.

Huda, K.M.K., Bhuiyan, M.S.R., Zeba, N., Banu, S.A., Mahmud, F., and Khatun, A. 2009. Effect of $FeSO_4$ and pH on shoot regeneration from the cotyledonary explants of Tossa Jute. Plant Omics J., 2(5): 190-196.

IBPGR [International Board for Plant Genetic Resources]. 1976. Report on the IBPGR working group on engineering, design and cost aspects of long-term seed storage facilities. International Board for Plant Genetic Resources, Rome. 109p.

Ikhlaq, M., Hafizl, I.A., Micheli, M., Ahmad, T., Abbasi, N.A., and Standardi, A. 2010. *In vitro* storage of synthetic seeds: Effect of different storage conditions and intervals on their conversion ability. African J. Biotech. 9(35): 5712-5721.

Ipekci, Z. and Gozukirmizi, N. 2003. Direct somatic embryogenesis and synthetic seed production from *Paulownia elongata*. Plant Cell Rep., 22(1): 16-24.

Janick, J., Kitto, S., and Kim, Y. H. 1989. Production of synthetic seed by desiccation and encapsulation. In vitro Cell. Dev. Bio., 25: 1167-1172.

Jaydip, M., Sitakanta, P., and Chand, P.K. 2000. Alginate encapsulation of axillary buds of *Ocimum americanum* L., *Ocimum basilicum* L., *Ocimum gratissimum* and *Ocimum sanctum*. In-vitro Cell. Dev. Biol. Plant, 36(4): 287-292.

Karssen, C.M., Zagorski, S., Kepczynski, J., and Groot, S.P.C. 1989. A key role for endogenous gibberellins in the control of seed germination. Ann. Bot., 63: 71-80.

Kavyashree, R., Gayatri, M.C., and Revanasiddaiah, H.M. 2006. Propagation of mulberry variety - S54 by synseeds of axillary buds. Plant Cell Tissue. Organ. Cult., 84:245-249.

Kawakami, N., Miyake, Y., and Noda, K. 1997. ABA insensitivity and low ABA levels during seed development of non-dormant wheat mutants. J. Exp. Bot., 48: 1415-1421.

Kende, H., Ninnemann, H., and Lang, A. 1963. Inhibition of gibberellic acid biosynthesis in *Fusarium moniliforme* by Amo-1618 and CCC. Naturwiss., 50: 599-600.

Khan, A.A. and Zeng, G.W. 1985. Dual action of respiratory inhibitors: Inhibition of germination and prevention of dormancy induction in lettuce seeds. Plant Physiol., 77: 817-825.

Khan, M.A. 2002. Halophyte seed germination: Success and pitfalls. In: Hegazi, A.M., El-Shaer, H.M., and El-Demerdashe, S. (eds), International symposium on optimum resource utilization in salt affected ecosystems in arid and semi arid regions, Desert Research Centre, Cairo, pp 346-358.

King, M.W. and Roberts, E.H. 1979. The storage of recalcitrant seeds: achievements and possible approaches. International Board for Plant Genetic Resources, Rome, 96p.

King, M.W. and Roberts, E.H. 1980. Maintenance of recalcitrant seeds in storage. In: Chin, H.F. and Roberts, E.H. (eds.), Recalcitrant Crop Seeds, Tropical Press, Kuala Lumpur, pp 53-89.

Kitto. S.L, and Janick, J. 1982. Polyox as an artificial seed coat for asexual embryos. Hort Sci., 17: 448.

Kitto, S.L. and Janick, J. 1985. Production of synthetic seeds by encapsulating asexual embryos of carrot. J. Amer. Soc. Hort. Sci., 110: 277-282.

Knypl, J.S. 1967. Synergistic inhibition of Kale seed germination by coumarin and (2-Chloroethyl)- trimethylammonium chloride, and its reversal by kinetin and gibberellic acid. Planta (Berl.), 72: 292-296.

Kucera, B., Cohn, M.A., and Leubner-Metzger, G. 2005. Plant hormone interactions during seed dormancy release and germination. Seed Sci. Res., 15: 281-307.

Kumaran, K., and Palani, M., and Jerlin, R., and Surendran, C., 1994. Effects of growth regulators on seed germination and seedling growth of neem (*Azadirachta indica*). J. Trop. For. Sci., 6: 529-532.

Kusumoto, D., Chae, S.H., Mukaida, K., Yoneyama, K., Yoneyama, K. Joel, D.M., and Takeuchi, Y. 2006. Effects of fluridone and norflurazon on conditioning and germination of *Striga asiatica* seeds. Plant Growth Reg., 48: 73-78.

Li, L. and Pan, R.C. 1996. Increasing yield and drought resistance of groundnut using plant growth regulators. In: Renard (ed.), Achieving High Groundnut Yields. Proceedings of an International Workshop, Andhra Pradesh, India, pp 147-155.

Liu, Y., Bergervoet, J.H.W., Ric De Vos, C.H., Hilhorst, H.W.M., Kraak, H.L., Karssen, C.M., and Bino, R.J. 1994. Nuclear replication activities during imbibition of abscisic acid- and gibberellin-deficient tomato (*Lycopersicon esculentum* Mill.) seeds. Planta., 194: 368-373.

Lupini, A., Sorgonà, A., Miller, A.J., and Abenavoli, M.R. 2010. Short-term effects of coumarin along the maize primary root axis. Plant Signal Behav., 5(11): 1395-1400.

Machii, H. 1992. In-vitro growth of encapsulated adventitious buds in mulberry *Morus alba* L. Japanese J. Breed., 42: 553-559.

Maithani, G.P., Bahuguna, V.K., Rawat, M.M.S., and Sood, O.P. 1989. Fruit maturity and interrelated effects of temperature and container on longevity of neem (*Azadirachta indica*) seeds. The Indian Forester, 115(2): 89-96.

Mariani, P. 1992. Eggplant somatic embryogenesis combined with synthetic seed technology. Capsicum News lett., 289-294.

Mathur, A.K. and Ahuja, P.S. 1991. Artificial seeds - Some emerging trends. Curr. Res. Medic. Aromatic Pl., 13: 119-127.

McCarty, D.R. 1995. Genetic control and integration of maturation and germination pathways in seed development. Annu. Rev. Plant Physiol. Plant Mol. Biol., 46: 71-93.

McKersie, B., Senaratna, T., Bowley, S., Brown, D., Krochko, J., and Bewley, J.I. 1989. Application of artificial seed technology in the production of hybrid alfalfa (*Medicago sativa* L.). In vitro Cell De. Biol., 25: 1183-1188.

McKersie, B.D., Hoekstra, F., and Krieg, L. 1990. Differences in the susceptibility of plant membrane lipids to peroxidation. Biochim. Biophys. Acta., 1030: 119-126.

Mehra, V., Tripathi, J., and Powell, A.A. 2003. Aerated hydration treatment improves the response of *Brassica juncea* and *Brassica campestris* seeds to stress during germination. Seed Sci. Technol., 31: 57-70.

Micheli, M., Mencuccini, M., and Standardi, A. 1998. Encapsulation of *in-vitro* proliferated buds of olive. Adv. Hort. Sci., 12(4): 163 168.

Millar, A.A., Jacobsen, J.V., Ross, J.J., Helliwell, C.A., Poole, A.T., Scofield, G., Reid, J.B., and Gubler, F. 2006. Seed dormancy and ABA metabolism in Arabidopsis and barley: the role of ABA 8'-hydroxylase. Plant J., 45(6): 942-54.

Misra, N. and Dwivedi, U.N. 2004. Genotypic difference in salinity tolerance of green gram cultivars, Plant Sci., 166: 1135

Misra, N., Murmu, B., Singh, P., and Misra, M. 1996. Growth and proline accumulation in mungbean seedlings as affected by sodium chloride, Biol. Plant, 38: 531.

Mohammad, S. and Sen, D.N. 1990, Germination behaviour of some halophytes in Indian desert. Ind. J. Exp. Biol., 28(5): 546-549.

Moreland, D.E. and Novitzky, W.P. 1987. Effects of phenolic acids, coumarins and flavonoid in isolated chloroplasts and mitochondria. In: Waller, G.R. (ed.), Allelochemicals: Role in agriculture and forestry, American Chemical Society: Washington, DC, 330: 247-261.

Mumford, P.M. and Brett, A.C. 1982. Conservation of cacao seed. Trop. Agric. (Trinidad), 59: 306-310.

Murashige, T. 1977. Plant cell and organ culture as horticultural practices. Acta Hortic., 78: 17-30.

Myers, B.A. and Couper, D.I. 1989. Effects of temperature and salinity on the germination of *Puccinellia ciliata* (Bor.) cv. Menemen. Aust. J. Agric. Res., 40: 561-571.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plant., 15: 473-497.

Naidoo, G. and Naicker, K. 1992. Seed germination in the coastal halophytes *Triglochin bulbosa* and *Triglochin striata.* Aquatic Bot., 42: 217-229.

Nair, R.V. 1987. Kerala agricultural development project compailation report cocoa 1977-86, Kerala agricultural university, Thrissur, 104p.

Nambara, E. and Marion-Poll, A. 2005. Abscisic acid biosynthesis and catabolism. Annu. Rev. Plant Biol., 56: 165-85.

Naqvi, S. M., Sultana, R. and Rasheed, H. 2005. Tissue culture studies in *Oryza sativa* l. Cvs. Basmati 385 and super basmati. Pak. J. Bot., 37(4): 823-828.

Ni, B.R. and Bradford, K.J. 1992. Quantitative models describing the sensitivity of tomato seed germination to abscisic acid and osmoticum. Plant Physiol., 98: 1057-1068.

Ninnemann, H., Zeevaart, J.A.D., Kende, H., and Lang, A. 1964. The plant growth retardant CCC as an inhibitor of gibberellin biosynthesis in *Fusarium moniliforme*. Planta (Bert.), 61: 229-235.

Nyende, A.B., Schittenhelm, S., Mix-Wagner , G., and Greef, M.J. 2005. Yield and canopy development of field grown potato plants derived from synthetic seed. Euro. J. Agron., 22: 175-184.

Nyende, A.B., Schittenhelm, S., Wagner, G.M., and Greef, J.M. 2003. Production, storability, and regeneration of shoot tips of potato (*Solanum tuberosum* L.) encapsulated in calcium alginate hollow beads. In-vitro Cell Dev. Biol. Plant, 39: 540-544.

Ogilvy, S. 1985. Changing the shape of oilseed rape. Ann. Rev. of High Mowthrope Experimental Husbandry Farm, Eng. Publisher, Malton, 8: 24-27.

Okoloko, G.E. and Lewis, L.N. 1968. Enhancement of lateral bud growth in *Coleus blumei* Benth. by (2-chloroethyl) trimethylammonium chloride (CCC). Plant Cell Physiol., 9(2): 259-266.

Olson, D.F. and Gabriel, W.J. 1974. Seeds of woody plants in the United States, Agriculture Handbook 450, U.S. Department of Agriculture, Washington, 883p.

Olympic Horticultural Products. 2005. Cycocel®. Plant growth regulant. Product information bulletin, 301p.

Onishi, N., Sakamoto, Y., and Hirosawa, T. 1994. Synthetic seeds on application of mass production of somatic embryos. Plant Cell Tissue Organ Cult., 39: 137-145.

Pammenter, N.W. and Berjak, P. 1999. A review of recalcitrant seed physiology in relation to desiccation-tolerance mechanisms. Seed Sci. Res., Cambridge University Press, pp 13-37.

Pammenter, N.W., Berjak, P., Farrant, J.M., Smith, M.T., and Ross, G. 1994. Why do stored, hydrated recalcitrant seeds die? Seed Sci. Res., 4: 187-191.

Pammenter, N.W., Vertucci, C.W., and Berjak, P. 1993. Responses to desiccation in relation to non-freezable water in desiccation-sensitive and -tolerant seeds. In: Côme, D. and Corbineau, F. (eds.), Fourth International Workshop on Seeds: Basic and Applied Aspects of Seed Biology, Université Pierre et Marie Curie, Paris, pp 867-872.

Pattnaik, S. and Chand, P.K. 2000. Morphogenic response of the alginate encapsulated axillary buds from in-vitro shoot cultures of six mulberries. Plant Cell Tiss. Org. Cult., 60: 177-185.

Peal, L. and Williams, R.D. 2002. The Effects of coumarin on radish seed germination and radicle elongation. Research Day Abstracts: Regional Universities Research Day, 74p.

Pearse, H.L. 1974. Hibiskus. Boerdery in Suid-Africa. No. J. 5: 1-7.

Prasannakumari amma, S., Nair, R.V., Lalitha bai, E.K., Malika, V.K., Minimol, J.S., and Abraham, K. 2009. Cocoa in India. Kerala Agricultural University, 72p.

Promila, K. and Kumar. S. 2000. *Vigna radiata* seed germination under salinity, Biol. Plant., 43: 423.

Pujol, J.A., Calvo, J.F., and Diaz, L.R. 2000. Recovery of germination from different osmotic conditions by four halophytes from southeastern Spain. Ann. Bot., 85(2): 279-286.

Qin, X., Yang, S.H., Kepsel, A.C., Schwartz, S.H., and Zeevaart, J.A.D. 2008. Evidence for abscisic acid biosynthesis in *Cuscuta reflexa*, a parasitic plant lacking neoxanthin. Plant Physiol., 147: 816-822.

Rademacher, W. 2000. Growth retardants: effects on gibberellin biosynthesis and other metabolic pathways. Ann. Rev. Plant Physiol. Plant Mol. Biol., 51: 501-531.

Ramos, M.L.G., Gordon, J.A., Minchin, R.F., Sprent, I.J., and Parsons, R. 1999. Effect of water stress on nodule physiology and biochemistry of a drought tolerant cultivar of common bean (*Phaseolus vulgaris*). Ann. Bot., 83: 57-63.

Ramprakash and Mangalprasad. 2000, Effect of nitrogen, chloromequate chloride and farmyard manure applied to cotton (*Gossypium hirsutum*) and their residual effect on succeeding wheat (*Triticum aestivum*) crop. Indian J. of Agron., 45(2): 263-268.

Ravindra, B. and Staden, V. 2005. Storability and germination of sodium alginate encapsulated somatic embryos derived from the vegetative shoot apices of mature *Pinus patula* trees. Plant Cell Tiss. Org. Cult., 82: 259-265.

Reddy, G.S., Kumar, D.D., Babu, R.S., and Madhavi, M. 2005. Callus culture and synthetic seed production in *Rauvolfia serpentina*. In. J. Hort., 62(1): 102-103.

Redenbaugh, K. 1990. Application of artificial seed to tropical crops. Hort. Sci., 25(3): 251.

Redenbaugh, K. 1993. Hydrogel encapsulated synseed. In: Redenbaugh K., (ed.).Synseeds. CRC, Press, London, 199p.

Redenbaugh, K., Fujii, J.A., and Slade, D. 1993. Hydrated Coating for Synthetic Seeds. In : Redenbaugh, K. (Ed.) Synseeds, CRC Press, Boca Raton, pp 35-46.

Redenbaugh, K., Paasch, B.D., Nichel, I.W., Kosslu, M.W., Viss, P.R., and Walker, K.A. 1986. Somatic seeds : Encapsulation of asexual embryos. Biotch, 4: 797-801.

Redenbaugh, K., Slade, D., Viss, P., and Fuji, J. 1987. Encapsulation of somatic embryos in synthetic seed coats. Hort. Sci., 22(5): 803-809.

Rice, E.L. 1984. Allelopathy. Orlando: Academic Press Inc. 422p.

Roberts, E.H. 1973. Predicting the storage life of seeds. Seed Sci. and Technol., 1: 499- 514.

Roberts, E.H. and King, M.W. 1980. The characteristics of recalcitrant seeds. In: Chin, H.F. and Roberts, E.H. (eds), Recalcitrant Crop Seeds Tropical Press, Kuala Lumpur, Malaysia. pp 1-5.

Rodríguez-Gacio, M.C., Matilla-Vázquez, M.A., and Matilla, A.J., 2009. Seed dormancy and ABA signaling: The breakthrough goes on, Plant Signaling Behavior, 4: 1035-1048.

Rowland, P.E.M. 1974, A summary of growth regulant studies on cotton *Gossypium hirsutum* in the lowland. Rhodesia Agric. J., 71(4): 99-101.

Sakamoto, Y., Mashiko, T., Suzuki, A., and Kawata, H. 1992. Development of encapsulation technology for synthetic seeds. Acta Hortic., 319: 71-76.

Savmell, H. 1972. Responses of sunflower to application of chloromequat. Field Crop Abstr., 25: 804.

Schopfer, P. and Plachy, C. 1984. Control of seed germination by abscisic acid. II. Effect on embryo water uptake in *Brassica napus* L. Plant Physiol., 76: 155-160.

Schopfer, P., Bajracharya, D., and Plachy, C. 1979. Control of seed germination by abscisic acid. Plant Physiol., 64: 822-827.

Senaratna, T, Saxena, P.K, Rao, M.V., and Afele, J. 1995. Synthetic seeds prospects and limitations. Plant Cell Rep., 14: 375-379.

Senaratna, T., Mckersie, B.D., and Bowley, S.R. 1990. Artificial seeds of alfalfa (*Medicago sativa*) induction of dessication tolerance in somatic embryos. In-vitro Cell Dec. Biol., 26: 356-359.

Sengupta, V.K., Pandey, M., and Sirohi, G.S. 1979. Effect of growth regulators on seed germination in groundnut interaction studies. Seed Res., 7: 114-119.

Seong, R.C., Chung, H.J., and Hong, E. H. 1988. Varietal responses of soybean germination and seedling elongation to temperature and polyethylene glycol solution. Korean J. Crop Sci., 33: 31-37.

Sharma, A., Tandon, P., and Kumar, A. 1992. Regeneration of *Dendrobium wardianum* Warner (Orchid) from synthetic seeds. Indian J. Exp. Biol., 30: 747-748.

Singh, F. 1991. Encapsulation of *Spathoglottis plicata* protocorms. Lindleyana, 6(2): 61-63.

Singh, J. and Brar, Z.S. 1999. Effect of growth regulators and defoliant on yield and maturity of upland cotton (*Gossypium hirsutum*) under irrigated conditions. Indian J. Agron., 44(1): 179-184.

Sondheimer, E., Galson, E.C., Tinelli, E., and Walton, D.C. 1974. The Metabolism of hormones during seed germination and dormancy: IV. The Metabolism of (S)-2-C-abscisic acid in ash seed. Plant Physiol., 54(6): 803-808.

Souhail, M. and Chaabane, R. 2009. Toxicity of the salty and pericarp inhibition on the germination of some *Atriplex* species. Am-Euras. J. Toxicol. Sci., 1: 43-49.

Sudhakara, K., Nagaraj, B.N., Santhoshkumar, A.V., Sunilkumar, K.K., and Vijaykumar, N.K. 2000. Studies on the production and storage potential of synthetic seeds in cocoa (*Theobroma cacao* L.). Seed Res., 28(2): 119-125.

Sujatha, G.R. and Kumari, B.D. 2007. Micropropagation, encapsulation and growth of *Artimisia vulgaris* node explants for germplasm preservation. Plant Cell Tissue Organ Cult., 74(1): 93-100.

Sunilkumar, K.K., Sudhakara, K., and Vijayakumar, N.K. 2000. An attempt to improve storage life of *Hopea parviflora* seeds through synthetic seed production. Seed Res., 28(2): 126-130.

Sushmita, B., Khan, H.A., and Reddy, P.V. 1998. In-vitro seed germination, production of synthetic seed and regeneration of plantlets of *Phalaenopsis* hybrid. Ann. Agric. Sci., Cairo, 43(2): 539-543.

Svensson, S.B. 1971. The effect of coumarin on root growth and root histology. Physiol. Plantar., 24: 446-470.

Swiatek, A., Lenjou, M., Van Bockstaele, D., Inzé, D., and Van Onckelen, H. 2002. Differential effect of jasmonic acid and abscisic acid on cell cycle progression in tobacco BY-2 cells. Plant Physiol., 128: 201-211.

Timbert, R., Barbotin, J.N., Kersulec, A., Bazinet, C., and Thomas, D. 1995. Physicochemical properties of the encapsulation matrix and germination of carrot somatic embryos. Biotech. Bioeng., 46 (6): 573-578.

Tompsett, P.B. 1984a. Desiccation studies in relation to the storage of *Araucaria* seed. Ann. Appl. Biol. 105: 581-586.

Tompsett, P.B. 1984b. The effect of moisture content and temperature on the seed storage life of *Araucaria columnaris*. Seed Sci. Technol., 12: 801-816.

Toruan, M. and Sumaryono, N. 1994. Application of synthetic seed technology for mass clonal propagation of crop plants. Bull. Bioteknologi-Perkebunan, 1(1): 10-16.

Ulitzur, S. and Poljakoff-mayber, A. 1963. Oxidative phosphorylation in germinating Lettuce seeds. J. Exp. Bot., 14: 95-100.

Ungar, I.A. 1995. Seed germination and seed-bank ecology in halophytes. In: Kigel, J. and Galili, G. (eds), Seed Development and germination, Marcel Dekker, New York, pp 599-628.

Valio, I.F.M. 1976. Germination of coffee seeds (*Coffea Arabica* L. cv. Mundo Novo). J. Exp. Bot., 27: 983-991.

Van Sumere, C.F., Cottenie, J., De Greef, J., and Kint, J. 1972. Biochemical studies in relation to the possible germination regulatory role of naturally occurring coumarin and phenolics. Recent Adv. Phytochemistry, 4: 165-221.

Vertucci, V.W. and Roos, E.E. 1990. Theoretical basis of protocols for seed storage. Plant Physiol., 94(3): 1019-1023.

Williams, R.D. and Bartholomew, P.W. 2011. Variation of hairy vetch seed weight alters germination and seedling growth response to an allelochemical. Proceedings of Southern Weed Science Society. 64: 134.

Withers, L.A. 1988. Germplasam conservation. In: Block, G. and Marsh, J. (eds). Applications of plant cell and tissue culture, Wiley, Chinchester, UK., pp 163-177.

Zeevaart, J.A.D. and Osborne, H.D. 1965. Comparative effects of some Amo-1618 analogs on gibberellin production in *Fusarium moniliforme* and on growth in higher plants. Planta., 66: 320-330.

Zhang, W. 2008. A Comprehensive study on the role of hormones, seed coat and genes during the germination of canola (*Brassica napus*) seed under adverse environmental conditions. Ph. D. thesis, University of Saskatchewan, Saskatoon, Canada.167p.

Zobel, A.M. and Brown, S.A. 1995. Coumarins in the interaction between the plant and its environment. Allelopathy J., 2: 9-22.

Zobel, A.M., Wang, J., March, R.E., and Brown, S.A. 1991. Identification of eight coumarins occurring with psoralen, xanthotoxin, and bergapten on leaf surfaces. J. Chem. Ecol., 17: 9.

ENHANCEMENT OF STORAGE LIFE OF COCOA (*Theobroma cacao* L.) SEEDS THROUGH ENCAPSULATION AND GERMINATION INHIBITION

By

SHIRAN KALAPPURAKKAL

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ABSTRACT

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DEPARTMENT OF TREE PHYSIOLOGY AND BREEDING
COLLEGE OF FORESTRY
KERALA AGRICULTURAL UNIVERSITY
VELLANIKKARA, THRISSUR-680 656
KERALA, INDIA

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ABSTRACT

Studies on enhancement of storage life of cocoa (*Theobroma cocoa* L.) seeds through encapsulation and germination inhibition were carried out at the Department of Tree Physiology and Breeding , College of Forestry, Kerala Agricultural University, Vellanikkara, during 2010-12. 100-120 days old cocoa pods of, collected from polyclonal cocoa garden of Kerala Agricultural University were utilized for the standardization of microencapsulation techniques. To enhance longevity, germination inhibitor, osmoticum and desiccation were used and longevity of embryonic axes and synthetic seeds were observed. Cotyledon attachment on embryonic axis influenced the germination of embryo. The absence of cotyledon had checked the shoot regeneration of embryonic axes. Embryonic axes with ¼ cotyledon had good root and shoot regeneration with higher germination percentage. The storage medium influenced the longevity of embryonic axes. Higher longevity and viability was observed in ½ MS media while least longevity was observed in dry cotton. The presence of 10^{-3}M coumarin in the basal media (½ MS) retarded the root growth of embryonic axes. ABA added to media in 10^{-4}M concentration had increased the longevity of embryonic axes up to 56 days. Cycocel did not have influence on longevity of embryo. No combination effect by the germination inhibitor was seen in the experiment. Osmotic concentrations had positive influence on enhancing longevity of embryonic axes. Media with 250 mM sorbitol enhanced the longevity of embryonic axes to 65 days. Mannitol added media had crystallization during the storage which resulted in low longevity. Sodium chloride in higher concentration had checked the growth of embryonic axes and had shorter longevity.

The encapsulation of embryonic axes had increased the longevity of seeds to 40 days while embryonic axes had longevity of 29 days. Maximum longevity of 70 days was observed in synthetic seeds stored in 250 mM sorbitol added media. Longevity was found less than 10 days in dry cotton due to absence of moisture content. The incorporation of MS media in the encapsulation, reduced the longevity of synthetic seeds. In addition, MS media and inhibitor combination in the encapsulation did not

have influence on longevity. The MS media reduced the activity of inhibitor action. The 10^{-3}M ABA, 250 mM sobritol and 500 mM sobritol in the encapsulation enhanced longevity to 65 days. Desiccation had a little effect on longevity and it had a negative correlation between RH level and longevity. Longevity had a positive correlation with duration of desiccation. The maximum longevity was possible by storing synthetic seeds in 250 mM sorbitol added media for 55 days and transferring to wet cotton for germination. It had a longevity of 89 days with 80 percent germination.

The results of present study indicated that it is possible to store recalcitrant seeds by encapsulation and altering surrounding condition of embryo. The storage potential can be increased up to 3 months by the encapsulation and osmotic environment.