ENHANCEMENT OF STORAGE LIFE OF SYNTHETIC SEEDS OF COCOA (*Theobroma Cacao* L.) THROUGH GERMINATION INHIBITION, DESICCATION AND LOW TEMPERATURE TREATMENTS

By

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(2012 – 17 – 108)

THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"Enhancement of storage life of synthetic seeds of cocoa (*Theobroma cacao* L.) through germination inhibition, desiccation and low temperature treatments"** 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 of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Place: Vellanikkara,

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Mobin, K. M.

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ABBREVIATIONS

%	Percent
ABA	Abscisic Acid
CCC	Chloro Choline Chloride
et al	Co-workers
KIN	Kinetin
M	Molar
mM	Milli molar
mm	Milli meter
MS	Murashige and Skoog medium
NAA	Naphthalene Acetic Acid
°C	Degree celsius
PEG	Poly Ethylene Glycol
RH	Relative Humidity
UV	Ultra Violet

INTRODUCTION

1 INTRODUCTION

Botanically, a seed is defined as a 'mature ovule' or a reproductive unit formed from a fertilized ovule, consisting of an embryo, reserve food, and a protective cover. Seeds are the major medium for propagation in a wide variety of plant and tree species. Hence, storage of seed has got prime attention ever since humans began to domesticate plants. Since agriculture began, farmers have learned to maintain viable seeds from one growing season to the next. Seed storage is concerned with the preservation of seed in adequate means until their planting season without compromising its viability. The longevity of seeds is a species- specific characteristic. The duration of successful storage of seed depends upon both the objectives and the species concerned.

Roberts (1973) has defined two categories of seed storage behaviour: orthodox and recalcitrant. Orthodox seeds are seeds that acquire desiccation tolerance during development and can be dried to low water contents (generally less than 5%), without loss of viability for a predictable period. Hence, storage of orthodox seed has never been a problem. However, recalcitrant seeds which are shed at high water contents, are sensitive to desiccation, and are also metabolically active on shedding. This makes them difficult to store. A third category with properties in between the orthodox and recalcitrant has since been identified and was termed as intermediate (Ellis et al., 1990).

Literally, recalcitrant means obstinate, hard to control, obstinately disobedient, does not obey the normal rules etc. This definition itself explains the general behaviour of recalcitrant seeds. Seeds with recalcitrant nature exhibit a great range of variation in shape, size, colour and behaviour. Recalcitrant seeds are mainly found in trees from tropical humid forests, but relatively few temperate tree species also produce recalcitrant seeds. In the tropics, many economically important plantation crops like cocoa, rubber, coconut; timber species belonging to the family Dipterocarpaceae,

Araucariaceae and fruit species such as mango, jack fruit, rambuttan, mangosteen etc. with recalcitrant seeds form the backbone of developing countries. Besides producing short-lived seeds, many of the recalcitrant-seeded species are threatened by overexploitation, indiscriminate harvesting and habitat loss (Berjak, 2005). These seeds are difficult to store due to varying desiccation sensitivity and intolerance to low temperature.

Due to various complex problems, storage of the recalcitrant seed becomes difficult in different species. Gene banks such as seed banks and *in vitro* gene banks have been established fairly all over the world. They are limited to orthodox seeds while it is scarce for recalcitrant seeds. Hence, understanding the phenomenon of seed recalcitrance, and consequently developing sound storage protocols for species producing such seeds, is of major scientific and practical importance.

As like most of the tropical evergreen species, *Theobroma cacao* L. also produces seeds with recalcitrant nature. It is widely domesticated for the commercial purpose and is grown as a plantation crop and agroforestry species. The recommended polyclonal plantations are very less in number which makes a need for the viable cocoa seed to be stored for a longer period. Usually the viability of the cocoa seed last only for one week which makes the planting difficult. Among the various approaches of storage, traditional techniques were found to be a failure, because it could not maintain the viability for a considerable period. However, synthetic seed technology has been proved to be a progressive approach for extending storage life, germplasm conservation and transfer of cocoa seeds to far areas (Shiran, 2012).

Synthetic seeds are defined as artificially encapsulated somatic embryos, shoot tips, axillary buds or any other meristematic tissue, used for sowing as seed and possess the ability to convert into whole plant under *in vitro* and *in vivo* conditions and keep its potential also after storage (Capuano et al., 1998). These artificial seeds are believed to be the manmade counter part of true seeds. The encapsulation provided around the

embryonic axes helps to overcome the recalcitrance which is supposed to be due to seed coat and storage tissue.

The possibilities of incorporation of germination inhibitors in storage media, alteration of seed moisture content by desiccation treatments and low temperature treatments to minimize or avoid fungal growth has great practical relevance in the field of recalcitrant seed storage. In this context, synthetic seed technology coupled with germination inhibitors, desiccation treatments and low temperature treatments were attempted in cocoa seed, which can be practically explored for other forest tree species also.

The present study was carried out with the objectives to enhance the storage potential of synthetic seeds of cocoa through the use of germination inhibitors, desiccation treatments and low temperature treatments. The study also aimed to develop desiccation protocol and low temperature protocol for the storage of synthetic seeds of cocoa.

REVIEW OF LITERATURE

2 REVIEW OF LITERATURE

Seed storage is imperative, not only to provide good-quality planting material from season to season, as well as inter seasonal food reserves and feedstock, but also in the long term as base and active collections conserving genetic resources. 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 accession maintained world-wide (Withers, 1988; Bonner, 1990; Vertucci and Roos, 1990; Hong and Ellis, 1996; Engels and Engelmann, 1998). Given appropriate facilities, storage for all these purposes can readily be achieved only if the seeds exhibit orthodox post-harvest physiology (Roberts, 1973). Based on their storage behaviour, seeds can be divided into three groups: orthodox, intermediate and recalcitrant.

Orthodox seeds are seeds that acquire desiccation tolerance during development, can dry to low water contents (generally less than 5%), and retain viability in the dry state for predictable periods (Roberts, 1973). Recalcitrant seeds, on the other hand, are shed at high water contents, ranging from 0.4 to 4.0 g water per g dry matter (gg^{-1}), are sensitive to desiccation, and are also metabolically active on shedding (Roberts, 1973; Ellis et al., 1990; Ellis, 1991). A group of species which can be dried to a moisture content low enough to qualify as orthodox, but are sensitive to low temperatures typical for orthodox seeds has been termed 'intermediate' (Ellis et al., 1990). Because many economically important tropical tree species have recalcitrant seeds, a prevailing problem is how to successfully store them (Daws et al., 2004; Finch-Savage, 1996; Pammenter and Berjak, 1999; Tommasi et al., 1999; Tompsett, 1992). Further, since many recalcitrant seeds are damaged at storage temperatures < 15°C, their storage lifespan is quite short, varying from two weeks to several months (Chin and Roberts, 1980; Tompsett and Kemp, 1996; King and Roberts, 1982).

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.

There is a huge number of species with recalcitrant or suspected recalcitrant seeds, and the majority are wild species. The traditional method for *ex situ* conservation of genetic resources of recalcitrant seed species is as field gene banks. There are, however, several serious problems with field gene banks, including exposure to natural disasters, attacks by pests and pathogens, and high maintenance costs. *In vitro* culture techniques offer alternative approaches to improve the safety and cost-effectiveness of conservation of these problem species (Engels and Engelmann, 1998). For medium-term conservation, the growth of the plant material is reduced by modifying the environmental conditions and/or the culture medium, thus increasing the intervals between subcultures (Drew et al., 2000). The development of *in vitro* conservation techniques for recalcitrant seed species is currently limited for various reasons. Recalcitrant seeds have characteristics which make their introduction and conservation *in vitro* difficult, such as their usually large size, the important variation between and among seed lots in their moisture content and developmental stage, the very complex tissue composition of embryos and the high level of associated contaminants, particularly with tropical species (Berjak and Pammenter, 2008). Research is underway to develop *in vitro* culture and conservation protocols for additional species.

2.1 RECALCITRANT SEED STORAGE

Recalcitrant seeds, are characterized by post- harvest life spans of the order of days to months, or, for temperate species, perhaps a year or two, as long as such seeds will tolerate low temperatures (e.g. Chin and Roberts, 1980). Besides producing short-lived seeds, many of the recalcitrant-seeded species are threatened by over exploitation, indiscriminate harvesting and habitat loss (Berjak, 2005). Among their traits, recalcitrant seeds are very variable both within and across species as well as inter and intra-seasonally. For example, seeds of *Camellia sinensis* harvested from the same tree population had mean water contents as wide as 4.4 and 2.0 g g^{-1} in consecutive years; Berjak and Pammenter (2008) and Xia et al. (2012), investigating range of species of *Quercus* and *Cyclobalanopsis* seeds, reported wide differences in the rates at which the seeds are dried when dehydrated from 1.0 g g^{-1} under identical conditions. Recalcitrant seeds either lack, or do not express the various processes and mechanisms typifying the acquisition of desiccation tolerance as occurs in orthodox seeds. Hence, understanding the phenomenon of seed recalcitrance, and consequently developing sound conservation practices for species producing such seeds, are major scientific and practical challenges.

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). They cannot tolerate water loss and so cannot be stored using conventional seed bank conditions. Particularly with respect to storage, recalcitrant seeds do not undergo intracellular dedifferentiation nor any significant metabolic shutdown. Embryos of recalcitrant seeds remain metabolically active, with little or no reduction in extent of the extensive intracellular membranes. They can be stored intact only until germination is initiated, which can range from a few days to several months, depending on species (Berjak et al., 1990). Recalcitrant seeds cannot be dried beyond a critical moisture content without damage. When fresh recalcitrant seeds begin to dry, viability

also gets reduced. Loss of viability gets increased as the moisture content get reduced and at a certain moisture content termed "critical moisture content" (Kings and Roberts, 1979; 1980) or "lowest safe moisture content" (Tompsett, 1984) viability get completely reduced. If drying continues further, viability is eventually reduced to zero. Moist storage of 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.

Because they are shed at a range of species-related high water contents and will not tolerate dehydration to levels appropriate for low relative humidity (RH)/sub-zero temperature storage, recalcitrant seeds cannot be conserved using standard gene bank approaches. Short/medium- term storage is possible, but the conditions must preclude water loss. However, as recalcitrant seeds are not only metabolically active, but will germinate at the water contents typifying shedding, such hydrated storage is strictly a short-term option (Berjak and Pammenter, 2008). Additionally, proliferation of seed-associated fungi under hydrated storage conditions is an enduring problem, especially for seeds of tropical/sub-tropical provenance, which almost invariably harbor fungal inoculum internally (Sutherland et al., 2002). One of the major difficulties in the short term storage of recalcitrant 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). Fungicide treatment has been shown to be highly effective in extending storage life span of recalcitrant seeds in hydrated storage, e.g. for *A. marina* (Calistru et al., 2000) and *Hopea parviflora* (Sunilkumar and Sudhakara, 1998). While short-term storage of recalcitrant seeds under the best conditions is possible, currently the only feasible option for long-term storage of the germplasm is by cryopreservation (Engelmann, 2011), which entails complex processing and *in vitro* recovery presently requiring to be refined on an individual species basis.

2.2 SYNTHETIC SEED TECHNOLOGY

The concept of synthetic seed was given by Murashige (1997), but first report on the development of synthetic seed was published by Kitto and Janik (1982). Later, Redenbaugh et al. (1989) were successful in producing synthetic seeds of alfalfa by encapsulating somatic embryos with alginate hydrogel. Since then several research groups have been working on synthetic seeds with different plant species including cereals, fruits, vegetables, ornamentals, medicinal plants, forest trees and orchids (Rai et al., 2009).

A synthetic seed or artificial seed is referred to as artificially encapsulated somatic embryo, shoot bud or any other meristematic tissue that can be used to functionally mimic seed for sowing and possesses the ability to convert into a plant under *in vitro* or *ex vitro* conditions and that can retain this potential even after storage (Capuano et al., 1998; Ara et al., 2000). Based on technology established there are two types of synthetic seeds: hydrated and desiccated. Although the most studied method involves the encapsulation of propagules in hydrogel for synthetic seed production (Redenbaugh and Walker, 1990). A number of coating agents such as sodium alginate, potassium alginate, carrageenan, sodium alginate with gelatin, sodium pectate, carboxyl methyl cellulose etc. are used for encapsulation and among these substances sodium alginate has been extensively used (Redenbaugh et al., 1987: Rao et al., 1993; Ara et al., 2000). Mathur and Ahuja (1991) 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. Addition of nutrients, carbon sources, growth regulators and antimicrobial agents such as antibiotics fungicides etc. in the gel matrix which apparently served as a synthetic endosperm, facilitated growth and survival of encapsulated propagules (Redenbaugh et al., 1987; Gray, 1987; Bapat and Minal, 2005).

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 or bead around the embryo. The capsule hardness of 0.5-2.0 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).

Encapsulation technology is an exciting and rapidly growing area of seed biotechnological research. It is an excellent technique for propagation of rare hybrids, elite genotypes, genetically engineered plant, and rare and endangered plants for which the seeds are either very expensive and are not available (Mandal et al., 2000). Conservation is an important aspect of encapsulation technology. *In vitro* conservation involves the maintenance of explants in a pathogen free environment for short to medium or long-term (Engelmann, 2011). Various approaches have been applied for slow-growth maintenance of cultures (Gupta and Mandal, 2003). These include techniques like:

- Maintenance under reduced temperature and/or reduced light intensity
- Use of growth retardants such as ABA, coumarin, cycocel etc.
- Use of minimal growth medium
- Use of osmoticum (ie. Mannitol, sorbitol, high sucrose concentrations etc.)
- Reduction in oxygen concentration
- Combination of more than one treatment

Long term storage of synthetic seeds can be achieved through storage at ultra-low temperature, termed as cryopreservation, is usually carried by using liquid nitrogen at -196°C (Engelmann, 2011).

Studies on synthetic seed production using somatic embryos have been reported in a few forest species, such as *Paulownia elongata* (Ipekci and Gozukirmizi, 2003), *Eucalyptus citriodora* (Muralidharan and Mascarenhas, 1995) and *Chamaecyparis pisifera* (Maruyama et al., 1997). The concentration of sodium alginate and calcium chloride used as the encapsulation matrix varies depending upon the species or the explant used. Sodium alginate 2% and calcium chloride 75 mM was best for encapsulation of indigenous production of synthetic seeds in *Daucus carota* (Zakia et al., 2007). As per Ibrahim et al. (2003) encapsulated somatic embryos of date palm (*Phoenix dactylifera* L.) coated with 4% (wv^{-1}) sodium alginate dissolved in distilled water, and complexing in a 100 mM $CaNO_3$ solution for an ion exchange duration of 30 minutes resulted in extending the conversion frequency duration. Sudhakara et al., (2000) found out that the ideal concentration for encapsulation of cocoa seed is by encapsulating in a medium containing 4% sodium alginate and 75 mM $CaCl_2$. A 3% sodium alginate with 100 mM $CaCl_2$ has been found to be optimum concentration for the production of uniform synthetic seeds of *Vitex negundo* (Ahmad and Anis, 2010). For germination, the synthetic seeds were cultured on Murashige and Skoog (MS) basal medium supplemented with kinetin (KIN) and α-naphthalene acetic acid (NAA) either singly or in various combinations. MS medium containing 2.5 µM KIN in combination with 1.0 µM NAA was found to be the optimum for maximum (92.6±3.71%) plantlet conversion frequency (Ahmad and Anis, 2010).

2.2.1 Advances in Synthetic Seed Technology

Piatczak and Wysokinska (2013) observed that synthetic seeds of *Centaurium erythraea* encapsulated with 3% sodium alginate and 3% sucrose stored at 4°C

remained viable for 6 weeks. Adding nutrient medium and growth regulator to the alginate matrix increased plantlet recovery from both non-stored and stored synthetic seeds: synthetic seeds retained their viability and ability to form plantlets even after 14 weeks of storage. Kulus and Zalewska (2014) have found out that synthetic seed technology can be valuable in storage of *Chrysanthemum grandiflorum*. A study on alginate encapsulation of *Begonia* micro shoots for short-term storage revealed that when both control and encapsulated shoots were transferred into sterile petri dishes and stored at 4°C and 22°C for 0, 2, 4, 6 and 8 weeks, encapsulation of explants improved survival rate over time irrespective of the medium type or storage environment (Sakhanakho et al., 2013). Datta et al., (1999) reported that synthetic seeds of *Geodorum densiflorum* showed 100% 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. John and Kumar (2006) found out that in tea (*Camellia sinensis*) germination rate of synthetic seeds was significantly higher in acidic pH while, near neutral pH enhanced the growth rate of germinated synthetic seeds.

In an investigation by Mohammad et al. (2013), synthetic seeds of *Rauvolfia tetraphylla* were produced using *in vitro*- proliferated shoots upon complexation with 3% sodium alginate and 100 mM $CaCl_2$. The encapsulated buds were stored at 4, 8, 12, and 16°C and high conversion to plantlets was observed in synthetic seeds stored at 4°C for 4 weeks. 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, 1998; 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 and 25°C. *Pinus patula* synthetic seeds could be stored at 2°C for 120 days without reduction in germination as opposed to non-encapsulated somatic embryos which showed only 9% germination after 20 days at 2°C (Ravindra and Staden, 2005).

Sunilkumar et al. (2000) made an attempt to improve the storage life of synthetic seeds of *Hopea parviflora*, a typical tropical evergreen forest tree species. In this experiment, a viability of up to one and four weeks were obtained for the intact and synthetic seeds stored at temperatures of 0°C and 4°C respectively. But within a period of one week only 30% germination was observed for synthetic seeds and intact seeds stored at room temperature. Synthetic seeds obtained from seeds pre-treated with 2 and 3 mgl^{-1} of ABA showed tolerance to low storage temperature and retained higher percentage germination.

Synthetic seeds can thus be used efficiently to improve the storage potential and as a universal delivery system of *in vitro* plantlets to greenhouse or field.

2.3 COCOA SEED STORAGE

Theobroma cacao is a tropical evergreen tree native to Amazon basin. It is a widely domesticated tree for the commercial purpose and grown as a plantation crop or agroforestry species. The cocoa tree flourishes in the dense shade of warm rain forests in its natural habitat and hence can be cultivated in all similar climatic conditions. 'The best soil for cocoa is forest soil rich in humus. Though three varietal types viz., Criollo, Forastero and Trinitario are recognized, only Forastero types are known to perform well under Indian conditions. Cocoa can be propagated by seeds and vegetative means. As like most of the tropical species recalcitrant nature of the seeds is a limiting factor in the plantation raising. The recommended polyclonal plantations are very less in number which makes the need for the viable seed to store for a long time. Seeds lose viability within a week of harvest of pods. Seeds are to be sown immediately after extraction from these pods' (KAU Package of Practices Recomendations: Crops, 2003 edn.).

The recalcitrant nature of cocoa seeds is supposed to be due to seed coat and storage tissue (King and Roberts, 1980). Prasannakumariamma et al. (2009) reported

that cocoa seeds showed difference in germination depending on the sowing months. Higher germination was seen during March, whereas December, January and April months showed low germination. Vanitha et al. (2008a) on their studies on seed coat colour variation revealed that pink coloured seeds registered higher recovery of good-quality seeds. Loss of moisture and leakage of electrolytes increased with the increase in the duration of storage, accompanied by loss of viability. The rate of decline was higher when seeds were stored instead of pods. The study also pointed out that cocoa seeds mixed with moist charcoal and stored at 10°C minimized the rate of drying and loss of viability up to 40 days. The lowest safe moisture content to retain 50% viability and above appeared to be 29% (Vanitha et al., 2008b).

In an experiment conducted by Sudhakara et al. (2000), synthetic seeds of 4.5-5 mm were prepared from excised embryos of mature seeds (~120 days old pods), collected from 10-year old cocoa trees, by encapsulating in a medium containing 4% sodium alginate and 75 mM $CaCl_2$. After storage for 0-25 days at 10°C in either wet or dry cotton medium under aseptic conditions, seeds were tested for germination. The germination of synthetic seeds was 97.3% after encapsulation, and decreased to 71 and 49% when stored for 25 days in wet or dry cotton medium, respectively. The germination of seeds extracted from fresh pods was 90%, and decreased to 76% at the end of a 5 day storage at 27°C. Complete mortality was observed at the end of a 10 day storage period. The time taken from initiation of seed germination to complete root formation and shoot emergence was shorter in synthetic than in normal seeds.

Shiran (2012) found that embryonic axes having ¼ cotyledon stored in ½ MS media had higher germination and longevity compared to seeds with full and ½ cotyledon stored in full and ½ MS media. The minimum attachment of endosperm ensured high germination. It also helped in the easy making of synthetic seeds. Embryonic axes stored in germination inhibitors had significant difference in root, shoot regeneration and longevity. Various germination inhibitors and their varied concentrations showed significant difference in the longevity of the embryonic axes of

cocoa seeds. Higher osmotic levels of the osmoticum in the media controlled the water uptake and thus had an influence on the growth of embryonic axes. The investigation also found out that encapsulated embryonic axes encapsulation enhanced the longevity of embryonic axes from 29 to 40 days. 250 mM sorbitol added media enhanced the longevity of synthetic seeds to 70 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. As a whole the experiment indicated that it is possible to store recalcitrant seeds by encapsulation and altering surrounding condition of embryo.

Liang and Sun (2000) examined the effect of drying rate on desiccation tolerance of *Theobroma cacao* seed axes at 16°C. They found out that rapid drying at low RH and slow drying at high RH were harmful to cocoa axes because electrolyte leakage began to increase and axes viability began to decrease at high water contents. These findings confirmed that the physiological basis of the optimal drying rates is related to both mechanical stress during desiccation and the length of desiccation duration during which deleterious reactions may occur.

2.4 ABSCISIC ACID (ABA)

Abscisic acid (ABA) is a plant hormone commonly 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). ABA plays important roles in many aspects of seed development, including accumulation of storage compounds, acquisition of desiccation tolerance, induction of seed dormancy and suppression of precocious germination. ABA is implicated in the control of many processes during embryogenesis and germination of zygotic embryos (Skriver and Mundy, 1990). These processes include accumulation of storage reserves and maintenance of embryos in a maturation stage by the prevention of precocious

germination (Kermode, 1990). ABA levels in plants increase in response to abiotic stresses such as heat, freezing or drought, triggering specific biochemical responses (Leung and Giraudat, 1998). When the stress is alleviated, the hormone is metabolized to inactive products. ABA levels in plant cells are dynamically maintained by continual synthesis, transport and degradation (Cutler and Krochko, 1999).

A review of recent progress in the role of ABA in plant tissue culture (Rai et al., 2011) suggest that ABA plays a significant role in the regulation of many physiological processes of plants. It is often used in tissue culture systems to promote somatic embryogenesis and to enhance somatic embryo quality by increasing desiccation tolerance and preventing precocious germination. ABA is also employed to induce somatic embryos to enter a quiescent state in plant tissue culture systems and during synthetic seed research. Application of exogenous ABA improves *in vitro* conservation and the adaptive response of plant cell and tissues to various environmental stresses. ABA can act as anti-transpirant during the acclimatization of tissue culture-raised plantlets and reduces relative water loss of leaves during the *ex vitro* transfer of plantlets even when non-functional stomata are present.

Xiaowen et al. (2013) in their studies on seed dormancy in four Tibetan Plateau *Vicia* species reported that fresh seeds were more sensitive to exogenous ABA than stored seeds, indicating that storage decreased embryo sensitivity to ABA. Data on the study by Kanno et al. (2010) indicates that ABA metabolism depends on developmental stages and tissues, and that ABA interacts with other hormones to regulate seed developmental processes. 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 Giberellic acid (GA) were involved in seed germination and the dormancy in response to fluctuating temperature. In plants, ABA is synthesized through the cleavage and oxidation of carotenoids, and is catanolized via hydroxylation or by

conjugation to glucose (Nambara and Marion-Poll, 2005). In an experiment conducted by Shiran (2012), the cocoa seeds were able to store in 10^{-4} M ABA for 56 days without germination. ABA concentrations of 10^{-5} M and 10^{-6} M were found to be ineffective to inhibit the growth of embryonic axes of cocoa. The study revealed that ABA in exogenous condition as in dormant seeds effect the growth of seeds.

2.5 COUMARIN

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, 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). Coumarin affects root formation and function (Abenavoli et al., 2001a, 2004), decreases respiration and photosynthesis (Moreland and Novitzky, 1987), and influences nitrogen uptake and metabolism (Abenavoli et al., 2001b, 2003). Coumarin (1, 2 benzopyrone), is a strong inhibitor of seed germination and root growth. It is known that coumarin is modulated by light (Svensonn 1971, Aliotta et al., 1992, Abenavoli et al., 2001).

Coumarin also is involved in cell differentiation, acting as a plant growth regulator and affecting auxin metabolism (Abenavoli et al., 2001a). Coumarin is able to promote or inhibit plant growth, the response being species-specific and concentration-dependent (Aliotta et al., 1992). Exogenous application of a coumarin derivative, 4-methylumbelliferone (4-MU), in *Arabidopsis thaliana* inhibits seed germination by mainly reducing primary root growth (Li and Gao, 2011). In drum wheat seeds, coumarin inhibited seed germination at concentrations above 200 mM (Abenovoli et al., 2006). A study on the effects of coumarin on radish seed germination and radicle elongation by Peal and Williams (2002) found that at all concentrations of

coumarin had a negative effect on radish seed germination and radicle growth. The process of hydration and dehydration of radish seed in the soil in the presence of coumarin may provide further insight as to the soil-seed dynamics in allelopathic interactions among plants. Shiran (2012) found that root growth of cocoa embryonic axes was retarded by the medium containing 10^{-3}M coumarin. The elongated embryonic axes also produced abnormal swelling.

2.6 CYCOCEL

(2-chloroethyl) trimethylammonium chloride (CCC/cycocel) has a growth retardant property on seeds (Abenavoli et al., 2001; Aliotta et al., 1993). Application of CCC inhibited seed germination of non-dormant groundnut cultivar (Sengupta et al., 1979).

Cycocel has been found to give good results in height control. Application of cycocel decreased plant height in sunflower (Savmall, 1972; Dorell, 1973; Bhattacharjee and Gupta, 1981). Plant height can be effectively decreased by the application of CCC (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. A study on *Fusarium moniliforme* indicated that addition of CCC to the culture medium can suppress gibberellin production. A concentration of 0.1 mg l^{-1} of CCC causes 5% inhibition whereas 10 mgl^{-1} and higher concentrations fully suppress GA production (Ninnemann et al., 1964). As per the study by Shiran (2012) cycocel at 10^{-2} M, 10^{-3} M and 10^{-4} M did not show any inhibitory influence on root and shoot regeneration in cocoa embryonic axes.

2.7 OSMOTICA

Water availability is one of the main environmental factors able to influence the germinative process of seeds. In these conditions, the ability of seeds to maintain their viability during the storage can be an adaptive advantage of species found in different biomes. Water relations between the embryo and its environment play regulatory role in embryo development, particularly during maturation (Bradford and Chandler, 1992). Osmotic agents not only act as a common source of carbon in cell culture media, 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.

Sorbitol plays a crucial role not only on cell growth and ethanol production but also on the protection of cellular proteins from stress responses (Sootsuwan et al., 2013). Marquez et al. (2011) revealed that the osmotic agents such as sorbitol and PEG did not have positive effects on embryo maturation of avocado somatic embryos. The study also showed that medium water potential was influenced by sorbitol concentration. A study on *in vitro* conservation of germplasm of *Asparagus racemosus* (Ankita and Animesh, 2013) pointed out that mannitol and sorbitol (10 g and 80 mg respectively) helped to reduce the growth considerably. Temperature also served as a big influencing factor. Regeneration frequency in Super Basmati decreased by the addition of sorbitol (Naqvi et al., 2005). Shiran (2012) identified that application of 250 mM and 500 mM sorbitol inhibited the root development in cocoa embryonic axes. The embryonic axes were able to survive up to 70 days without affecting there viability. This study has proved the osmotic effect of sorbitol.

Polyethylene glycol (PEG) solutions can be used to promote early and synchronous germination of seeds at cool temperatures. Duan et al. (2004) noted a recovery response in germination when seeds of *Chenopodium glaucum* were transferred from PEG solutions to water.

2.8 RECALCITRANT SEED DESICCATION TOLERANCE

A characteristic of recalcitrant seeds is that they are metabolically active when they are shed. However, the type and intensity of metabolism differ among recalcitrant seeds of different species, depending on the developmental status and water concentration at shedding. Variation in desiccation sensitivity between species have been reported in many genera of the family Dipterocarpaceae (Thompsett, 1984), and other genera like *Acer* (Olsen and Gabriel, 1974) and *Araucaria* (Tompsett, 1984). To explain this, it must be appreciated that unlike the situation in orthodox seeds, there is no cessation of metabolism in recalcitrant seeds (reviewed by Finch-Savage, 1996). Instead, developmental events progress, without any outward signs, into those of germination, without an exogenous water supply (Berjak et al., 1989). As discussed by those authors, in some cases germination will ensue in a matter of days after shedding; seeds of some species may be poised for immediate germination; while seeds of yet other species are shed with embryos still having to undergo considerable pre-germination development. These differences have marked effects on the degree of desiccation that the seeds will tolerate, thereby contributing to unpredictable variability. For example, Lin and Chen, (1995), working with *M. thunbergii*, showed that developing seeds lost viability within 30 days when dried at 73% relative humidity and 25°C, while those that were mature were able to tolerate 19% loss of water before germinability declined. Differing degrees of desiccation sensitivity have been similarly correlated with embryo/seed developmental status for *Landolphia kirkii* and *Camellia sinensis* (Pammenter et al., 1991; Berjak et al., 1992). It appears generally that for recalcitrant seeds of most species, the least desiccation sensitive stage occurs when the metabolic rate is lowest, which usually coincides with natural shedding. The axes of the recalcitrant seeds are at considerably higher water content than the cotyledons (Berjak et al., 1989; Fu et al., 1993).

Lack of the ability for metabolic switch-off as occurs in orthodox seeds, is one of the possible basic reasons that recalcitrant seeds are desiccation sensitive. Metabolism progresses without any obvious punctuation from development into germination, the onset of which strictly curtails the period for which the seeds can be stored. Nevertheless, hydrated storage under RH conditions, is necessary to maintain viability in the short- to medium-term. However, the seeds will sooner or later germinate in storage, and because those of many tropical/sub-tropical species are chilling-sensitive (Berjak and Pammenter, 2008), preliminary trials must be undertaken to ascertain the lowest temperature which will limit metabolic progress while having no adverse effects on viability. In this context also, dehydration to 'sub-lethal' water contents–originally suggested as a means to slow or obviate germination in storage (King and Roberts, 1980)-actually enhances the rate of germination (e.g., Fu et al., 1993; Tompsett and Pritchard, 1998). As the processes involved in germination progress, recalcitrant seeds become increasingly desiccation sensitive (Farrant et al., 1989; Pammenter and Berjak, 1999) the finding that both germination rate and desiccation sensitivity were enhanced when the seeds of several species were subjected to mild dehydration (Drew et al., 2000; Eggers et al., 2007) is in agreement with those earlier observations.

Liang and Sun (2000) did study on *Theobromo cacao* to examine the hypothesis that drying rate and dehydration duration could interact to determine desiccation tolerance through different physico-chemical mechanisms. The effect of drying rate on desiccation tolerance of cocoa seed axes at 16°C was examined. They found out that rapid drying at low RH and slow drying at high RH were more harmful to cocoa axes, because electrolyte leakage began to increase and axes viability began to decrease at high water contents. Maximum desiccation tolerance was observed with intermediate drying rates at RH between 88% and 91%, indicating the existence of an optimal drying rate or optimal desiccation duration. The optimal drying rate represents

a situation where combined damages from mechanical and metabolic stresses become minimal.

The critical moisture content below which *Quercus coccifera* and *Quercus pubescens* seeds start losing their viability is approximately 26%, which is achieved after a drying period of 5 and 7 days respectively (Ganatsas and Tsakaldami, 2013). Redenbaugh (1990) observed that desiccated artificial seeds had other problems that needed resolution. The desiccation process itself damages the embryos.

2.9 LOW TEMPERATURE STORAGE

2.9.1 Short to medium-term storage of recalcitrant seed

The only way in which vigor and viability of recalcitrant seeds can be maintained is to keep them at the lowest temperature they will withstand, under conditions not permitting water loss, and to eliminate or at least to minimize the seed-associated mycoflora. The latter objective is actually difficult to achieve, but the storage parameters can be optimized once preliminary trials have been conducted on a species basis. Nevertheless, storage of whole seeds is strictly a short to medium term option. This is because the seeds are metabolically active, and will progress from development to germination at the water content typifying shedding (Berjak and Pammenter, 2008). In the case of recalcitrant seeds that are not chilling sensitive (which would be expected for temperate species), storage longevity can be further optimized (Bharuth et al., 2007). However, the nature of the chilling injury is still under investigation. It has become increasingly apparent that the means to optimize short to medium term storage of recalcitrant seeds is to maintain the shedding water content and impose the lowest temperature tolerated without chilling damage. Nevertheless, proliferation of microorganisms particularly fungi will almost inevitably occur, as recalcitrant seeds are seldom free of inoculum that is often located within the seed tissues. Fungicide treatment has been shown to be highly effective in extending storage life span of

recalcitrant seeds in hydrated storage, e.g. for *A. marina* (Calistru et al., 2000) and *Hopea parviflora* (Sunilkumar and Sudhakara, 1998). However, application of non-penetrating fungicides will be effective only in situations where the inoculum is primarily located on the seed surfaces.

2.9.2 Long-term storage of recalcitrant germplasm

Extension of storage life span of recalcitrant seeds remains a short to medium term option, because of the fact that germination at the shedding water content will inevitably occur. Seedling slow growth does offer an alternative to hydrated storage of seeds (Chin, 1996), but this is less than ideal as a long-term means of conservation. Hence cryostorage generally in liquid nitrogen at -196° C or, less ideally, at some temperature below -80° C presently appears to offer the only option for long-term storage. It would be ideal if whole seeds could be cryopreserved, although this is generally not possible because recalcitrant seeds of most species are large, and at high water contents when shed. As discussed above, large seeds cannot be dried rapidly, and slow dehydration to water contents commensurate with efficient cooling (freezing) is lethal. However, for survival at cryogenic temperatures, water content must be reduced to a level obviating lethal ice crystallization during cooling. While reduction of water content to, or near to, the level where only non-freezable water is present achieves this for orthodox seeds, there are only a few documented cases of non-orthodox seeds transiently surviving such drastic levels of dehydration (reviewed by Berjak and Pammenter, 2004).

Successful cryopreservation of small non-orthodox seeds has been achieved in cases where dehydration could be achieved rapidly, e.g. *Azadirachta indica* (Berjak and Dumet, 1996), *Warburgia salutaris* (Kioko et al., 1999; 2003) and *Wasabia japonica* (Potts and Lumpkin, 2000). The most favourable storage temperatures for *Hopea hainanensis* were 15°C and 20°C (Lan et al., 2012).

If whole seeds are optimally thawed and rehydrated after retrieval from cryostorage, seedlings should, theoretically, be able to be generated in a greenhouse without an intervening in vitro stage. However, in the great majority of cases, recalcitrant seeds are far too large, necessitating the use of the excised embryonic axes as explants for cryopreservation. The great advantage offered by excised axes (which generally constitute only an insignificant component by volume or mass of the entire seed) is that they are very small and amenable to rapid dehydration by flash drying (Pammenter et al., 2002). A further aspect that could be profitably pursued is to induce a measure of axes desiccation and chilling tolerance prior to cryopreservation, as carried out for the temperate species, *A. saccharinum* (Beardmore and Whittle, 2005). However, whether or not this would be successful for highly recalcitrant tropical species is a matter of conjecture. Developing sound storage practices for recalcitrant seeds are of much relevant in the present scenario.

2.10 WORKS DONE AT COLLLEGE OF FORESTRY, KAU RELATED TO RECALCITRANT SEED STORAGE

Studies done on *Hopea parviflora* (Sunilkumar and Sudhakra, 1998; Sunilkumar et al, 2000) revealed that, moisture content of intact seeds/seeds without seed coat/excised embryonic axes decreased with increase in intensity and duration of desiccation levels and only the seeds without seed coat showed significant difference in moisture content due to maturity level. Increasing the duration of desiccation significantly increased the leachate conductivity of intact seeds/seeds without seed coat/embryonic axes. Synthetic seeds stored in 4°C retained maximum viability after four weeks, compared to those stored at 20°C and 27°C, however after cryopreservation the propagules invariably failed to regenerate but could retain green colour for two to three days.

Sunilkumar et al, (2000) examined that storing the de-winged *Hopea parviflora* seeds with a moisture below 30% can result in rapid decline in seed viability due to dehydration injuries irrespective of storage temperature. Storing fungicide treated winged seeds collected just before natural seed shedding, at 10°C retained high germination percentage up to 40 days. Synthetic seeds were also successfully stored up to 1 month at 10°C without significant reduction in germination percentage.

A study on the production and storage potential of synthetic seeds in cocoa (Sudhakara et al, 2000) showed that encapsulated embryos stored in wet cotton was more effective than that stored in dry cotton. By this, the embryos keep viability for more than 25 days with 71.2% germination (wet cotton) and 49.9% germination (dry cotton). In the case of intact seeds, the seeds lost viability within 10 days even though its initial germination was 90%. In the case of artificial seed, initial germination was 97.43%.

Shiran (2012) on his study to enhance storage life of embryonic axes of cocoa found that, embryonic axes having ¼ cotyledon stored in ½ MS media had higher germination and longevity compared to seeds with full and ½ cotyledon stored in full and ½ MS media. Embryonic axes stored in germination inhibitors had significant difference in root, shoot regeneration and longevity. Various germination inhibitors and their varied concentrations showed significant difference in the longevity of the embryonic axes of cocoa seeds. Higher osmotic levels of the osmoticum in the media controlled the water uptake and thus had an influence on the growth of embryonic axes. 250 mM sorbitol added media enhanced the longevity of synthetic cocoa seeds to 70 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.

MATERIALS AND METHODS

3 MATERIALS AND METHODS

The research entitled "Enhancement of storage life of synthetic seeds of cocoa (*Theobroma cacao* L.) through germination inhibition, desiccation and low temperature treatments" was undertaken during the period 2012-2014. The investigation was carried out in the Tissue Culture Laboratory, Department of Tree Physiology and Breeding, College of Forestry, Kerala Agricultural University, Vellanikkara, Thrissur district, Kerala.

A description of the materials used and the methodology followed for the conduct of the investigation are described below.

3.1 PROPAGULE

Synthetic seeds of cocoa were used as the propagule for this research. Cocoa pods collected from the nine year old cocoa trees in polyclonal seed garden maintained by Cocoa Research Centre, KAU were used for the study (10° 31'N latitude and 76° 13' E longitude). Polyclonal seed garden involves superior self- incompatible parents and it ensures genetic superiority of seeds. Polyclonal seed garden consist of clones of CCRP 1, CCRP 2, CCRP 4, CCRP 7, CCRP9 and TISSA of Forastero variety. The pods were collected based on the visual observation mainly on colour of pods. The yellow ridged cocoa pods, aged between three to four months old were found to be best for the extraction of embryonic axes for the experiment [Plate 1 (A)] (Shiran, 2012). The following criteria recommended in KAU Package of Practices was also ensured while collecting good cocoa pods:

- Husk thickness of pods to be not more than 1 cm
- Pod value (number of pods to give 1 kg wet beans) to be not more than 12
- Number of beans per pod to be not less than 35
- Bean dry weight to be not less than 1 g

Fresh pods collected were brought immediately to the laboratory for conducting the experiments.

Cocoa pods were washed in tap water to remove dust and other foreign particles. It was then wiped with a towel. The pods were then cut open with a sharp knife to take out the beans [Plate 1 (B)]. The pulp surrounding each bean was removed carefully using a stainless steel blade without damaging the embryonic axes. Pulp removed beans were then washed with distilled water several times to make the beans clean [Plate 1 (C)]. Beans/seeds were then treated with 50% WP Carbendazim (Bavistin), for 30 minutes and washed thoroughly to remove the fungicide. It was then brought to the laminar air flow cabinet (which has been UV sterilized for 15 minutes) to extract the embryonic axes. Surgical blade and forceps were used for the purpose. Excision of embryonic axes and inoculation were carried out in a sterile environment with utmost care to avoid any contamination during storage. The embryonic axes with ¼ cotyledon were used for the preparation of synthetic seeds (Shiran, 2012).

3.2 LABWARE STERILISATION

Steel petri plates, forceps, glassware like petri plates, conical flask, blotting papers, tissue papers and other accessories were used. The labwares used for tissue culture were wrapped in aluminium foil and were sterilized in a standard pressure cooker at 15 psi pressure for 20 minutes. Flame sterilization using 70% ethanol in the flames of a bunsen burner was also done at the time of use.

3.3 ENCAPSULATION MEDIA

Sodium alginate encapsulation method was used in this research to make encapsulated synthetic seeds or synthetic cocoa seeds. 4% sodium alginate and 1.1% calcium chloride mixture was used for synthetic seed production (Sudhakara et al., 2000).

3.3.1 Preparation of encapsulation media

Four grams of sodium alginate was weighed and added to 100 ml of distilled water taken in a conical flask with constant stirring to avoid clumping of sodium alginate powder. Sodium alginate solution was then subjected to mild heating in a microwave oven to obtain a gel consistency. Calcium chloride ($CaCl_2$) solution was simultaneously prepared by dissolving 1.1 grams of calcium chloride in 100 ml distilled water. The encapsulation medium, i.e., calcium alginate is made by the combination of these two solutions. These two solutions were sterilized in an autoclave at 15 psi pressure for 30 minutes prior to the preparation of synthetic seeds.

3.4 PREPARATION OF SYNTHETIC COCOA SEEDS

The embryonic axes with ¼ cotyledon extracted using surgical blade and forceps were used for the preparation of synthetic cocoa seeds [Plate 1 (D)]. The whole process was carried out inside the laminar air flow to provide an aseptic condition. The embryonic axes were dropped into sodium alginate solution and was stirred well. These sodium alginate coated embryos were carefully sucked with the help of micropipette shaped so that the diameter is 6 mm and was added one by one into the $CaCl_2$ solution in the conical flask. It was then kept as such for around 20-30 minutes. The encapsulated embryos with a transculent bead like appearance were removed from $CaCl_2$ after 20-30 minutes [Plate 1 (E)]. Seeds become harder due to the formation of

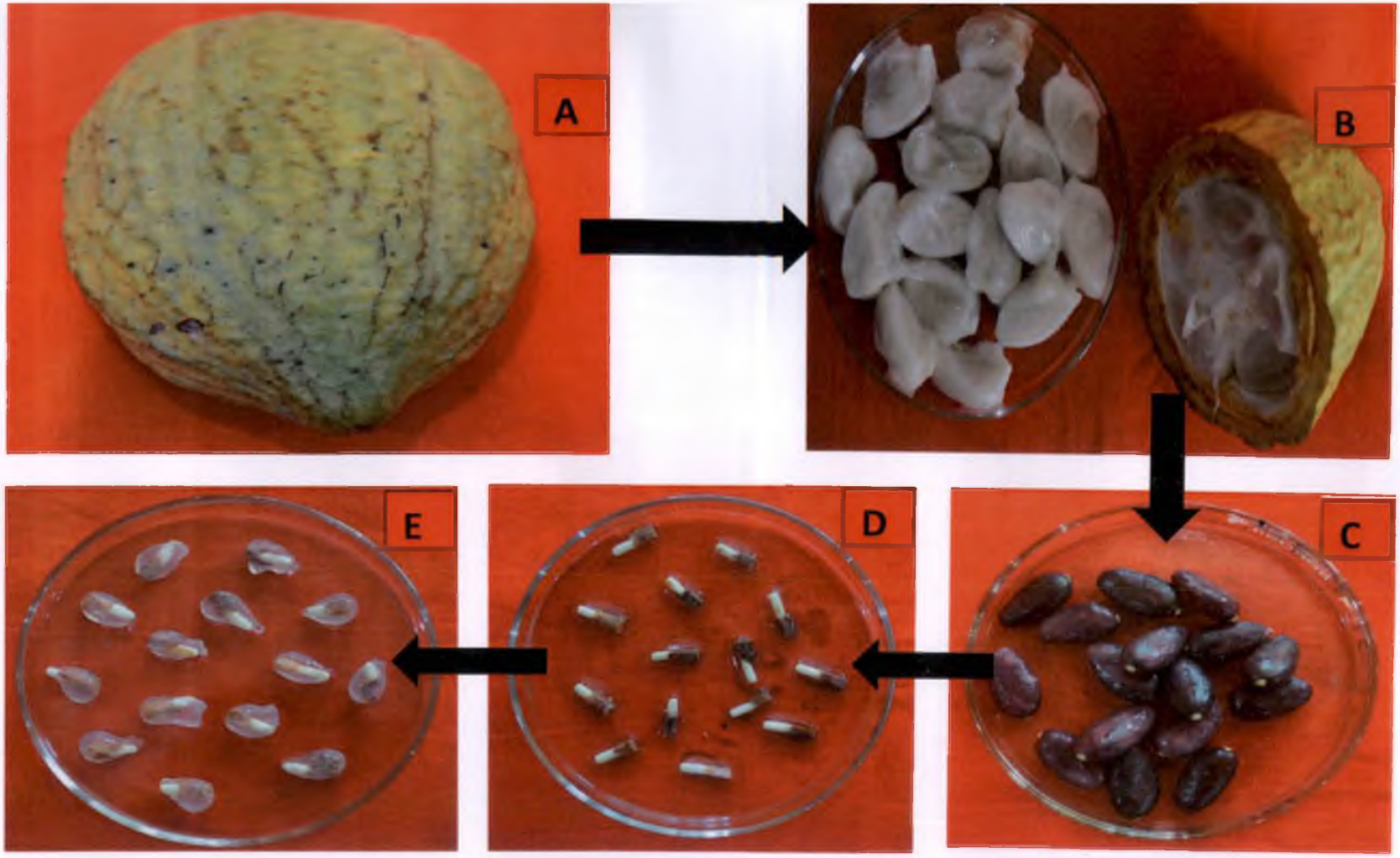

Plate 1. Stages of preparation of synthetic cocoa seed: A) Yellow ridged cocoa pod (~120 days old) B) Cocoa beans with pulp C) Embryonic axes with full cotyledon D) Embryonic axes with ¼ cotyledon E) Synthetic cocoa seeds

ionic bond. The synthetic seeds were then transferred to a petridish containing double distilled water to remove excess calcium ions and were then wiped out using sterilized tissue paper to remove excess water.

3.5 INOCULATION AND STORAGE OF SYNTHETIC COCOA SEEDS

Test tubes, glass jam bottles and petri plates were used for the storage of synthetic cocoa seeds which were subjected to germination inhibitor treatments, desiccation treatments and low temperature treatments respectively. Sterilized cotton plug and cling film were used to seal the mouth of the culture vessel to prevent the entry of microbes. At the time of inoculation, the cotton plug of the culture vessel was removed and the vessel neck was first flamed over a Bunsen burner. The synthetic seed were quickly transferred into the medium using sterile forceps. The neck of the culture vessel was again flamed and the cotton plug was replaced.

The test tubes and culture vessels with synthetic seeds were stored in culture racks in the tissue culture lab. Artificial illumination was provided using white fluorescent lamps for a photoperiod of 9 hours per day. The cultures were incubated at a temperature of 20±2°C.

3.6 CULTURE MEDIA

MS tissue culture medium at 50% level (Murashige and Skoog, 1962) was used as the basal medium for the investigation in this research on synthetic cocoa seeds. The selection was done based on its effect on delayed shoot formation of synthetic cocoa seeds (Shiran, 2012).

Standard procedure (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 chemicals in distilled water (Table 1).

Vitamin stock was also prepared by dissolving the required quantity of vitamins (Table 2). Iron stock was prepared with great care to avoid precipitation (Table 2). To avoid precipitation Na_2EDTA and $FeSO_4.7\ H_2O$ were dissolved in separate beakers with 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. 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 stock number and date of preparation. The stock solutions were stored in amber coloured bottles under refrigerated conditions at 4°C. The stock solutions of nutrients were prepared fresh every four weeks and that of vitamins and amino acids every week.

The pH of the solution was adjusted using 1 N NaOH or 1 N HCl. Standard pH meter (Eutech pH tutor) was used for the purpose. In order to dissolve agar, the solution was boiled in microwave oven. 10-15 ml of freshly prepared melted media was poured hot to the oven dried test tubes. The test tubes with medium were then tightly plugged with non-absorbent cotton plugs. The media was then autoclaved in a standard autoclave for 30 minutes at approximately 15 psi pressure. After sterilization, the culture test tubes were immediately transferred to the culture room and maintained at a controlled temperature of 25±2° C and a light intensity of 1000 lux.

Table 1. Composition of MS major salts and minor salts for the preparation of stock solution (Murashige and Skoog, 1962)

Stock-1 Conc. 10x				Volume required for 1L medium
MS major salts	**mg/1 L medium**	**Wt. required for 1 litre stock**	**Wt. required for 500ml stock**	
1. NH_4NO_3	1650 mg	16.5 g	8.25 g	100 ml
2. KNO_3	1900 mg	19.0 g	9.5g	
3. $CaCl_2 2H_2O$	440 mg	4.4 g	2.2 g	
4. $MgSO_4.7H_2O$	370 mg	3.7 g	1.85 g	
5. KH_2PO_4	170 mg	1.7 g	0.85 g	
Stock-2 Conc. 100x				**Volume required for 1L medium**
MS minor salts	**mg/1 L medium**	**Wt. required for 1 litre stock**	**Wt. required for 500ml stock**	
1. H_3BO_3	6.2 mg	620 mg	310 mg	100 ml
2. $MnSO_4.4H_2O$	22.3 mg	2230 mg	1115 mg	
3. $ZnSO_4.4H_2O$	8.6 mg	860 mg	430 mg	
4. KI	0.83 mg	83 mg	41.5 mg	
5. $Na_2MoO_4.2H_2O$	0.25 mg	25 mg	12.5 mg	
6. $CoCl_2.6H_2O$	0.025 mg	2.5 mg	1.25 mg	
7. $CuSO_4.5H_2O$	0.025 mg	2.5 mg	1.25 mg	

Table 2. Composition of MS vitamins and Iron stock for the preparation of stock solution (Murashige and Skoog, 1962)

Stock-3 **Conc. 100x**				**Volume required for 1L medium**
MS Vitamins	**mg/1 L medium**	**Wt. required for 1 litre stock**	**Wt. required for 500ml stock**	
1. Thiamine	0.1 mg	10 mg	5 mg	10 ml
2. Niacine (Nicotinic acid)	0.5 mg	50 mg	25 mg	
3. Glycine	2.0 mg	200 mg	100 mg	
4. Pyrodoxine	0.5 mg	50 mg	25 mg	
Myo inositol (Cyclohexane 1,2,3,4,5,6-hexol)- 100 mg/1L, Add at the time of media preparation, it is a carbohydrate.				
Stock-4 **Conc. 100x**				**Volume required for 1L medium**
Iron Stock salts	**mg/1 L medium**	**Wt. required for 1 litre stock**	**Wt. required for 500ml stock**	10 ml
1. Na_2EDTA	37.3 mg	3.7 g	1.85 g	
2. $FeSO_4.7H_2O$	27.8 mg	2.78 g	1.3 g	

Half MS medium in 1000 ml with the growth regulator of interest was prepared (Table 3).

Table 3. Procedure for making 1000 ml of ½ MS medium with the growth regulators of interest

Take 600ml distilled H_2O	
Stock –I MS Major (10x)	50 ml
Stock-II MS Minor (100x)	5 ml
Stock-III MS Vitamin (100x)	10 ml
Stock-IV Iron (100x)	5 ml
Myoinositol	100 mg
Sucrose (3%)	30 g
Plant growth regulators	?
Make the final volume to 1000ml distilled H_2O	
Set PH at 5.8	
Add agar (75%)	7.5 g
Agar completely dissolved by heating	
Sterilize the media at 15 psi/121°C for 15min	

3.7 STORAGE OF SYNTHETIC COCOA SEEDS IN GERMINATION INHIBITOR MEDIUM

3.7.1 Storage of synthetic cocoa seeds in ½ MS basal medium with chemical inhibitor

Experiment was conducted to study the effect of various chemical inhibitors on the growth of synthetic cocoa seeds (radicle emergence and shoot sprout). ½ MS basal medium and the chemical inhibitor at the designed level was used for the conduct of the experiment. The desired amount of chemical inhibitors were measured using a precise weighing balance.

Three chemical inhibitors [ABA, Coumarin, Chlorocholine Chloride (CCC)] at different concentrations were tried for the experiment (Table 4). Observations on the root, shoot and growth pattern were observed. Details were recorded at an interval of two days till the complete viability of the synthetic seeds were lost

Table 4. Chemical inhibitors and their varied concentrations used for the study

Chemical inhibitor	Concentration
ABA	10^{-2} M
	10^{-3} M
	10^{-4} M
Coumarin	10^{-2} M
	10^{-3} M
CCC	10 M
	1 M
	10^{-1} M

3.7.2 Storage of synthetic cocoa seeds in ½ MS basal medium with osmotica

Sorbitol and Polyethylene glycol (PEG) were tried inorder to study the response of synthetic seeds to osmotica at different levels. The osmotica used and their levels followed are tabulated (Table 5).

Table 5. Osmotica and their varied concentrations used for the study

Osmoticum	Concentration
Sorbitol	100 mM
	250 mM
	500 mM
PEG (Molecular weight: 6000-7500 g)	5%
	10%
	15%

3.8 DESICCATION TREATMENTS ON SYNTHETIC COCOA SEEDS

Synthetic cocoa seeds were subjected to different desiccation treatments. Eight inch diameter desiccator was used for the experiments. Synthetic seeds were prepared fresh and were kept in sterilized petri plates over a glass sheet inside the desiccator and was covered using glass lid (Plate 2). In order to make the desiccator air tight, the edge of the lid was coated with petroleum jelly. The synthetic seeds were desiccated in desiccators set at relative humidities of 100%, 85.3%, 75.6%, 46.6% and 30% for 12, 18, 24 and 36 hours using standard solutions (Agrawal, 1987). Synthetic seeds pretreated for different time durations inside the desiccator were later inoculated into

glass jam bottles. The entire work was done under sterile conditions inside a laminar air flow cabinet. Germination characteristics of the synthetic seeds were studied before and after the treatments.

Different relative humidities were achieved in glass desiccators of uniform size (160 mm) by pouring 100 ml each of the solutions (Agarwal, 1987). Distilled water (100 ml) was used to obtain a 100% RH (Table 6).

Table 6. Schedule of chemicals and their specified concentration for achieving desired desiccation levels (Agarwal, 1987)

Relative Humidity	Chemical	Concentration
100%	Distilled water	100 ml
85.3%	KCl	100 ml saturated solution
75.6%	NaCl	100 ml saturated solution
46.6%	$Ca(NO_3)_2\ 4H_2O$	100 ml saturated solution
30%	KOH	423 gl^{-1} distilled water

3.9 LOW TEMPERATURE TREATMENT ON SYNTHETIC COCOA SEEDS

Synthetic cocoa seeds transferred to petri plates after desiccation in desiccator was subjected to low temperature treatments under refrigerated condition at 4°C. Transferring of synthetic seeds from desiccator to petri plates were carried out in laminar air flow in aseptic condition. The best desiccated synthetic seeds (desiccated synthetic seeds with comparatively more storage life) as identified from previous experiments were selected for low temperature treatment. They were kept in petri plates and were sealed using cling film. Observations were taken daily until complete

Plate 2. Desiccator with desiccation medium and synthetic cocoa seeds stored in it

viability of the cultures were lost. The cultures were periodically taken out at every 24 hours and subcultured to ½ MS basal media to examine its viability.

3.10 OBSERVATIONS

Each trial for germination inhibitors was carried out with 18 tubes or 18 cultures. For desiccation treatments each trial was conducted with 6 seeds. The observations from various experiments were recorded from 2nd day of inoculation and was taken in regular intervals. The observations were taken until the entire synthetic seeds lost viability. The morphological changes were also recorded during the storage period.

Major observations that were recorded from various experiments include the following:

- Number of synthetic seeds germinated: Number of synthetic seeds that showed radicle emergence were counted and expressed in percentage of total number of cultures in that replication
- Number of synthetic seeds that showed shoot sprout and were expressed in percentage
- Number of mortal or contaminated synthetic seeds and were expressed in percentage. The type of contamination was also recorded
- Number of days taken for shoot emergence: Average number of days taken by each replication was recorded
- Visual changes that happened to the synthetic seeds were observed and were recorded using photograph
- Changes happened to the storage media were also observed

3.11 VIABILITY CHECK OF SYNTHETIC COCOA SEED DURING STORAGE

Viability checking of the synthetic seeds were done by taking one or two synthetic seed at random from the treatments which doesn't show shoot regeneration in the storing medium and culturing them into ½ MS medium.

3.12 STATISTICAL ANALYSIS

The data recorded were Arc-sin transformed wherever necessary and statistically analyzed using the ANOVA tests and t-tests. Treatment means were compared using Duncan's multi range test (DMRT). The analysis was done using statistical package SPSS 20.0. Microsoft excel was utilized for making graphs.

RESULTS

4 RESULTS

The present study on the enhancement of storage life of synthetic cocoa (*Theobroma cacao*) seeds through germination inhibition, desiccation and low temperature treatments was conducted at College of Forestry, Vellanikkara during the period 2012-2014. The results of the study are presented in this chapter.

The results of various experiments are presented in the following order:

- Effect of germination inhibitors on *in vitro* germination and storage of synthetic cocoa seeds
  - Chemical inhibitors
  - Effect of chemical inhibitors on *in vitro* radicle emergence of synthetic cocoa seeds
  - Effect of chemical inhibitors on *in vitro* shoot emergence of synthetic cocoa seeds
  - Effect of chemical inhibitors on *in vitro* mortality of synthetic cocoa seeds
  - Osmotica
  - Effect of osmotica on *in vitro* radicle emergence of synthetic cocoa seeds
  - Effect of osmotica on *in vitro* shoot emergence and mortality of synthetic cocoa seed
- Effect of desiccation on *in vitro* storage and germination of synthetic cocoa seeds
  - Effect of desiccation on *in vitro* radicle emergence of synthetic cocoa seeds
  - Effect of desiccation on *in vitro* shoot emergence of synthetic cocoa seeds
- Effect of low temperature on *in vitro* storage and germination of synthetic cocoa seeds
- Transplantation of seedlings developed from synthetic cocoa seeds

4.1 EFFECT OF GERMINATION INHIBITORS ON *IN VITRO* GERMINATION AND STORAGE OF SYNTHETIC COCOA SEEDS

4.1.1 Chemical inhibitors

The storage media (½ MS basal media) was fortified with chemical inhibitors to examine the effect on *in vitro* germination and storage of synthetic cocoa seeds. Three chemical inhibitors: ABA (10^{-2} M, 10^{-3} M, 10^{-4} M), coumarin (10^{-2} M, 10^{-3} M) and cycocel [CCC (10 M, 1 M, 10^{-1} M)] were tried for the experiment. The results of the experiments are presented herein.

4.1.1.1 Effect of chemical inhibitors on in vitro radicle emergence of synthetic cocoa seeds

The data on radicle emergence of synthetic cocoa seeds as influenced by different concentrations of chemical inhibitors in the storage media are given in Table 7. Radicle emergence significantly differed among different treatments after 35 days of storage. Synthetic cocoa seeds stored in 10^{-2} M coumarin and 1 M CCC survived only for a short duration. In treatments containing 10^{-2} M coumarin and 1 M CCC, all synthetic seeds were found to be dead by 10 days and 15 days after culturing respectively.

Early radicle emergence (5 days) was observed for synthetic seeds stored in 10^{-3} M ABA (13.33%) and 10^{-4} M ABA (16.67%). About ¾th (72.21%) of the cultures stored in 10^{-4} M ABA were able to produce roots by the 20th day of storage (Fig. 1). Among the cultures stored in 10^{-3} M ABA, radicle emergence was obtained only in ¼th (24.44%) of the cultures. The radicle which emerged dried on contact with the media and further elongation was stopped.

Coumarin initially delayed radicle emergence in 10^{-3} M level, but from the 15th day of storage, abnormal bulging and curving of embryonic axes and radicle emergence was observed in some cultures (29.6%) [Plate 3 (a)]. However, the root growth was

retarded by the medium as seen for storage in 10^{-3} M ABA. Browning and subsequent darkening of the embryonic axes was observed as encapsulation split open and when it came in contact with the storage media.

Radicle emergence in storage media with 10^{-1} M CCC started from the 20th day of storage and peaked by the 35th day (71.76%). Among the three chemical inhibitors tried out at different levels, CCC (10^{-1} M level) and coumarin (10^{-3} level) were found to be better to induce delayed radicle emergence of synthetic cocoa seeds.

4.1.1.2 Effect of chemical inhibitors on in vitro shoot emergence of synthetic cocoa seeds

Shoot emergence was not observed in any of the cultures subjected to different chemical inhibitor treatments up to 60 days in inhibitor containing media. In treatments except 10^{-3} M coumarin and 10^{-1} M CCC, all synthetic cocoa seeds died within 60 days without showing shoot emergence. Those synthetic seeds which were still viable after 60 days of storage in chemical inhibitor media (10^{-3} M coumarin and 10^{-1} M CCC) were later subcultured to ½ MS storage media and shoot emergence was observed after a maximum of 32 and 38 days, respectively (Plate 4).

4.1.1.3 Effect of chemical inhibitors on in vitro mortality of synthetic cocoa seeds

The mortality rate of synthetic cocoa seeds were influenced by the presence of chemical inhibitor in the media. There was a significant difference among the treatments (Table 8). All synthetic seeds stored in 10^{-2} M coumarin, 1 M CCC, 10^{-3} M ABA and 10^{-4} M ABA were found mortal by 10th, 15th, 20th and 30th day respectively. The mortality rate was found significantly different in 10^{-3} M coumarin and 10^{-1} M CCC compared to all other treatments (Fig. 2). The presence of 10^{-3} M coumarin and 10^{-1} M CCC in the storage media helped to extend the storage life of synthetic cocoa seeds [25.07% (10^{-3} M coumarin) and 34.03% (10^{-1} M CCC)] for 60 days.

Plate 3. Bulging and abnormal growth by synthetic cocoa seed subjected to treatments in media fortified with:

a) 10^{-3} M coumarin

b) 10^{-3} M coumarin +10^{-1} M CCC

Table 7. Effect of chemical inhibitors and their concentrations on radicle emergence of synthetic cocoa seeds at different days after culturing

Chemical Inhibitors		Percentage of cultures											
		05 DAC	10 DAC	15 DAC	20 DAC	25 DAC	30 DAC	35 DAC	40 DAC	45 DAC	50 DAC	55 DAC	60 DAC
Coumarin	10^{-2} M	0	-	-	-	-	-	-	-	-	-	-	-
	10^{-3} M	0	0	7.4[a]	29.60	29.60	29.60	29.60[a]	29.60[a]	29.60[a]	29.60[a]	29.60[a]	29.60[a]
CCC	1 M	0	0	-	-	-	-	-	-	-	-	-	-
	10^{-1} M	0	0	0[a]	27.47	53.23	68.73	71.76[b]	71.76[b]	71.76[b]	71.76[b]	71.76[b]	71.76[b]
ABA	10^{-3} M	13.33	24.44	24.44[a]	-	-	-	-	-	-	-	-	-
	10^{-4} M	16.67	44.44	66.66[b]	72.21	-	-	-	-	-	-	-	-

DAC – Days After Culturing

Figures with same letter superscripted in a column do not differ significantly

Independent t-test used for comparing two treatments alone

'-' indicates complete mortality of synthetic cocoa seed

Higher concentrations of all chemicals tried out resulted in early mortality of synthetic cocoa seeds.

The investigation on the effect of chemical inhibitors to extend storage life of synthetic cocoa seeds revealed that 10^{-3} M coumarin and 10^{-1} M CCC in the storage media have significant influence. A maximum of 60 days of storage can be obtained by treating the synthetic seeds in above concentrations. ABA was found to be unsuitable for storage, because it caused early mortality of synthetic seeds.

Based on the results obtained on different chemical inhibitors, 10^{-3} M coumarin and 10^{-1} M CCC was found to get an extended storage life of synthetic cocoa seeds. Therefore, a combination treatments of the inhibitors coumarin (10^{-3} M) and CCC (10^{-1} M) were tried to control the growth and enhance the storage life of synthetic cocoa seeds.

During the whole period of storage in 10^{-3} M coumarin + 10^{-1} M CCC + ½ MS media, no synthetic seed was able to produce radicle as well as shoot. The combination treatment was able to delay germination in the cultures. In this treatment, embryonic axes were found to become thicker in size compared to all other treatments involving chemical inhibitors. Abnormal bulging of embryonic axes was observed at the 40^{th} day of storage [Plate 3 (b)]. Bulging of embryonic axes was also observed for storage in 10^{-3} M coumarin. Synthetic seeds started turning brownish/blackish in colour from the 55^{th} day of storage. The viable cultures after 60 days of storage were later subcultured to ½ MS storage media and shoot emergence was observed in a maximum of 7 days.

4.1.2 Osmotica

Sorbitol (100 mM, 250 mM and 500 mM) and PEG (5%, 10% and 15%) were added to the storage medium (½ MS) in order to study the effect of osmotica on the shoot emergence and radicle emergence of the synthetic cocoa seeds. The effect of

Table 8. Effect of chemical inhibitors and their concentrations on mortality of synthetic cocoa seeds at different days after culturing

Chemical inhibitors		Percentage of cultures											
		05 DAC	10 DAC	15 DAC	20 DAC	25 DAC	30 DAC	35 DAC	40 DAC	45 DAC	50 DAC	55 DAC	60 DAC
Coumarin	10^{-2} M	87.17^{b}	100^{c}	100^{b}	100^{b}	100^{c}	100^{c}	100^{c}	100^{c}	100^{b}	100^{b}	100^{b}	100^{b}
	10^{-3} M	0^{a}	7.36^{a}	12.93^{a}	25.9^{a}	29.6^{b}	51.83^{b}	68.46^{b}	74.03^{b}	74.03^{a}	74.03^{a}	74.03^{a}	74.03^{a}
CCC	1 M	31.33^{a}	77.43^{b}	100^{b}	100^{b}	100^{c}	100^{c}	100^{c}	100^{c}	100^{b}	100^{b}	100^{b}	100^{b}
	10^{-1} M	0^{a}	0^{a}	6.06^{a}	6.06^{a}	6.06^{a}	12.12^{a}	21.81^{a}	54.49^{a}	63.07^{a}	63.07^{a}	63.07^{a}	63.07^{a}
ABA	10^{-3} M	0^{a}	0^{a}	20^{a}	100^{b}	100^{c}	100^{c}	100^{c}	100^{c}	100^{b}	100^{b}	100^{b}	100^{b}
	10^{-4} M	5.53^{a}	16.64^{a}	16.64^{a}	22.20^{a}	83.33^{c}	100^{c}	100^{c}	100^{c}	100^{b}	100^{b}	100^{b}	100^{b}

DAC – Days After Culturing

Figures with same letter superscripted in a column do not differ significantly

these chemicals on the mortality of synthetic seeds were also examined during the storage period.

4.1.2.1 Effect of osmotica on in vitro radicle emergence of synthetic cocoa seeds

Data regarding the radicle emergence of synthetic cocoa seeds treated with different osmotic levels are presented in Table 9. Radicle emergence pattern varied significantly during the early period of storage (up to 15 days). Low level of sorbitol (100 mM) did not inhibit radicle emergence of synthetic seeds. Curving of the embryonic axes and its break out from the encapsulation was observed within 4 days of its storage [Plate 5 (a)]. Roots were formed in clusters from the tip of the embryonic axes of the synthetic cocoa seed [Plate 5 (b)]. Tap root reached 5 cm length within 15 days of storage. Maximum radicle emergence (80.37%) was observed within 10 days of storage in 100 mM sorbitol fortified medium (Fig. 3), while radicle emergence was observed within 5 days of storage in 100 mM sorbitol. Higher concentration of sorbitol (250 mM) in the media had an effect on radicle emergence. Radicle emergence started only from the 10^{th} day (42.22%), in 250 mM sorbitol fortified media (Table 9). 500 mM sorbitol added storage media had a negative influence on the storage of synthetic cocoa seeds. All the cultures lost viability within 20 days of storage [Plate 6 (b)]. No synthetic seeds were able to produce radicle at higher concentration of sorbitol. Radicle emergence was inhibited at 500 mM sorbitol induced high osmotic condition.

PEG (5%) supplemented media showed the maximum (81.24%) rooting among all the treatments tried. Synthetic cocoa seeds were completely killed in PEG (15%) fortified media. All seeds turned black in colour and were found mortal within 10 days of storage. PEG at 10% had a good impact in the storage of synthetic cocoa seeds. Seeds were able to survive in the media without shoot emergence for 40 days (Table 10). Only 29.26% of cultures were able to produce radicle until 40^{th} day of storage.

Table 9. Effect of osmotica and their concentrations on radicle emergence of synthetic cocoa seeds

Osmotica		Percentage of cultures									
		05 DAC	10 DAC	15 DAC	20 DAC	25 DAC	30 DAC	35 DAC	40 DAC	45 DAC	50 DAC
Sorbitol	100 mM	17.77	80.37^{c}	80.37^{b}	80.37	80.37	80.37	80.37	80.37	80.37	80.37
	250 mM	0	42.22^{b}	57.03^{b}	57.03	57.03	57.03	57.03	57.03	57.03	57.03
	500 mM	0	0^{a}	0^{a}	-	-	-	-	-	-	-
PEG	5%	3.70	81.24^{bc}	81.24^{b}	81.24	81.24	81.24	81.24	81.24	81.24	81.24
	10%	0	0^{a}	1.93^{a}	16.67	27.35	27.35	27.35	29.26	-	-
	15%	0	-	-	-	-	-	-	-	-	-

DAC – Days After Culturing

Figures with same letter superscripted in a column do not differ significantly

'-' indicates complete mortality of synthetic cocoa seeds

4.1.2.2 Effect of osmotica on in vitro shoot emergence and mortality of synthetic cocoa seeds

A maximum of 80.37% (35th day), 32.22% (45th day) and 43.05% (25th day) shoot emergence was observed for cultures stored in 100 mM sorbitol, 250 mM sorbitol and 5% PEG respectively. Shoot emergence in cultures from the 20th day to 40th day of storage showed significant difference (Table 10). Synthetic seeds cultured in 500 mM sorbitol, 10% PEG and 15% PEG was not able to produce shoots during its storage period.

The mortality rate of the cultures showed significant difference from the 10th day of storage (Table 11). Complete mortality (100%) was observed in the media fortified with 500 mM sorbitol, 10% PEG and 15% PEG within 20, 45 and 15 days respectively. Least mortality was observed in 100 mM sorbitol (17.40%) followed by 250 mM sorbitol and 5% PEG (Fig. 4).

Altogether, the experiments indicated that osmotica influence the germination and longevity of synthetic cocoa seeds. The synthetic seeds survived up to 50 days in sorbitol (100 mM and 250 mM) and PEG (5%) contained media. The viability of surviving seeds were tested by subculturing to ½ MS basal storage media. It is notable that synthetic seeds stored in higher concentrations of osmotica showed greater mortality rate compared to all other treatments.

4.2 EFFECT OF DESICCATION ON *IN VITRO* STORAGE AND GERMINATION OF SYNTHETIC COCOA SEEDS

In order to study the effect of desiccation on *in vitro* storage and germination of synthetic cocoa seeds, the synthetic seeds were treated in desiccator set at different relative humidities (30%, 46.6%, 78.6%, 85.3% and 100%) for 12, 18, 24 and 36 hours. Schedule of chemicals and their specified concentration for achieving desired desiccation levels (Agarwal, 1987) are given in Table 6. Synthetic seeds pretreated in

Plate 4. Shoot initiation in synthetic cocoa seed treated with 10^{-1} M CCC which was later subcultured to ½ MS storage media

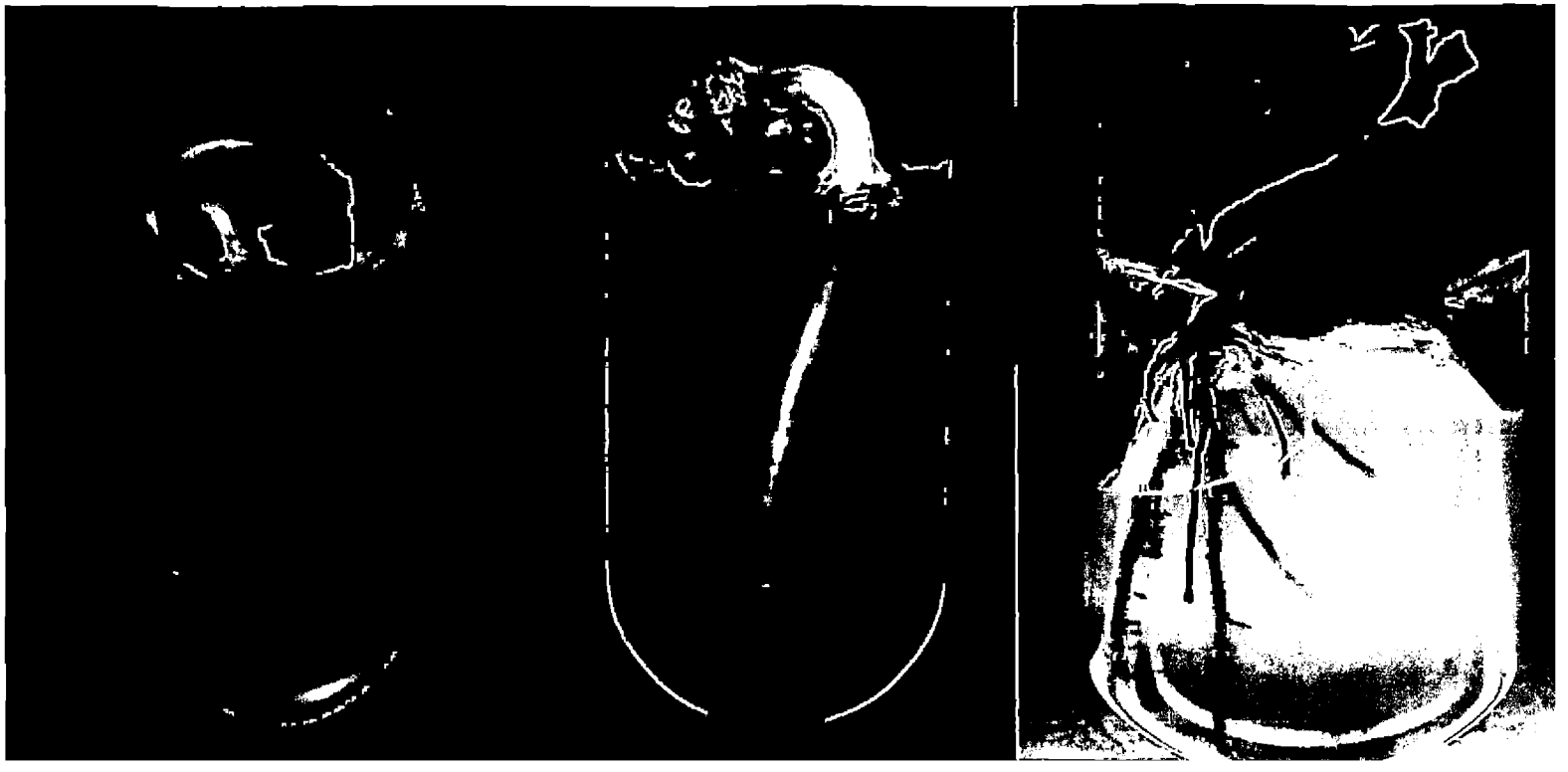

Plate 5. Different stages of growth of a synthetic cocoa seed subjected to treatment in 100 mM sorbitol containing media

a) Embryonic axis curvature (Day 3) b) Root elongation (Day 14) c) Leaf formation (Day 45)

Table 10. Effect of osmotica and their concentrations on shoot emergence of synthetic cocoa seeds

Osmotica		Percentage of cultures									
		05 DAC	10 DAC	15 DAC	20 DAC	25 DAC	30 DAC	35 DAC	40 DAC	45 DAC	50 DAC
Sorbitol	100 mM	0	0	0	16.66^{ab}	33.70^{ab}	61.85^{b}	80.37^{c}	80.37^{b}	80.37	80.37
	250 mM	0	0	0	0^{a}	0^{a}	0^{a}	15.18^{a}	28.51^{ab}	32.22	32.22
	500 mM	0	0	0	-	-	-	-	-	-	-
PEG	5%	0	0	3.70	34.72^{b}	43.05^{b}	43.05^{b}	43.05^{b}	43.05^{ab}	43.05	43.05
	10%	0	0	0	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	-	-
	15%	0	0	0	-	-	-	-	-	-	-

DAC – Days After Culturing

Figures with same letter superscripted in a column do not differ significantly

'-' indicates complete mortality of synthetic cocoa seeds

Table 11. Effect of osmotica and their concentrations on mortality of synthetic cocoa seeds

Osmotica		Percentage of cultures									
		05 DAC	10 DAC	15 DAC	20 DAC	25 DAC	30 DAC	35 DAC	40 DAC	45 DAC	50 DAC
Sorbitol	100 mM	5.55^{a}	17.40^{a}	17.40^{a}	17.40^{ab}	17.40^{ab}	17.40^{ab}	17.40^{a}	17.40^{a}	17.40^{a}	17.33^{a}
	250 mM	4.07^{a}	22.58^{a}	22.58^{a}	22.58^{b}	22.58^{b}	22.58^{b}	22.58^{a}	22.58^{a}	22.58^{a}	22.58^{a}
	500 mM	9.25^{a}	42.59^{ab}	80.95^{a}	100^{c}	100^{c}	100^{c}	100^{b}	100^{b}	100^{b}	100^{b}
PEG	5%	3.70^{a}	20.60^{a}	20.60^{b}	20.60^{ab}	20.60^{ab}	20.60^{ab}	20.60^{a}	20.60^{a}	20.60^{a}	20.60^{a}
	10%	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	43.53^{a}	91.87^{b}	100^{b}	100^{b}
	15%	8.58^{a}	97.9^{b}	100^{b}	100^{c}	100^{c}	100^{c}	100^{b}	100^{b}	100^{b}	100^{b}

DAC – Days After Culturing

Figures with same letter superscripted in a column do not differ significantly

desiccators were then transferred to ½ MS storage media to estimate their storage behaviour. Effect of desiccation on *in vitro* radicle emergence and shoot emergence of synthetic cocoa seeds are presented in Table 12 and 13 respectively.

4.2.1 Effect of desiccation on *in vitro* radicle emergence of synthetic cocoa seeds

The level of desiccation and duration of desiccation treatments significantly influenced the radicle emergence of synthetic cocoa seeds. Exposure of synthetic seeds for longer period of desiccation (24 and 36 hours) compared to shorter period of desiccation (12 and 18 hours) influenced the radicle emergence (Table 12). Percentage of cultures that showed radicle emergence was significantly lesser for synthetic seeds subjected to longer desiccation duration at all desiccation RH. Desiccation at lower RH was found to inhibit radicle emergence. Even at the shortest desiccation duration (12 hours), in the 20th day, only 44.44% and 27.77% of the cultures were able to produce roots for desiccation RH of 46.60% and 30% respectively. Lower desiccation combined with higher duration can kill the seed. All the cultures subjected to desiccation for 36 hours at 46.60% RH and 30% RH were found mortal within 4th day of storage. Cultures which were subjected to desiccation at a RH of 30% for 18, 24 and 36 hours were also found mortal within 4th day of storage.

Under normal *in vitro* condition (100% RH) maximum radicle emergence was completed within 8 days after culturing. At higher RH, the time do not seem to affect radicle emergence. It was also found that, synthetic seeds by itself do not extend storage life in higher desiccation RH. At 100% RH, all the cultures showed radicle emergence in the 8th day for treatments involving desiccation durations of 12 and 18 hours. For cultures exposed to 36 hours duration, it took 16 days for completion of maximum radicle emergence (Fig. 6). Days taken for the completion of maximum radicle emergence in synthetic cocoa seeds subjected to different desiccation treatments varied among treatments (Table 14). Desiccation for 12 hours had the least

Plate 6.

a) Viable synthetic cocoa seed in 250 mM sorbitol (Day 6) b)

Mortal synthetic cocoa seed in 500mM sorbitol (Day 40)

Plate 7. a) and b) Leaf formation in synthetic cocoa seeds subjected to desiccation (100% RH) c) Absence of leaves in synthetic cocoa seed which was subjected to desiccation (46.6% RH

influence on radicle growth compared to all other desiccation durations in different levels (Fig. 6-9). It seems worth noting that in synthetic seeds subjected to desiccation for 24 and 36 hours, only less than 50% culture showed radicle emergence (except for 100% and 78.60% RH). Average days taken for completion of maximum radicle emergence at different desiccation levels were found to be inversely proportional to the desiccation levels carried out (Fig. 10).

4.2.2 Effect of desiccation on *in vitro* shoot emergence of synthetic cocoa seed

Shoot emergence in synthetic cocoa seeds were also significantly influenced by the desiccation treatments (Table 13). Seeds stored in 100% RH had lower longevity. In 100% RH, shoot emergence was observed in all cultures within 32 days after culturing. Cultures kept in storage media after desiccation at 46.6% RH and 30% RH failed to produce shoots (except for treatment with 12 hours 46.6% RH, where a maximum of 30.67% shoot formation was observed). All the cultures subjected to desiccation for 36 hours (46.60% RH and 30% RH) and cultures which were subjected to desiccation at a RH of 30% (18, 24 and 36 hours) were found mortal within 4th day of storage. Hence, no shoot emergence was obtained in them. In normal condition (100% RH), maximum shoot emergence was completed by 36th day after desiccation treatment. In cultures subjected to desiccation treatment at 78.60% RH, shoot emergence was observed only for 12 hours and 18 hours treatment durations. The maximum percentage of cultures with shoot emergence in 78.60% RH at 12 hours and 18 hours were 50% and 20% respectively.

Table 12. Effect of desiccation on radicle emergence of synthetic cocoa seeds

RH (%)	Duration (hour)	Percentage of cultures				
		04 DAC	08 DAC	12 DAC	16 DAC	20 DAC
100	12	23.33^{b}	100^{d}	100^{d}	100^{e}	100^{e}
	18	5.55^{a}	100^{d}	100^{d}	100^{e}	100^{e}
	24	50^{b}	88.88^{d}	88.88^{cd}	88.88^{de}	88.88^{de}
	36	0^{a}	46.66^{bc}	46.66^{b}	66.66^{cde}	66.66^{cde}
85.30	12	0^{a}	58.88^{c}	100^{d}	100^{e}	100^{e}
	18	0^{a}	26.66^{ab}	68.88^{bcd}	68.88^{cde}	68.88^{cde}
	24	0^{a}	0^{a}	22.21^{ab}	22.21^{abc}	22.21^{ab}
	36	0^{a}	5.55^{a}	5.55^{a}	5.55^{ab}	5.55^{ab}
78.60	12	0^{a}	0^{a}	94.44^{cd}	100^{e}	100^{e}
	18	0^{a}	22.22^{abc}	61.11^{bc}	66.66^{cde}	66.66^{cde}
	24	0^{a}	0^{a}	94.44^{cd}	100^{e}	100^{e}
	36	0^{a}	16.66^{ab}	44.33^{bc}	44.33^{bcde}	44.33^{bcde}
46.60	12	0^{a}	0^{a}	0^{a}	44.44^{abcd}	44.44^{abc}
	18	0^{a}	0^{a}	42.22^{ab}	47.77^{abcd}	47.77^{abc}
	24	0^{a}	0^{a}	0^{a}	22.22^{abc}	22.22^{abc}
	36	-	-	-	-	-
30	12	0^{a}	0^{a}	0^{a}	27.77^{abc}	27.77^{abc}
	18	-	-	-	-	-
	24	-	-	-	-	-
	36	-	-	-	-	-

DAC – Days After Culturing

Figures with same letter superscripted in a column do not differ significantly; '-' indicates complete mortality of synthetic cocoa seeds

Table 13. Effect of desiccation on shoot emergence of synthetic cocoa seeds

RH (%)	Duration (hour)	Percentage of cultures			
		28 DAC	32 DAC	36 DAC	40 DAC
100	12	11.11^{b}	63.33^{c}	94.44^{b}	94.44^{d}
	18	0^{a}	24.99^{b}	80.55^{b}	80.55^{cd}
	24	0^{a}	23.33^{ab}	58.77^{b}	58.77^{bc}
	36	0^{a}	5.55^{a}	35.66^{a}	35.66^{ab}
85.3	12	0^{a}	0^{a}	27.77^{a}	$50b^{c}$
	18	0^{a}	0^{a}	27.77^{a}	35^{a}
	24	0^{a}	0^{a}	5.55^{a}	5.55^{a}
	36	0^{a}	0^{a}	0^{a}	0^{a}
78.6	12	0^{a}	0^{a}	22.22^{a}	50^{bc}
	18	0^{a}	0^{a}	0^{a}	20^{a}
	24	0^{a}	0^{a}	0^{a}	0^{a}
	36	0^{a}	0^{a}	0^{a}	0^{a}
46.6	12	0^{a}	0^{a}	16.66^{a}	30.67^{ab}
	18	0^{a}	0^{a}	0^{a}	0^{a}
	24	0^{a}	0^{a}	0^{a}	0^{a}
	36	-	-	-	-
30	12	0^{a}	0^{a}	0^{a}	-
	18	-	-	-	-
	24	-	-	-	-
	36	-	-	-	-

Figures with same letter superscripted in a column do not differ significantly

'-' indicates complete mortality of synthetic cocoa seeds; DAC indicates Days After Culturing

Table 14. Days taken for the completion of radicle emergence in synthetic cocoa seeds subjected to different desiccation treatments

RH (%)	Duration (hour)	DAC
100	12	8
	18	8
	24	8
	36	16
85.3	12	12
	18	12
	24	8
	36	16
78.6	12	16
	18	16
	24	16
	36	12
46.6	12	16
	18	16
	24	16
	36	-
30	12	16
	18	-
	24	-
	36	-

'-' indicates complete mortality of synthetic cocoa seed

DAC indicates Days After Culturing

Duration of desiccation treatments influenced the longevity of synthetic seeds. As the duration of desiccation increased, the percentage of synthetic seeds that showed shoot emergence decreased (Fig. 9). In 100% RH, a gradual decrease in the percentage of cultures with shoot emergence was observed. At the 40th day after culturing in 100% RH, percentage of cultures with shoot emergence was 94.44%, 80.55%, 58.77% and 35.66% for desiccation duration of 12, 18, 24 and 36 hours respectively. A decrease in percentage of cultures showing shoot emergence from 50% to 0% (12 hours to 36 hours) was also noticed in synthetic seeds subjected to desiccation at 85.30% RH. Longevity of more than 40 days was observed for synthetic seeds subjected to desiccation treatment: 85.3% RH (36 hours), 78.6% RH (24 hours and 36 hours) and 46.6% RH (18 hours and 24 hours) [Plate 7 (c)].

Callus formation was observed in cultures subjected to desiccation treatments at 100% RH and 85.30% RH. Callus was observed in stem as well as roots (Plate 8). The callus began to appear in the cultures soon after leaf formation.

4.3 EFFECT OF LOW TEMPERATURE ON *IN VITRO* STORAGE AND GERMINATION OF SYNTHETIC COCOA SEEDS

Synthetic seeds subjected to desiccation at 85.3% RH, 78.6% RH and 46.6% RH at different durations (12, 18, 24 and 36 hours) were stored in refrigerator (4°C) to find out the effect of low temperature on longevity of synthetic seeds.

Synthetic cocoa seeds subjected to treatments in desiccators were transferred to petri plates and were sealed with paraffin film. This was done inside a laminar air flow. The petri plates were then immediately transferred to refrigerator. After 10th day of storage, seeds were periodically taken out every 24 hours and subcultured in ½ MS basal media to know the viability.

Low temperature storage of synthetic seeds under refrigerated conditions harmfully affected its viability. No synthetic seeds subjected to desiccation treatments

Plate 8. Callus formation in shoots and roots subjected to storage after desiccation of 18 hours (100 % RH)

were able to germinate at this temperature. Seeds which were subcultured to ½ MS basal media were found mortal within 5 days. A longevity of only 15 days was obtained for synthetic seeds subjected to low temperature treatment in refrigerator. After 15 days, all seeds turned dark brown/black in colour indicating loss of viability (Plate 9).

4.4 TRANSPLANTATION OF SEEDLINGS DEVELOPED FROM SYNTHETIC COCOA SEEDS

Synthetic cocoa seeds subjected to treatments with osmotica (100 mM sorbitol, 5% PEG) and desiccation (100% RH, 85.3% RH) which showed germination in storage media were transplanted into paper glasses- half filled with vermiculite. The paper glasses were covered with polythene cover for 2 days. Later the cover was removed and transferred out of the culture room to acclimatize with the external environment. MS medium (¼ level) containing macro and micro nutrients was sprayed daily for one weeks, which was later replaced by water. Leaf formation was observed in the seedlings after 18 days of transplantation (Plate 10).

Plate 9. Mortal synthetic cocoa seeds after low temperature treatment at refrigerated condition (Day 15)

Plate 10. Leaf formation in seedlings which were transplanted to vermiculite

DISCUSSION

5 DISCUSSION

The results of the experiments conducted on enhancement of storage life of synthetic cocoa seeds are discussed in this chapter.

5.1 EFFECT OF GERMINATION INHIBITORS ON *IN VITRO* GERMINATION AND STORAGE OF SYNTHETIC COCOA SEEDS

5.1.1 Effect of chemical inhibitors

In orthodox seeds which are capable of storage for several months to years, the seed coat plays a prominent role (Ellis et al., 1990). The presence of growth inhibitors in the seed coat helps in inducing dormancy for these seeds. In the light of these concept, chemical inhibitors at different concentrations were incorporated into the storage media (½ MS basal media) to enhance the longevity of synthetic cocoa seed. Three chemical inhibitors: ABA (10^{-2} M, 10^{-3} M, 10^{-4} M), coumarin (10^{-2} M, 10^{-3} M) and cycocel [CCC (10 M, 1 M, 10^{-1} M)] were tried out for the experiment.

Radicle emergence (Fig. 1) and shoot emergence was significantly influenced by various chemical inhibitors tried. Shoot emergence was not observed in any of the cultures subjected to different chemical inhibitor treatments. All synthetic seeds stored in 10^{-3} M ABA and 10^{-4} M ABA were found mortal on 20^{th} and 30^{th} day respectively (Table 8). It is worth noting that as the concentration of ABA increased, the viability of synthetic seeds decreased (Fig. 2). Exogenous ABA treatment has been found to prevent precocious germination of immature somatic embryos in several plant species (Attree et al., 1994). A review of recent progress on the role of ABA in plant tissue culture (Rai et al., 2011) suggest that ABA plays a significant role in the regulation of many physiological processes of plants. These processes include accumulation of storage reserves and maintenance of embryos in a maturation stage by the prevention of precocious germination (Kermode, 1990). 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). ABA is known to regulate water uptake in the metabolically active embryos (Bewley, 1997; Kucera et al., 2005) through its influence on water relations (Schopfer et al., 1979; Schopfer and Plachy, 1984; Ni and Bradford, 1992). As germination proceeds, recalcitrant seeds are generally killed due to the lack of water availability. The reason for the early death of synthetic cocoa seeds stored in ABA fortified storage media might be due to this influence of ABA on water relations of the seed.

It was observed that CCC at a higher concentration (1 M) had a negative influence on survival of synthetic cocoa seed. All cultures died within 15 days of storage (Fig. 2). The synthetic seeds stored in 10^{-1} M CCC were able to remain viable in the inhibitor medium for 60 days. It suggested that, when the concentration of the same inhibitor was increased ten times, the cells became intolerant and natural mortality happened. Cycocel has been found to give good results in height control (Rowland, 1974). In a study done by Shiran (2012) in embryonic axes of cocoa, where he dealt with only lower concentrations of CCC (10^{-2} M, 10^{-3} M and 10^{-4} M), it was found out that CCC did not show any inhibiting action on germination. However, in the present investigation where higher concentrations of CCC was incorporated a notable inhibitory action was observed. Synthetic seeds stored in inhibitory media containing 10^{-1} M CCC for 60 days when transferred to basal media was able to produce shoots after 38 days (Table 15). This result was supported by the reports made by Abenovoli et al. (2001b) and Aliotta et al. (1993) who stated that CCC has a growth retardant property on seeds. A maximum of only 29.60% radicle emergence was observed for synthetic seeds stored in 10^{-3} M coumarin containing media. From the 15th day of storage, abnormal bulging and curving of embryonic axes was observed in 10^{-3} M coumarin containing media [Plate 3 (a)]. This happened when the embryonic axes emerged out of the encapsulation and came in contact with inhibitory medium. Similar observation was also made by Shiran (2012) who worked on the embryonic

axes of cocoa. Coumarin affects root form and function (Abenavoli et al., 2001a; 2004) decreases respiration and photosynthesis (Moreland and Novitzky, 1987); and influences nitrogen uptake and metabolism (Abenavoli et al., 2001b; 2003). No synthetic seed was able to survive in 10^{-2} M coumarin for more than 10 days. It denotes the killing effect of coumarin in its higher concentrations. Coumarin is an inducer of coat imposed dormancy through the inhibition of water uptake during seed imbibition (Aliotta et al. 1992; 1993). This inhibitory action of coumarin might have caused sudden mortality of synthetic seeds stored in 10^{-2} M coumarin.

Table 15. Potential storage period for synthetic cocoa seeds stored in chemical inhibitor fortified media

Chemical inhibitor	Shoot regeneration in basal media (Days)	Storage life (Days)
10^{-3} M coumarin	32	92
10^{-1} M CCC	38	98
10^{-3} M coumarin + 10^{-1} M CCC	7	67

As 10^{-3} M coumarin and 10^{-1} M CCC were found to extend the storage life of synthetic cocoa seeds when applied alone, a combination treatments of these inhibitors were tried to control the growth and enhance the storage life of synthetic cocoa seeds. 10^{-3} M coumarin + 10^{-1} M CCC combination was used for the purpose. No synthetic seed was able to produce radicle as well as shoot during its storage period. The cultures were found mortal by the 67th day after storage. As noticed for synthetic seeds stored in 10^{-3} M coumarin, the combination treatment also exhibited abnormal bulging of

embryonic axes at the 40th day of storage [Plate 3 (b)]. During the whole period of storage, no synthetic seed was able to produce radicle as well as shoot. Whereas, synthetic seeds stored in 10^{-3} M coumarin had minimal influence on radicle emergence with a maximum of only 29.60%. The results of these treatments indicated that when coumarin and CCC were used in combination, the inhibitory action of coumarin dominated over the inhibitory action of CCC. As observed for 10^{-3} M coumarin fortified media, the abnormal bulging of the cultures stored in 10^{-3} M coumarin + 10^{-1} M CCC containing media was an indication of the dominant action of coumarin over CCC. It is worth notable that a combination of the chemical inhibitors as such cannot enhance the longevity of synthetic seeds beyond a limit. Better results could be obtained if the inhibitors were used alone in the storage media. The findings of this experiment also supported the results made by Shiran (2012), where he observed a negative response to the longevity of embryonic axes of cocoa stored in combinations of various chemical inhibitors.

5.1.2 Effect of osmotica

Water relations between the embryo and its environment play regulatory role in embryo development, particularly during maturation (Adams et al., 1980; Bradford, 1994). Water availability is one of the main environmental factors which influence the germinative process of seeds. The osmoticum changes the water potential in the media and it reduces the intake of water by the seed for its germination (Mehra et al., 2003).

Inorder to study the effect of osmotica on the radicle and shoot emergence of the synthetic cocoa seeds, sorbitol (100 mM, 250 mM and 500 mM) and PEG (5%, 10% and 15%) were added to the storage medium (½ MS).

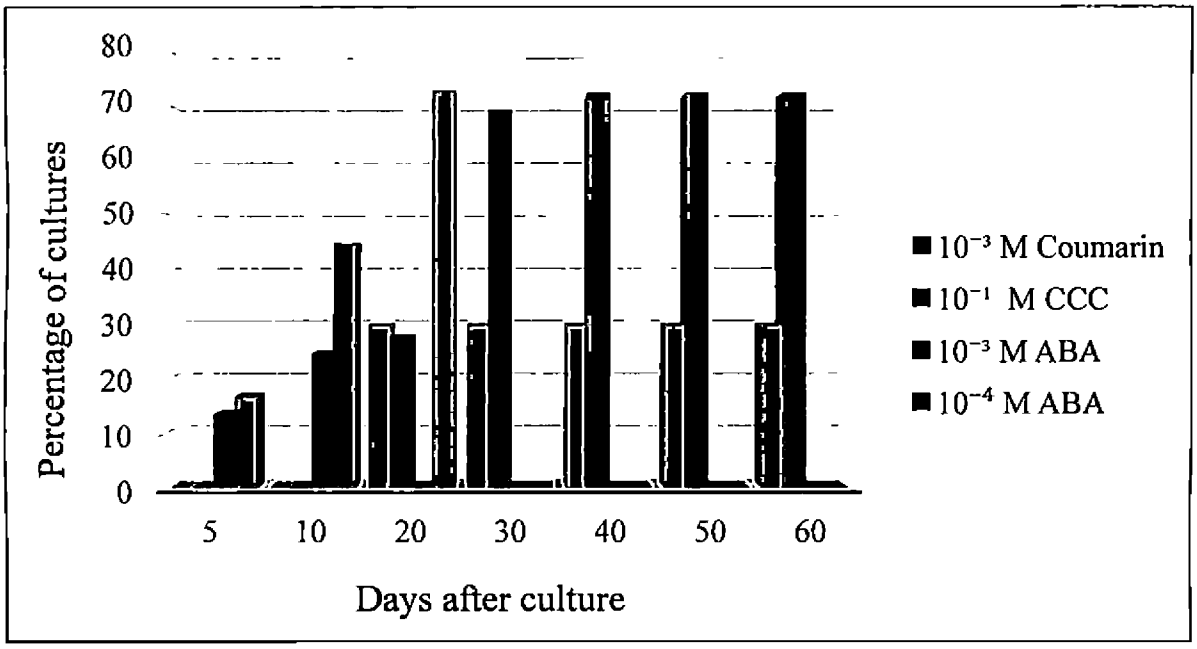

Fig. 1. Effect of chemical inhibitors and their concentrations on radicle emergence of synthetic cocoa seed (Basal media- ½ MS)

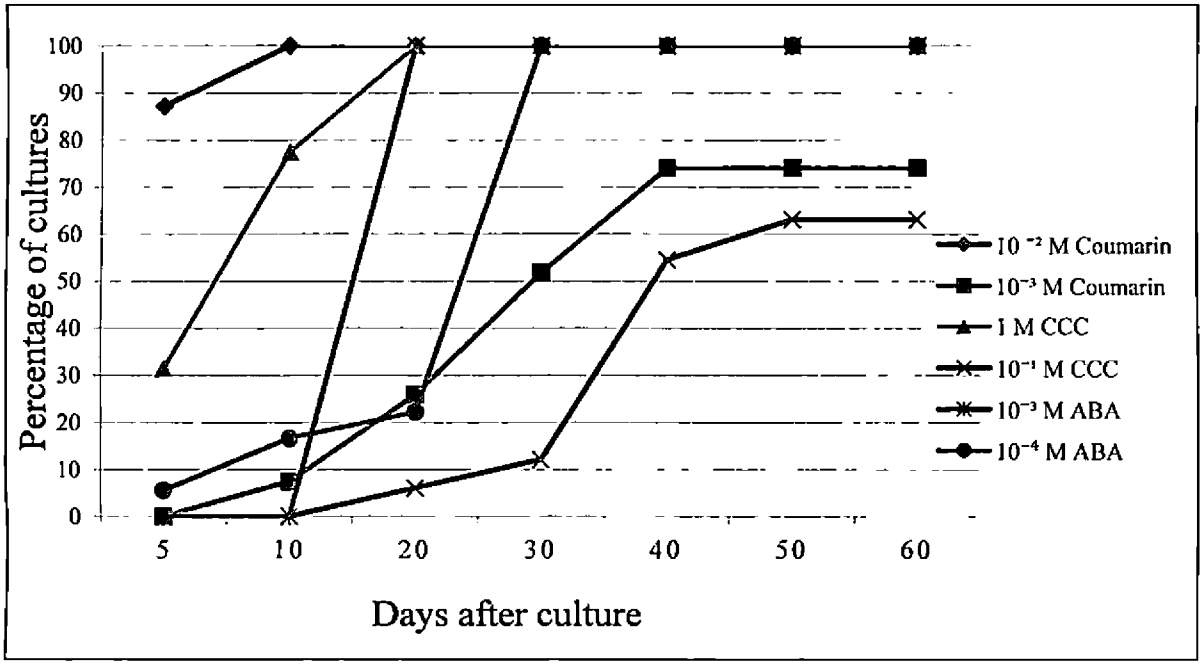

Fig. 2. Effect of chemical inhibitors and their concentrations on mortality of synthetic cocoa seed (Basal media- ½ MS)

The osmotica added media influenced the growth of synthetic seed. Low level of sorbitol (100 mM) had no influence on the root and shoot emergence of synthetic cocoa seeds (Fig. 3 and Fig. 4). Curving of the embryonic axes and its break out from the encapsulation was observed within 4 days of its storage [Plate 5 (a)]. All cultures which showed radicle emergence were able to produce shoot in 100 mM sorbitol (80.37%). Mortality rates of synthetic seeds stored in osmotica was found directly proportional to the concentration of chemicals used (Table 11). 100% mortality was observed for synthetic seeds stored in 500 mM sorbitol, 10% PEG and 15% PEG (Fig. 5). The reason for the sudden mortality of cultures can be related to the role of osmotica as an osmoregulator (Ramos et al., 1999). Higher concentration of osmotica changed the water potential in the media to a greater level such that it could have blocked the intake of water by the synthetic seeds (Marquez et al., 2011). Due to the lack of water around the embryonic axes, the cultures started losing its viability and subsequently undergone loss of viability.

Plasmolysing osmotica, such as sugar alcohols, readily pass through the cell wall and cause temporary plasmolysis, meanwhile PEG molecules are too large to move through the cell wall and do not cause plasmolysis (Attree and Fowke, 1993). Non-plasmolysing osmotica are more effective in controlling germination (Attree et al., 1991). Mexal et al. (1974) stated that beyond 5% PEG in embryos, O_2 concentration was found inversely proportional to PEG concentration. The shortage of O_2 availability to the synthetic seeds in the media may have accelerated the mortality of cultures stored in 10% and 15% PEG. In the present investigation, PEG (10%) had a good impact in the storage of synthetic cocoa seeds. Seeds were able to survive in the medium by arresting shoot formation for 40 days after which 100% mortality was observed. From the experiments conducted to find out the effect of osmotica on synthetic seed storage, it became evident that sorbitol and PEG at varying concentration played a crucial role in checking water availability of the cultures from the storage media. Hence, an

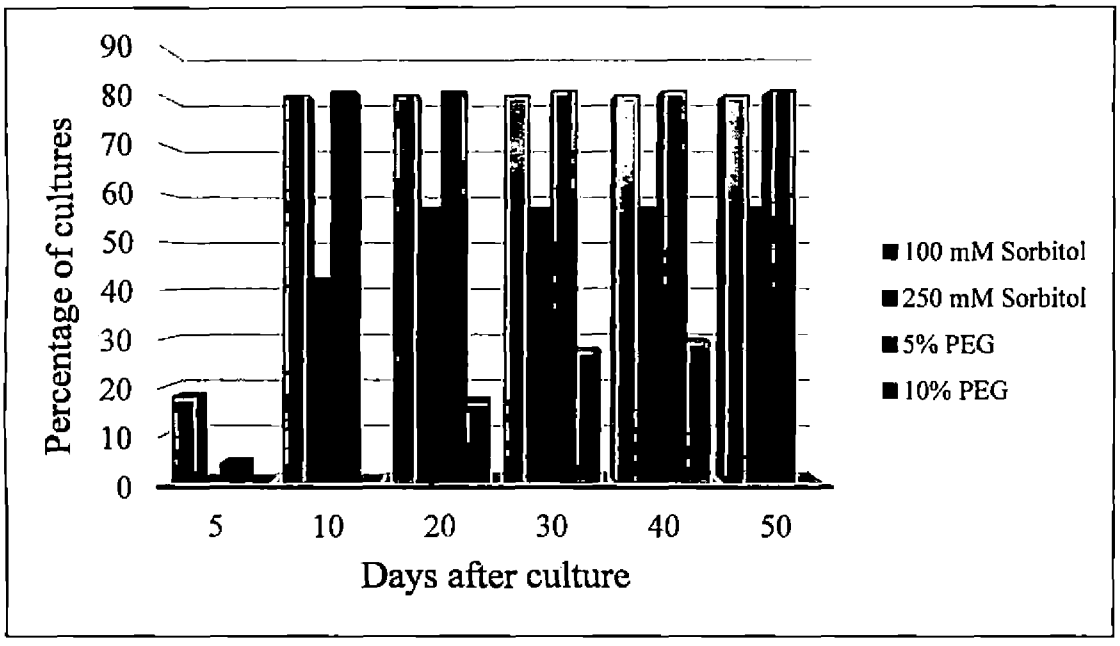

Fig. 3. Effect of osmotica and their concentrations on radicle emergence of synthetic cocoa seed (Basal media- ½ MS)

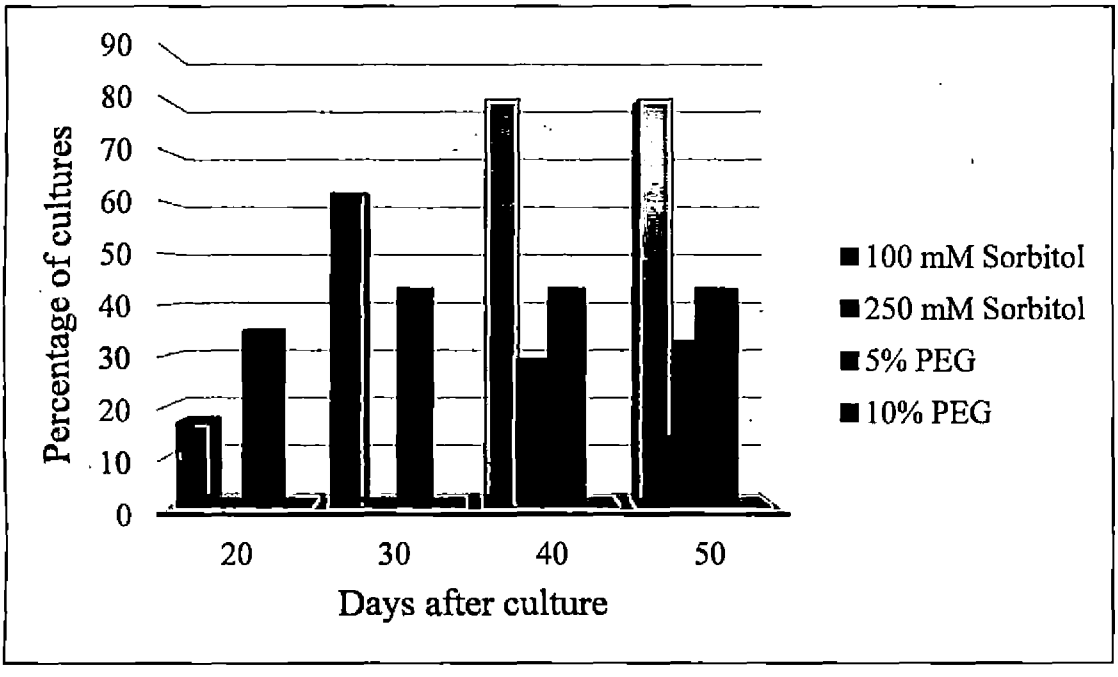

Fig. 4. Effect of osmotica and their concentrations on shoot emergence of synthetic cocoa seed (Basal media- ½ MS)

intermediate concentration of osmotica (10% PEG and 250 mM sorbitol) is effective for the medium term storage of synthetic seeds of cocoa.

5.2 EFFECT OF DESICCATION ON *IN VITRO* STORAGE AND GERMINATION OF SYNTHETIC COCOA SEEDS

Recalcitrant seeds are described so by the fact that they are metabolically active and are characterized with high water content at the time of shedding (Roberts, 1973). Recalcitrant seed survival is influenced by the level of desiccation and the rate of desiccation they can tolerate (Pammenter and Berjak, 1999). It was also shown that desiccation tolerance in seeds is highly associated with dormancy (Tweddle et al., 2003). Pammenter et al. (1991) reported for seeds of *Landolphia kirkii* that, when whole seeds are dried relatively slowly over several days, viability is generally lost at embryonic axes water contents in the range of 1.0 - 0.5 g H_2O g^{-1} dry mass (50 - 35% wet mass basis). However, if isolated embryonic axes are dried rapidly they survive much lower water contents (0.45 to 0.25 gg^{-1}) and possibly even lower.

In the present study, synthetic seeds were exposed to desiccation at different levels and durations. The synthetic seeds treated in desiccator set at different relative humidities (30%, 46.6%, 78.6%, 85.3% and 100%) for 12, 18, 24 and 36 hours were transferred to ½ MS basal media to study the effect of desiccation on germination. In the present investigation it was noticed that exposure of synthetic seeds for longer period of desiccation (24 and 36 hours) compared to shorter period of desiccation (12 and 18 hours) inhibited seed germination (Fig. 6-9). Cultures which were subjected to desiccation at a RH of 30% for 18, 24 and 36 hours were found mortal by 4^{th} day of storage (Table 12). As the duration of desiccation increased percentage of synthetic seeds that showed shoot emergence decreased (Fig. 9 and Fig. 11).

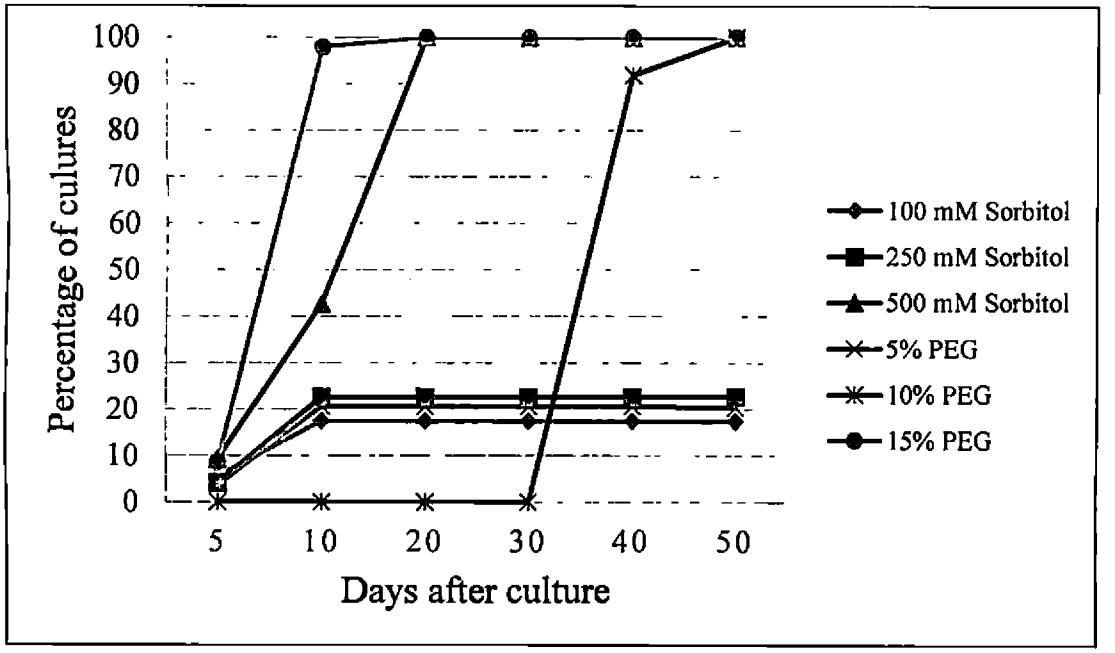

Fig. 5. Effect of osmotica and their concentrations on mortality of synthetic cocoa seed (Basal media- ½ MS)

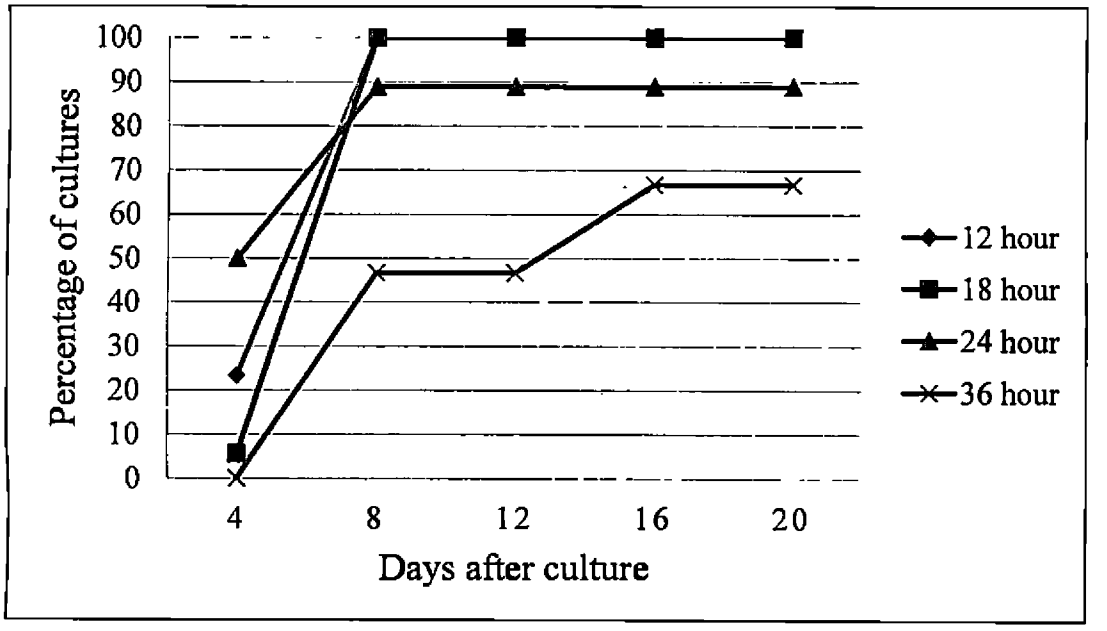

Fig. 6. Effect of different desiccation duration at 100% RH level on radicle emergence of synthetic cocoa seed (Basal media- ½ MS)

In a study conducted by Shiran (2012), where desiccation was carried out for durations of 3, 6, 9 and 12 hours, he found that maximum delay in radicle emergence was obtained for synthetic cocoa seeds subjected to desiccation at 20% RH for 12 hours. According to his findings, longevity of synthetic seeds increased at lower desiccation levels. However in the present study, synthetic seeds were not able to survive in desiccation durations of 36 hours for desiccation levels of 30% and 46.6% RH. This shows that apart from the desiccation level, the desiccation duration also plays a dominant role in maintaining the longevity of synthetic cocoa seeds. Days taken for completion of radicle emergence at different desiccation levels was found to be inversely proportional to the desiccation levels carried out (Fig. 10). When synthetic seeds were subjected to desiccation for 24 and 36 hours, less than 50% culture showed root formation (except for 100% and 78.60% RH). Differing degrees of desiccation sensitivity have been similarly correlated with embryo/seed developmental status for *Landolphia kirkii* and *Camellia sinensis* (Pammenter et al., 1991; Berjak et al., 1992).

Study done by Liang and Sun (2000) found that rapid drying at low RH and slow drying at high RH were more harmful to cocoa embryonic axes, because electrolyte leakage began to increase and axes viability began to decrease at high water contents. This finding gave an insight that the desiccation procedure followed is much relevant in experiments related to synthetic cocoa seed desiccation also. The alginate encapsulation enabled recalcitrant cocoa embryonic axes to withstand the desiccation. Encapsulation may be preventing the deleterious effects of desiccation on the embryo. The protective action of encapsulation on *in vitro* storage studies of synthetic seeds was reported earlier by Ikhlaq et al. (2010). The studies also show that survival of desiccated celery somatic embryos could be improved from 35% to 86% after encapsulation (Kim and Janick 1989; Janick et al. 1989). In the background of available literature, it can be stated that, the conversion of recalcitrant seed to synthetic seed may be inducing an orthodox nature in it after undergoing effective desiccation.

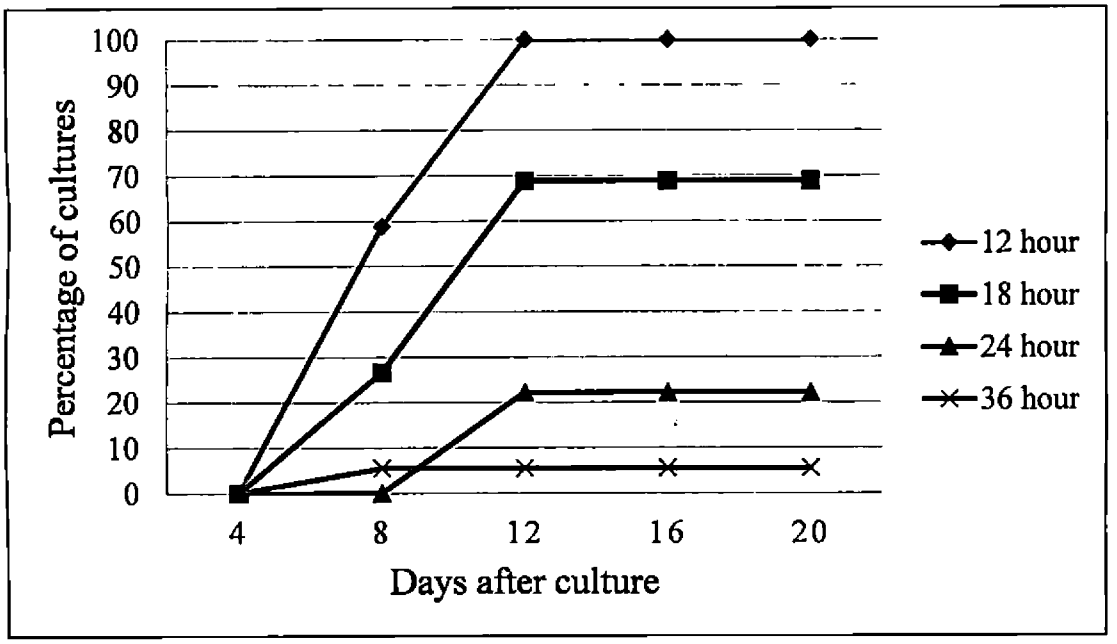

Fig. 7. Effect of different desiccation duration at 85.30% RH level on radicle emergence of synthetic cocoa seed (Basal media- ½ MS)

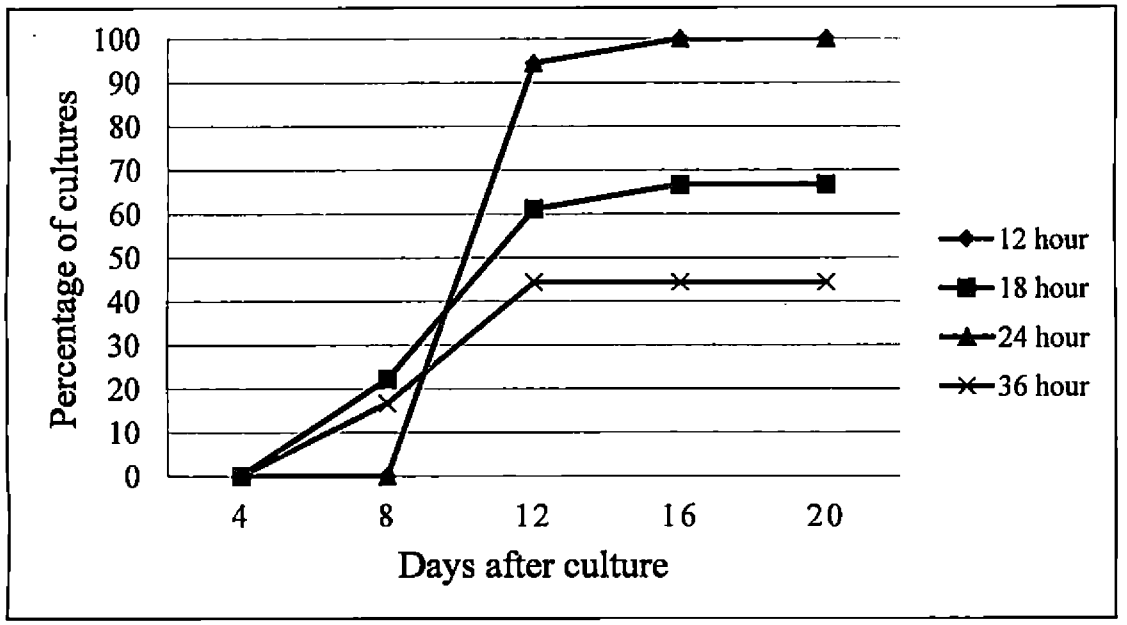

Fig. 8. Effect of different desiccation duration at 78.60% RH level on radicle emergence of synthetic cocoa seed (Basal media- ½ MS)

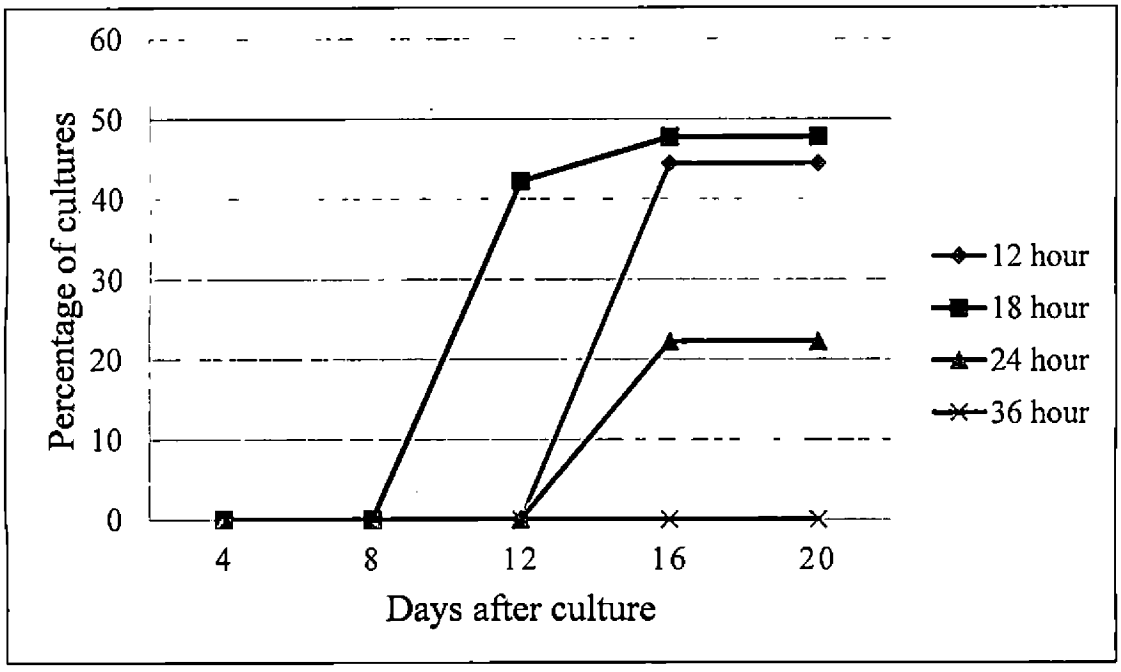

Fig. 9. Effect of different desiccation duration at 46.60% RH level on radicle emergence of synthetic cocoa seed (Basal media- ½ MS)

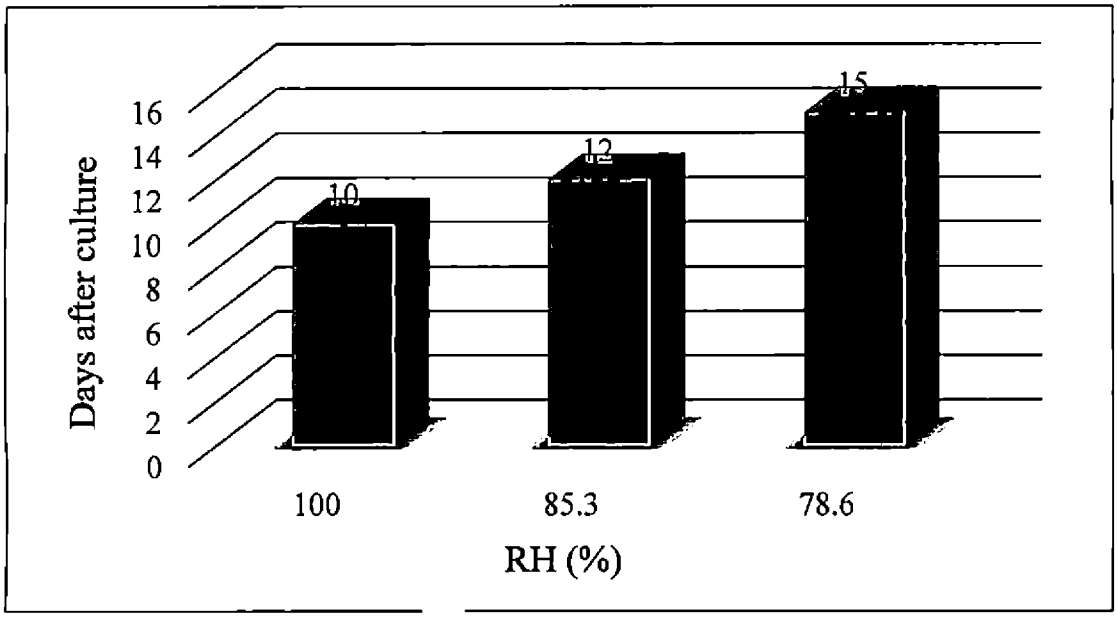

Fig. 10. Days taken for completion of radicle emergence at different desiccation levels

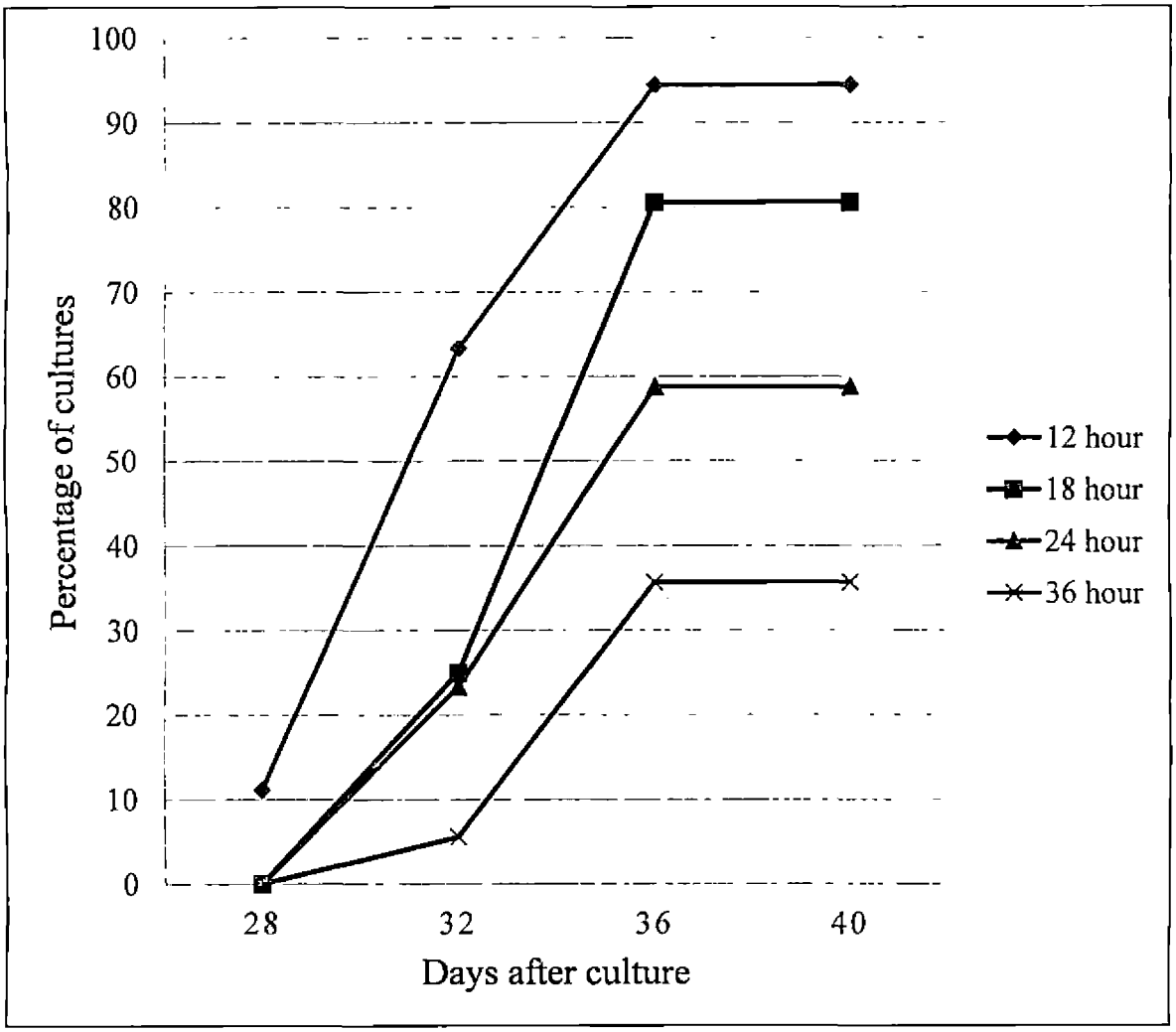

Fig. 11. Effect of different desiccation duration at 100% RH level on shoot emergence of synthetic cocoa seed (Basal media- ½ MS)

5.3 EFFECT OF LOW TEMPERATURE ON *IN VITRO* STORAGE AND GERMINATION OF SYNTHETIC COCOA SEEDS

According to Berjak and Pammenter (2008), the only way in which vigor and viability of recalcitrant seeds can be maintained, is keeping them at the lowest temperature that will withstand, under conditions not permitting water loss, and to eliminate or at least to minimize the seed-associated mycoflora. It has become increasingly apparent that the means to optimize short to medium term storage of recalcitrant seeds is to maintain the shedding water content and impose the lowest temperature tolerated without chilling damage.

In the present investigation pertaining to low temperature storage, synthetic seeds after desiccation at 85.3% RH, 78.6% RH and 46.6% RH for different durations (12, 18, 24 and 36 hours) were stored in refrigerator (4°C) to examine the longevity of synthetic seeds.

A longevity of only 15 days was observed for synthetic seeds subjected to low temperature treatment. After 15 days, seeds turned dark brown/black in colour indicating mortality (Plate 9). Chin and Roberts (1980) reported seeds of *Theobromo cacao* could not survive below 10°C. The result from this investigation has shown that as other recalcitrant seeds, synthetic cocoa seeds are also chilling sensitive. The calcium alginate encapsulation around the embryonic axes and desiccation was not helpful to overcome the damage caused by chilling effect in the refrigerator. As reviewed by Berjak and Pammenter (2004) there are only a few documented cases of non-orthodox seeds transiently surviving such drastic levels of low temperature after desiccation.

5.4 PROTOCOL FOR STORAGE OF COCOA SEED

Theobromo cacao is a tropical evergreen tree which produce recalcitrant seeds and the recalcitrant nature is supposed to be due to seed coat and storage tissue (King and Robert, 1980). In previous researches pertaining to cocoa seed storage, Nair (1987)

found that in harvested pods, longevity lasted up to 6-12 days. However, calcium alginate encapsulation of zygotic embryo had enhanced the longevity up to 3 weeks (Sudhakara et al., 2000). In a more recent study conducted by Shiran (2012), the maximum time of 89 days was recorded with synthetic seeds stored in 250 mM sorbitol added media for 55 days, and transferred to wet cotton for germination. In our investigation, a further enhancement in storage was tried by treatments involving higher concentrations of germination inhibitors, desiccation treatments and low temperature treatments.

From all the experiments conducted in the present study, it was proved that synthetic cocoa seeds are able to store for a considerable duration without losing its viability.

The protocol for storage of synthetic cocoa seeds can be categorised into two:

a) Short term storage of synthetic cocoa seeds
b) Medium term storage of synthetic cocoa seeds

5.4.1 Short term storage of synthetic cocoa seeds

In this study, synthetic seeds of cocoa which were able to store up to a maximum of 60 days without losing viability has been termed as short term storage. Low temperature storage of synthetic cocoa seeds in refrigerated condition was not a great success as far as longevity is considered. In refrigerated condition, synthetic seeds lost viability within 15 days of storage. Studies have revealed that storage in ½ MS medium after calcium alginate encapsulation can enhance the longevity for 20 days (Shiran, 2012). Hence, for short term storage of encapsulated embryonic axes (with ¼ cotyledon), storage in ½ MS medium is a recommendation.

Desiccation treatments in desiccators set at predetermined RH and transferring it to ½ MS storage medium is considered better for short term storage of synthetic cocoa

seeds. Storage in 10% PEG and 250 mM sorbitol also helped to enhance the longevity for 45 days and 50 days respectively. Synthetic seeds which were able to produce shoot after storage in basal medium for a considerable duration were taken into account. Those cultures which failed to regenerate shoot after desiccation treatments were discarded. Hence based on this study, for short term storage of synthetic cocoa seeds, the following desiccation treatments and osmotic treatment are being suggested:

- 85.3% RH (12, 24 and 36 hours)
- 78.6% RH (12 and 24 hours)
- 46.6% RH (12 hours)
- Storage in 10% PEG incorporated medium
- Storage in 250 mM sorbitol incorporated medium for 50 days

5.4.2 Medium term storage of synthetic cocoa seeds

Synthetic cocoa seeds which were able to store for more than 60 days in *in vitro* condition without affecting the viability were considered for the long term storage in this experiment. From all the experiments, it was evident that, chemical inhibitors at definite concentrations are useful for medium term storage of synthetic cocoa seeds.

From the experiments carried out, the best storage condition in the order of decreasing longevity for medium term storage of synthetic cocoa seeds are as follows:

- Storage in 10^{-1} M CCC + ½ MS media for 60 days and transferring to ½ MS basal medium. Shooting begins from 38th day (Longevity 98 days)
- Storage in 10^{-3} M coumarin + ½ MS medium for 60 days and transferring to ½ MS basal media. Shooting begins from 32nd day (Longevity 92 days)
- Storage in 10^{-1} M CCC + 10^{-3} M coumarin + ½ MS medium for 67 day

SUMMARY

6 SUMMARY

The objectives of the study "Enhancement of storage life of synthetic seeds of cocoa (*Theobroma cacao*) through germination inhibition, desiccation and low temperature treatments" was to enhance the storage potential of synthetic seeds of cocoa through the use of germination inhibitors, desiccation treatments and low temperature treatments. The study also aimed to develop desiccation protocol and low temperature protocol for the storage of synthetic cocoa seeds. The investigation was carried out at Plant Tissue Culture Laboratory, Department of Tree Physiology and Breeding, College of Forestry, Vellanikkara, during the period 2012-2014.

The response of synthetic cocoa seeds to varying concentrations of chemical inhibitors, osmotic conditions, desiccation at different RH for different durations and low temperature treatment under refrigerated conditions were examined. The salient findings of the study are summarized in this chapter.

1. Presence of different chemical inhibitors in storage medium significantly influenced radicle emergence of synthetic seeds of cocoa.

2. ABA containing ½ MS medium (10^{-3} M and 10^{-4} M level) did not inhibit the radicle emergence of synthetic cocoa seeds. Radicle emergence was observed within 5 days of storage in it.

3. All synthetic seeds stored in higher concentrations (10^{-2} M coumarin, 1 M CCC, 10^{-3} M ABA and 10^{-4} M ABA) of chemical inhibitor containing ½ MS basal medium were found mortal on 10^{th}, 15^{th}, 20^{th} and 30^{th} day respectively.

4. Synthetic cocoa seeds which were stored in 10^{-3} M coumarin and a combination of 10^{-3} M coumarin + 10^{-1} M CCC containing ½ MS basal media showed abnormal bulging and curving of embryonic axes.

5. CCC (10^{-1} M level) and coumarin (10^{-3} level) were found to be better in delaying radicle emergence of synthetic cocoa seeds.

6. Shoot emergence was not observed in any of the cultures which were subjected to different chemical inhibitor treatments up to 60 days.

7. The presence of 10^{-3} M coumarin or 10^{-1} M CCC in the storage medium helped to extend the longevity of synthetic cocoa seeds for 60 days.

8. The combination of 10^{-3} M coumarin and 10^{-1} M CCC in the storage medium extended the longevity of the cultures for 67 days.

9. Low levels of osmotica in the storage medium (100 mM sorbitol and 5% PEG) did not inhibit the germination of synthetic cocoa seeds.

10. 500 mM sorbitol and 15% PEG incorporated storage medium had a negative influence on the storage of synthetic cocoa seeds. Seeds lost complete viability within 20 and 15 days in this storage conditions respectively.

11. The level of desiccation and duration of desiccation treatments significantly influenced the radicle emergence of synthetic cocoa seeds.

12. All the cultures which were subjected to desiccation for 36 hours at 46.60% RH and 30% RH were found mortal within 4th day of storage.

13. Synthetic seed cultures which were subjected to desiccation at a RH of 30% for 18, 24 and 36 hours were found mortal within 4th day of storage.

14. Cultures which were kept in storage medium after desiccation at 46.6% RH and 30% RH failed to produce shoots except for treatment with 12 hours 46.6% RH, where a maximum of 30.67% shoot formation was observed.

15. Percentage of cultures that showed radicle emergence was significantly lesser for those synthetic seeds which were subjected to longer desiccation duration at all desiccation RH.

16. Average days taken for completion of maximum radicle emergence at different desiccation levels was found to be inversely proportional to the desiccation levels carried out.

17. Longevity of more than 40 days was observed for synthetic seeds subjected to desiccation treatment: 85.3% RH (36 hours), 78.6% RH (24 hours and 36 hours) and 46.6% RH (18 hours and 24 hours).

18. A longevity of only 15 days was obtained for synthetic seeds subjected to low temperature treatment in refrigerated conditions at 4°C. It proved that converting a seed to synthetic seed, along with desiccation cannot overcome the chilling effect at low temperatures.

19. The maximum storage time of 98 days and 92 days were obtained with synthetic seeds stored in 10^{-1} M CCC and 10^{-3} M coumarin added medium respectively, when the seed was transferred to ½ MS basal medium after 60 days.

20. The suggested protocol for storage of cocoa seed is as follows:

a) For short term storage, following desiccation treatments and osmotic treatment of synthetic cocoa seeds are being suggested:

- 85.3% RH (12, 24 and 36 hours)
- 78.6% RH (12 and 24 hours)
- 46.6% RH (12 hours)
- Storage in 10% PEG incorporated medium
- Storage in 250 mM sorbitol incorporated medium

b) For medium term storage, following storage techniques of synthetic cocoa seeds are being suggested:

- Storage in 10^{-1} M CCC + ½ MS medium for 60 days and transferring to ½ MS basal media. Shooting begins from 38th day (Longevity 98 days)
- Storage in 10^{-3} M coumarin + ½ MS medium for 60 days and transferring to ½ MS basal media. Shooting begins from 32nd day (Longevity 92 days)
- Storage in 10^{-1} M CCC + 10^{-3} M coumarin + ½ MS medium for 60 days and transferring to ½ MS basal media. Shooting begins from 7th day (Longevity 67 days)

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ENHANCEMENT OF STORAGE LIFE OF SYNTHETIC SEEDS OF COCOA (*Theobroma Cacao* L.) THROUGH GERMINATION INHIBITION, DESICCATION AND LOW TEMPERATURE TREATMENTS

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ABSTRACT

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ABSTRACT

The research work entitled "Enhancement of storage life of synthetic seeds of cocoa (*Theobroma cacao* L.) through germination inhibition, desiccation and low temperature treatments" was carried out at Plant Tissue Culture Laboratory, College of Forestry, Vellanikkara, during the period 2012-2014. The objectives of the research work were to enhance the longevity of synthetic cocoa seeds through the application of different concentrations of chemical inhibitors/osmotica, desiccation at different RH for varying durations and low temperature treatment under refrigerated conditions and hence to prepare a storage protocol for synthetic cocoa seed. Synthetic seeds using calcium alginate encapsulation technique were prepared from embryonic axes of cocoa containing ¼ cotyledon. The experiments revealed that, presence of different chemical inhibitors in storage medium significantly influenced radicle emergence of synthetic seeds of cocoa. ABA at 10^{-3} M and 10^{-4} M level did not inhibit the radicle emergence of synthetic cocoa seeds and seed mortality were observed by 20th and 25th day respectively in both the concentrations. All synthetic seeds stored in higher concentrations of coumarin (10^{-2} M) and CCC (1 M) containing ½ MS basal medium were found mortal on 10th and 15th day respectively. The presence of lower levels of coumarin (10^{-3} M) or CCC (10^{-1} M) in the storage medium helped to extend the longevity of synthetic cocoa seeds for 60 days without complete loss of viability. The combination of 10^{-3} M coumarin and 10^{-1} M CCC in the storage medium extended the longevity of the cultures for 67 days. Low levels of osmotica in the storage medium (100 mM sorbitol and 5% PEG) did not inhibit the germination of synthetic cocoa seeds whereas, higher levels (500 mM sorbitol and 15% PEG) had a negative influence on the cultures. Among the different osmotic treatments, 250 mM sorbitol fortified medium was found to be best as it could arrest radicle emergence in 42.97 % cultures. Longevity of more than 40 days were observed for synthetic seeds subjected to desiccation treatment: 85.3% RH (36 hours), 78.6% RH (24 hours and 36 hours) and 46.6% RH (18 hours and 24 hours). A longevity of only 15 days were obtained for synthetic seeds subjected to low temperature treatment in refrigerated conditions at 4°C. The synthetic seeds stored in 10^{-1} M CCC and 10^{-3} M coumarin added storage medium, when transferred to ½ MS basal medium after 60 days showed a maximum storage life of 98 days and 92 days respectively. The study was able to enhance the storage life of synthetic cocoa seeds by 9 days compared to a previous study by Shiran, (2012).

Based on the results of the experiments conducted, a protocol for short and medium term storage of cocoa seed was developed.