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Research Article

Growth inhibition as a viable technique to enhance the storage of synthetic seeds of cocoa (*Theobroma cocoa* L.)

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Abstract
Study was conducted to enhance storage life of cocoa (*Theobroma cocoa* L.) seeds through encapsulation and germination inhibition at College of Forestry, Kerala, India. The extracted embryonic axes of the seeds were encapsulated with calcium alginate and kept under different storage media alone as well as in combination with osmolytes under varying levels of relative humidity. Embryonic axes with quarter potion of cotyledon were ideal for preparation of synthetic seeds. Among different storage media, greater longevity and viability were observed in ½ MS media while least longevity was observed in dry cotton. Maximum longevity of 70 days was observed in synthetic seeds stored in cotton with 250 mM sorbitol. Longevity was less than 10 days in dry cotton due to absence of moisture content. The incorporation of MS media in the encapsulation reduced the longevity of synthetic seeds. In addition, MS media was found to reduce the activity of inhibitor. Addition sobitol (250 mM; 500 mM) in the encapsulation media enhanced longevity to 65 days. Duration of desiccation was positively correlated with seed longevity. Synthetic seeds stored with wet cotton and 250 mM sorbitol for 55 days and transferred to wet cotton for germination had longevity of 89 days with 80% germination. The results of present study indicate that it is possible to enhance the storage life of cocoa seeds from two weeks to three months by encapsulation and altering storage environment.

Keywords
Cocoa, Synthetic seeds, Recalcitrant seed, Seed longevity

Introduction

Cocoa (*Theobroma cacao* L.), an evergreen tree native to Amazon basin, is widely domesticated and grown in plantation and agroforestry programmes in the tropics for its beans. Recalcitrant nature of the seed is a limiting factor in plantation establishment of cocoa. Usually, the viability of cocoa seeds lasts only for a maximum of two weeks (Sudhakara *et al.*,2000). Several techniques have been tried to extend the longevity of cocoa seeds, including maintaining them in the pod or treating the extracted seeds with fungicides, osmotic, respiration inhibitors and hormones (Figueiredo, 1986). Partial drying of the seed for a few hours, treating with fungicide followed by sealed storage in thin polythene bags at low temperature at15°C could extend the storage life in cacao (King and Roberts, 1980). Storing cocoa seeds in pods coated with paraffin wax doubled the storage life of seeds to 28 days (Friend, 1964). Storage was further enhanced by leaving an unwaxed central band on the pod which presumably permitted respiratory exchange with minimal water loss. Isolated embryonic axes of cocoa could not survive either freezing or desiccation (Pence, 1991). Subsequently, Pence (1992) found that although cocoa embryos themselves do not survive liquid nitrogen treatment some of their cells do, allowing the technique to be used to regenerate somatic embryos with average survival of 38%.

Recalcitrant nature of seeds is supposed to be due to seed coat and storage tissue which could be overcome by the production of synthetic seeds from excised embryos (King and Roberts, 1980). Synthetic seed comprises of an embryo separated from its accessory structures and encapsulated in a hydrated gel capsule, which will permit the natural development of the embryo (Murashige,1997).Synthetic seed technology was found to be effective in increasing longevity of seeds of cocoa up to three weeks (Sudhakara *et al*.,2000). They developed synthetic seeds having bead size of 4.5 mm to 5 mm from excised embryos of mature seeds (approx. 120 days old pods) and encapsulating in a medium

containing 4% sodium alginate and 75 mM $CaCl_2.2H_2O$. Soon after encapsulation, germination of synthetic seeds was 97.3% which declined to 71% and 49% respectively, when stored in either wet or dry cotton medium for 25 days at 10^oC. On the other hand, germination percentage of the seeds extracted from fresh pods was 90 which declined to 76 at the end of five days storage of pods at 27 $\pm$ 2 ^oC; there was complete mortality at the end of 10 days' storage. Time taken for the initiation of germination and completion of root and shoot emergence were shorter in the case of synthetic seeds compared to the normal seeds.

Presence of certain chemical inhibitors or stress inducing substances in encapsulating material was found to decrease the growth of embryo and enhance storage life of synthetic seeds (Souhail and Chaabane, 2009; Huarte and Benech-Arnold, 2010). Role of Abscisic acid (ABA) in the promotion of seed desiccation tolerance and dormancy, and the inhibition of the phase transitions from embryonic to germinative growth is well documented (Finkelstein *et al*, 1986; Bewley *et al*, 2006).The importance of osmotic stress in the storage life of seeds is also well documented and exploited commercially in enhancing storage life and seed priming.

Considering all these we hypothesized that if plant growth inhibitors are incorporated while preparing the synthetic seeds from the zygotic embryos of cocoa with confirmed recalcitrant storage behavior and induce osmotic stress on them, the storage life could be extended., Hence an attempt was made to increase the storage life of cocoa synthetic seeds by minimizing precocious germination by using osmotic stress in the storage condition by storing in media containing the osmolytes or by coating synthetic seeds with them.

Material and Methods

Yellow ridged Cocoa pods aged between 100-120 days were collected for extraction of embryonic axes from polyclonal plantation situated in the research plots of Kerala Agricultural University, Thrissur, Kerala, India (10° 54` N and 76 °28` E.)

Pods after harvest were immediately washed in tap water, cleaned and dried with a towel, cut open using a sharp knife and seeds with pulp extracted. The pulp surrounding each seed was removed carefully without damaging the embryonic axis. This was subsequently washed in distilled water and soaked in 50% WP Carbendazim (Bavistin) fungicide for 30 minutes. The seeds were washed thoroughly again to remove the fungicide. Embryonic axes were excised under laminar air flow cabinet under aseptic conditions. Standardisation of size of explant for synthetic seed preparation was done by excising embryonic axes with different proportion of cotyledon attached ($^1/_2$cotyledon, ¼ cotyledon, without cotyledon) and inoculating it in the culture media and observing for root and shoot initiation (Figure 1). The embryonic axes of standardized size were converted to synthetic seeds through sodium alginate method (Sudhakara *et al.* 2000).

To standardise germination media for excised embryonic axis of cocoa, dry cotton, wet cotton, MS media, ½ MS media, ¼ MS media and 1/10 MS were tried. MS medium (Murashige and Skoog, 1962) and its variations (¼ MS and ½ MS) were prepared using standard procedure (Gamborg and Shyluk, 1981; Jana and Shekhawat, 2011). Excised embryonic axis (20 each) were stored in conical flasks (250 ml) with the different storage media *viz.*,The incubation was done at a temperature of 20 ± 2°C under artificial illumination (1000 lux) with a photo period of 9 hours per day. Each treatment was replicated thrice with one conical flask containing 20 embryonic axis serving as one replication. Observations were taken every two days on root and shoot initiation.

To standardize suitable media for *in vitro* storage of synthetic seeds, seeds were stored in conical flasks (250 ml) containing storage media *viz*., dry cotton , wet cotton , cotton wetted with ½ MS , with ¼ MS, with ½ MS + 250 mM sorbitol and with ½ MS+ 500 mM sorbitol . Each treatment was replicated thrice with one conical flask containing 20 synthetic seeds serving as one replication. The synthetic seeds in storage media was incubated at a temperature of 20 ± 2°C under artificial illumination (1000 lux) with a photo period of 9 hours per day. The synthetic seeds were stored until it germinated or died. Observations on root initiation and shoot initiation carried out at two days interval.

To evaluate the effect of osmolytes in the synthetic seed coat on enhancing storage life, the synthetic seeds were coated with MS media either alone or in combination with different concentrations osmoticum. Different treatment combinations for this study were MS, MS +250 mM Sorbitol, MS +500 mM Sorbitol, ½ MS, ½ MS +250 mM Sorbitol, ½ MS +500mM Sorbitol, 250 mM Sorbitol and 500 mM Sorbitol. The synthetic seeds (20 each) were the incubated in conical flasks (250 ml) lined with wet cotton at base and kept in dark. Each treatment was replicated thrice (Figure 2).

Synthetic seeds prepared from embryonic axis were subjected to desiccation in the desiccators set at relative humidity's of 100% , 85.3% , 75.6%, 46.6% , 30% and 20% for 3, 6, 9 and 12 hours(Agrawal, 1987). At the end of treatment, the seeds were transferred to conical flasks with wet cotton for further germination studies.Germination of synthetic seeds subjected to desiccation were compared with that of control at two days interval.

The observations on number of synthetic seeds germinated as evidenced through root initiation, shoot sprouting, number of dead synthetic seeds, number of days for shoot formation were recorded at an interval of two days. The viability of the synthetic seeds at the end of germination test were tested for viability by culturing two randomly selected synthetic seeds in ½ MS medium .

The data recorded were statistically analysed (transformed wherever necessary based on test of homogeneity of variance) using the ANOVA procedure. Treatment means were compared using Duncan's multiple range test. The analysis was done using statistical package IBM-SPSS 20.0.

Results and Discussion

The results indicated that the rooting started from second day and gave higher rooting in ½ MS (98.6%) ¼ MS (100%) and 1/10 MS (100%) by the end of 5th day. Rooting was not influenced by the culture media. Shoot initiation started from 10^{th}day in ¼ MS and 1/10 MS media. Higher percentage of shoot regeneration (85.4%, 98.4% and 96.5% respectively) was obtained in these media within 22 days. In ½ MS media, shoot formation was delayed up to 15 days. The mortality of embryonic axes were found to be very less in ¼ MS (1.6%) and 1/10 MS (3.5%) media. The formation of root and shoot and number of dead embryonic axes did not differ among the three MS media levels and wet cotton. Out of three MS media levels, ½ MS media was selected for further experiments due to delayed shoot formation.

All embryonic axes, irrespective of the size of cotyledons attached showed radicle emergence within five days of storage but shoot emergence was a slow process and took almost two weeks to start. Embryonic axes without a portion of cotyledon could survive only for a few days and showed mortality before the root formation. There was no significant difference between the performance of embryonic axes with half cotyledon and ¼ cotyledon in root and shoot formation as well as rate of mortality. It is evident from the present study that, presence of a portion of cotyledon is essential during the storage of excised cocoa embryonic axes. As shoots are formed from the point of attachment of cotyledon with the embryonic axis, excision at that position of embryo may be adversely affecting the germination of the excised embryonic axis.

Hence, embryonic axis with ¼ cotyledon was selected for the synthetic seed preparation as well as for the further experiments. It was convenient to prepare synthetic seeds in that size with reduced area of storage and without affecting the germination parameters.

Synthetic seeds stored in different media showed significant difference in radicle emergence, shoot regeneration and longevity (Table 1). Shoot emergence from synthetic seeds were completely inhibited in media containing sorbitol ($T_{5,}$ T_6) and dry cotton (T_1) . The shoot development of synthetic seeds stored in wet cotton started from 16th day and 70.8% of cultures produced shoot within 40 days. Highest shoot development was in wet cotton (T_2), while ½ MS (T_3) produced shoots in 57.4 and ¼ MS (T_4)in 38.9 per cent of cultures. Root development was on par in all treatments except 500 mM sorbitol (T6) and dry cotton (T1) at 25 days after storage. Synthetic seeds stored in media supplemented with 500 mM sorbitol (T_6) did not show root development during storage. In all other media, root development started from 5^{th} day and maximum root development was obtained within 20 days of storage.

The mortality of synthetic seeds varied significantly among the different storage media (Table 1). Synthetic seeds stored in dry cotton (T_1) was turned necrotic within 16 days of storage possibly due to desiccation. It is well known in recalcitrant seeds that, if drying continues and reaches below "critical moisture content" (King and Roberts, 1980) or "lowest safe moisture content" (Tompsett, 1984), viability is drastically reduced to zero.Storage potential of synthetic seeds in ½ MS (T_3) and ¼ MS (T_4) too was limited due to the complete germination of seeds (40 and 36 days respectively). However, synthetic seeds stored media containing 250 mM and 500 mM sorbitol were viable without germination up to 70 days and 60 days, respectively. Seeds were unable to grow in the sorbitol supplemented media indicating possibility of serving as a storage media for synthetic seeds of cacao. In all the treatments, synthetic seeds which did not germinate after 70 days of storage were turned necrotic.

Effect of growth inhibitors alone and in different combinations on *in vitro* germination and storage of cocoa seeds is given in Table 2. All treatments had more than 80 % root emergence within 20 days of storage of synthetic seeds. However, presence

of osmotica in the encapsulation media had significant effect on root formation. Shoot formation was significantly delayed up to 40 days in synthetic seeds which had osmotica alone (250 mM sorbitol and 500 mM sorbitol) in seed coat. In all treatments, more than 60 % of the seeds germinated by 70th day.

Sorbitol acts as a common source of carbon in cell culture media and energy and also as an osmoticum during organogenesis (Al-Khayri and Al-Bahrany, 2002). Osmotic agents accumulate in many plant tissues in response to environmental stress, including water deficit (Ramos *et al*.,1999). They play significant role in osmoregulation and cryoprotection. In experiments involving sorbitol as high osmoticum, it could suppress germination and maintain the storage protein synthesis under low moisture stress (Finkelstein and Crouch, 1986).

Desiccation levels and its duration influenced the longevity of synthetic seeds of cocoa significantly (Table 3). Among the desiccation environments, maximum seed longevity of 54.1 days was observed at 20% RH. Desiccation over the duration of 12 hrs was observed to give longer longevity (51.3 days). Among the various combinations of desiccating environment and duration, it was noticed that all durations at 20 % RH, all except 3 hrs under 30 % RH, 6 hrs, 9 hrs and 12 hrs under (46.6) % RH and 78.6 % RH all had longevity above 50 days (Figure 3).

Root regeneration was negatively influenced by desiccation treatments of synthetic seeds. Among different desiccation levels, 20, 30 and 46.6 percent of RH inhibited root germination. On an average, synthetic seeds took about 10 days for completing root regeneration under these changed relative humidity's, whereas under normal condition (100% RH), the process was completed within 5 days of storage. Exposing the synthetic seeds for longer duration of desiccation (12 hours) also delayed root emergence (11.94 days). There were no significant interactions between the desiccation levels and duration of desiccation on root emergence in synthetic seeds of cocoa.

Recalcitrant seed survival is influenced by the level of desiccation (Pammenter and Berjak, 1999). In cocoa too, the level of desiccation and duration influenced root emergence as well as longevity of synthetic seeds. This shows the effectiveness of desiccation for effective and long storage of synthetic cocoa seeds. Sudhakara *et al.,* (2000), reported that, embryonic axes of cocoa were desiccation sensitive and lost viability within 10 days when stored under 20% relative humidity. From the present study, it is found that synthetic seeds of cocoa can withstand desiccation. It seems that the alginate encapsulation enabled recalcitrant cocoa embryonic axis to withstand the desiccation. The protective action of encapsulation on *in vitro* storage of synthetic seeds has been reported earlier by Ikhlaq *et al*.(2010) . The encapsulation of embryonic axes enabled seeds to maintain viability even after high desiccation. The ability to withstand desiccation is a characteristic of orthodox seeds. The conversion of recalcitrant seed to synthetic seed may be inducing orthodox nature to recalcitrant seeds. The encapsulation might have played an important role in imparting desiccation tolerance. Effectiveness of the protective coating and possibility to store the propagules was also confirmed by Ballester *et al*.(1997), who reported that survival percentage of synthetic seeds was better than those of non-encapsulated ones.

In previous studies, viability of *Theobroma cacoa* seeds lasted up to 6-12 days in harvested pods (Nair, 1987) and encapsulation of zygotic embryo had enhanced the longevity up to 3 weeks (Sudhakara *et al*.2000). In the present experiments further enhancement in storage life was tried by storing synthetic seeds *in vitro* in selected media as well as directly incorporating osmoticum in to the encapsulation media. .

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Table 1. Effect of different storage media on *in vitro* germination of cocoa synthetic seeds

Synthetic Seed stored in	Root development (% cultures)					Shoot development (% cultures)						Longevity of seed (days)
	5 DAC	10 DAC	15 DAC	20 DAC	25 DAC	15 DAC	20 DAC	25 DAC	30 DAC	35 DAC	40 DAC	
Dry Cotton (T_1)	36.7^{e} (21.5)	47.8^{b} (28.5)	47.8^{b} (28.5)	47.8^{b} (28.5)	47.8^{b} (28.5)	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	15.0^{e}
Wet cotton (T_2)	14.6^{b} (8.3)	70.8^{c} (45.0)	88.9^{c} (62.7)	92.4^{c} (67.4)	92.4^{c} (67.4)	0.0	9.7 (5.5)	39.6^{c} (23.3)	64.6^{c} (40.2)	70.1^{d} (44.4)	70.8^{d} (45.0)	32.5^{d}
½ MS (T_3)	0.0^{a}	0.0^{a}	42.6^{b} (25.2)	88.9^{c} (62.7)	88.9^{c} (62.7)	0.0	0.0	0.0^{a}	0.0^{a}	29.6^{b} (17.2)	57.4^{c} (35.0)	40.0^{c}
¼ MS (T_4)	25.9^{d} (15.0)	96.3^{d} (74.3)	96.3^{d} (74.3)	96.3^{c} (74.3)	96.3^{c} (74.3)	0.0	0.0	18.5^{b} (10.6)	20.4^{b} (11.7)	38.9^{c} (22.8)	38.9^{b} (22.8)	36.6^{cd}
½ MS + 250 mM Sorbitol (T_5)	20.4^{c} (11.7)	57.4^{b} (35.0)	83.3^{c} (56.3)	87.0^{c} (60.4)	87.0^{c} (60.4)	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	70.0^{a}
½ MS+ 500 mM sorbitol (T_6)	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0	0.0	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	60.8^{b}
CD	5.16	11.93	12.82	12.99	12.99	-	-	4.45	7.30	7.48	7.65	6.25

(* p< 0.05; ns- non significant at 0.05 levels; DAC – Days after culture; Values in the parentheses are Arcsine transformed)

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Table.2. Effect of osmoticum in encapsulation media on *in vitro* germination of cocoa synthetic seeds

Synthetic Seed With encapsulation containing	Root development (% cultures)				Shoot development (% cultures)						
	5 DAC	10 DAC	15 DAC	20 DAC	10 DAC	20 DAC	30 DAC	40 DAC	50 DAC	60 DAC	70 DAC
MS	64.8^{c} (40.3)	87.0^{c} (60.4)	92.6^{b} (67.7)	95.6^{b} (72.9)	0.0^{a}	75.9^{e} (49.3)	85.2^{d} (58.4)	85.2^{c} (58.4)	85.2^{d} (58.4)	85.2^{d} (58.4)	85.2^{e} (58.4)
½ MS	75.9^{d} (49.3)	79.6^{b} (52.7)	79.6^{a} (52.7)	83.6^{a} (56.7)	14.8^{b} (8.5)	64.8^{d} (40.37)	68.5^{b} (43.2)	68.5^{b} (43.2)	68.5^{c} (43.2)	68.5^{c} (43.2)	68.5^{b} (43.2)
MS+250mM sorbitol	66.7^{c} (41.8)	100.0^{d} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)	0.0^{a}	48.1^{c} (28.74)	70.4^{b} (44.7)	70.4^{b} (44.7)	70.4^{c} (44.7)	70.4^{c} (44.7)	70.4^{b} (44.7)
½ MS +250Mm sorbitol	77.8^{d} (51.0)	100.0^{d} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)	0.0^{a}	79.6^{e} (52.7)	79.6^{cd} (52.7)	79.6^{c} (52.7)	79.6^{d} (52.7)	79.6^{cd} (52.7)	79.6^{d} (52.7)
MS+500Mm sorbitol	27.8^{a} (16.1)	94.4^{d} (70.7)	94.4^{b} (70.7)	94.4^{b} (70.7)	0.0^{a}	35.2^{b} (20.6)	68.5^{b} (43.2)	68.5^{b} (43.2)	68.5^{c} (43.2)	68.5^{c} (43.2)	68.5^{b} (43.2)
½ MS +500Mm sorbitol	88.9^{e} (62.7)	100.0^{d} (89.9)	100.0^{c} (89.9)	100.0^{c} (89.9)	0.0^{a}	0.0^{a}	63.0^{b} (39.0)	68.5^{b} (43.2)	68.5^{c} (43.2)	68.5^{c} (43.2)	68.5^{b} (43.2)
250Mm sorbitol	39.0^{b} (22.09)	64.0^{a} (39.7)	100.0^{c} (89.9)	100.0^{c} (89.9)	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	21.0^{a} (12.1)	54.3^{a} (32.8)	63.8^{a} (39.6)
500Mm sorbitol	31.8^{a} (18.5)	65.5^{a} (40.9)	100.0^{c} (89.9)	100.0^{c} (89.9)	0.0^{a}	0.0^{a}	0.0^{a}	2.8^{a} (1.6)	33.3^{b} (19.4)	62.5^{b} (38.6)	73.8^{c} (47.5)
CD	6.23	6.94	4.45	4.45	0.89	6.94	8.01	8.01	6.59	4.45	4.09

(* p< 0.05;DAC – Days after culture ; Values in the parentheses are Arcsine transformed)

Table 3. Effect of desiccation on *in vitro* germination and storage of synthetic seed

Desiccation RH (%)	Duration of desiccation (hour)				Mean	CD
	3	6	9	12		
20	51.2^{c}	55.0^{d}	55.0^{d}	55.0^{b}	54.1^{o}	1.07
30	45.0^{b}	51.6^{c}	51.6^{c}	53.3^{bc}	50.4^{n}	2.01
46.6	45.0^{b}	50.0^{c}	50.0^{c}	55.0^{c}	50.0^{n}	1.89
78.6	45.0^{b}	46.6^{b}	50.0^{c}	51.6^{b}	48.3^{n}	1.99
85.3	45.0^{b}	45.0^{b}	45.0^{b}	50.0^{b}	46.2^{m}	1.16
100	41.6^{a}	41.6^{a}	41.6^{a}	43.3^{a}	42.0^{l}	1.32
Mean	45.5^{x}	48.3^{y}	48.9z	51.3^{z}		
CD	1.34	2.03	2.10	1.87		

(* p< 0.05; ns- non significant at 0.05 level; Superscripts *a,b,c,d* are used for comparison of means of duration of desiccation treatments, *l,m,n,o* are used for comparison of means of desiccation levels)

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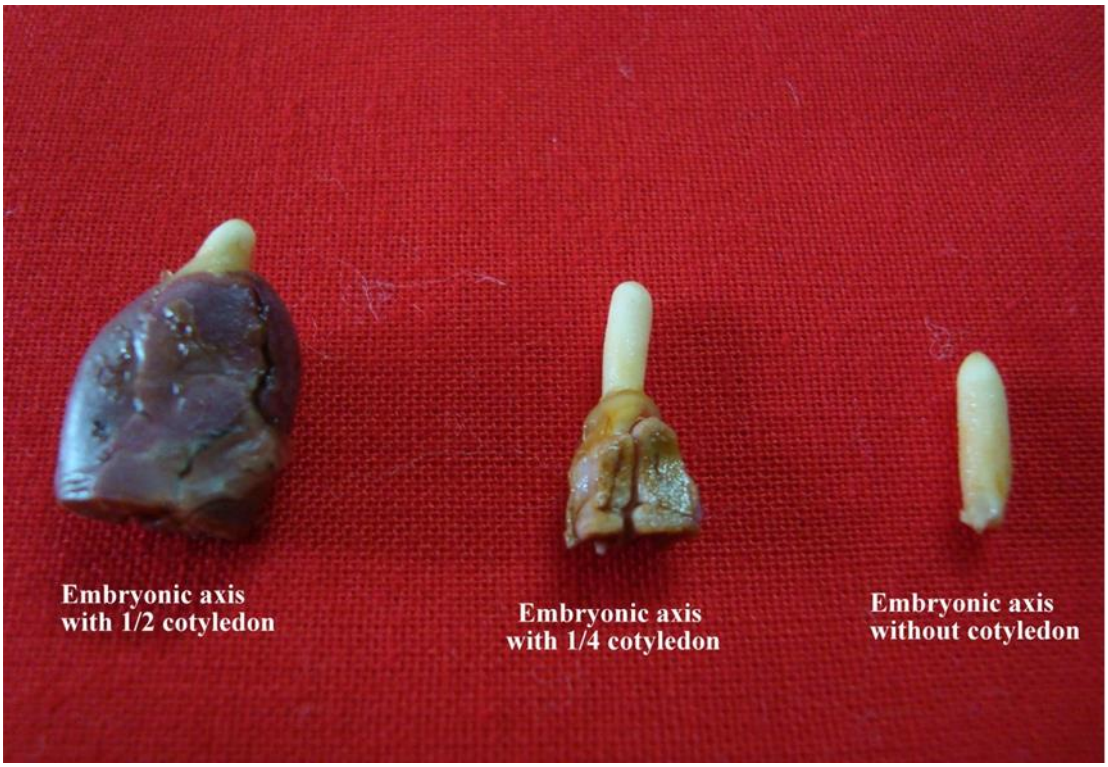


Fig. 1 Embryonic axis excised from seeds with different proportion of cotyledon

Fig. 2. Synthetic seeds made from cocoa embryonic axes.

DOI: 10.5958/0975-928X.2019.00028.0

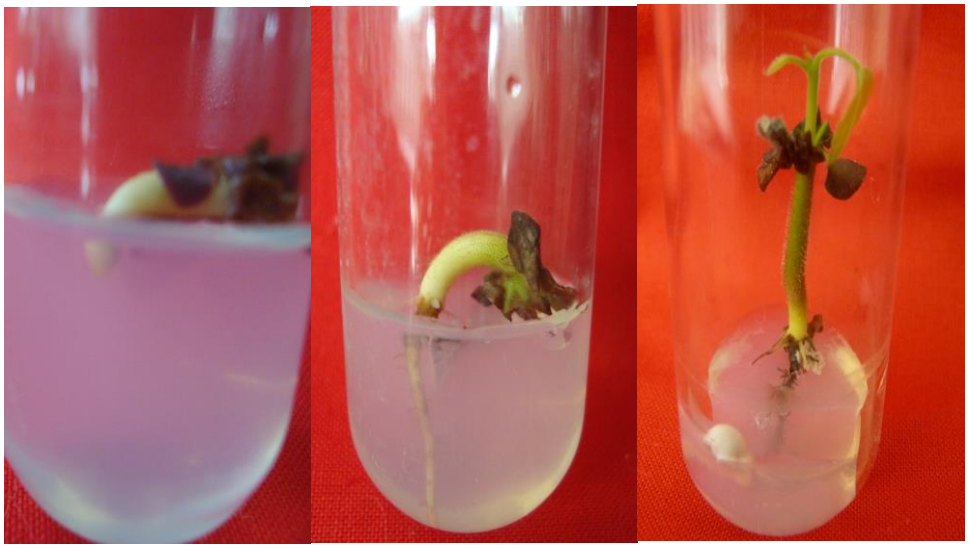
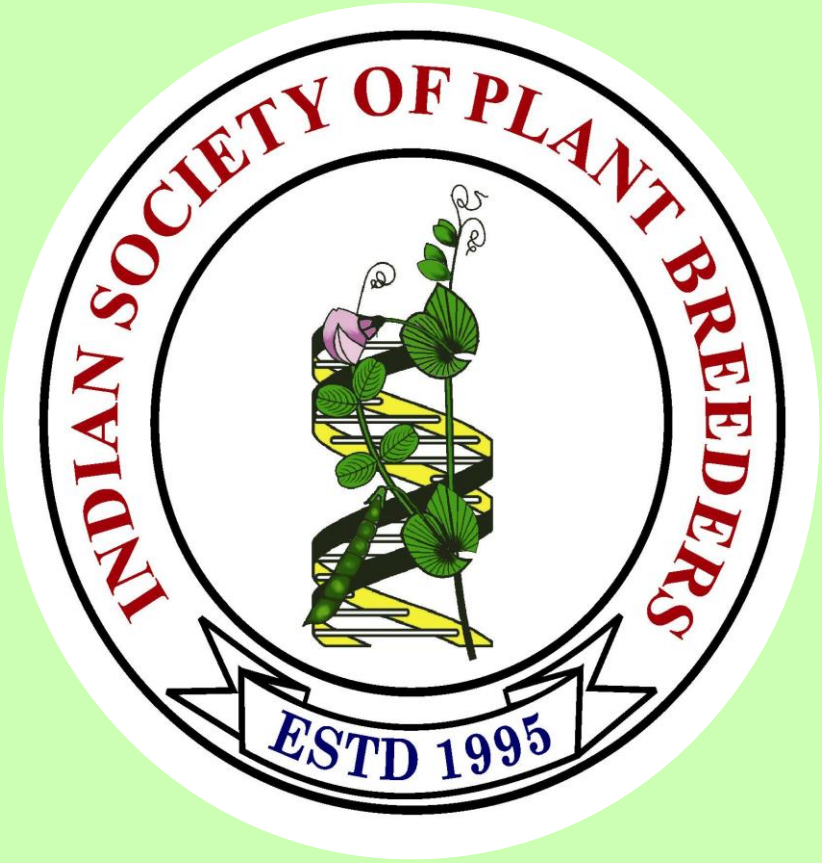


Fig. 3. Germination of cocoa embryonic axes in basal media (a) 2nd day , (b) 8th day and (c) 20th day of storage.



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