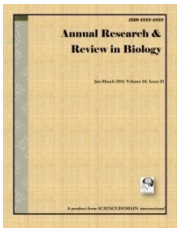

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# *In vitro* Seed Germination of *Coscinium fenestratum* (Gaertn.) Colebr

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Author PSW did the literature searches, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author SS designed the study and did necessary correction where necessary for the manuscript. Both authors read and approved the final manuscript.*

***Original Research Article***

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## ABSTRACT

The present investigation was aimed to study the *In vitro* seed germination potential of *Coscinium fenestratum* - An endangered woody climber with high medicinal value to ensure germplasm conservation and mass production of planting material to meet the unlimited demand. Mature seeds of *C. fenestratum* can be successfully surface sterilized by dipping explants in 0.1% Mercuric Chloride ($HgCl_2$) solution (w/v) for 2 min followed by 2 successive washings using sterilized distilled water. After surface sterilization, hard seed coats have to be damaged to overcome mechanical seed dormancy. After that seeds should be soaked 24h in filter sterilized 2,250 mg/L Gibberelic Acid ($GA_3$) solution to break the physiological dormancy. When mature seeds stored at 4ºC for 3- 6 months, in sealed cellophane after wrapping with wet cotton; soften the hard seed coats make it easy for mechanical damaging. The experiments were repeated in two fruiting seasons (September 2011 and June 2012).

Seeds have to be cultured into MS salt fortified sterilized sand: coir dust (1:1) incorporated culture vessels. To minimize fungal contaminations; 0.7g/L systemic fungicide - Thiophanate methyl 70% WP (w/v) has to be introduced into culture medium. To facilitate *in vitro* germination, seeds should be exposed to total darkness and temperature should be increased to 32±2ºC inside an incubator. Fresh seeds treated with 2,250mg/L $GA_3$ for 24 h before culture initiation achieved 80% mean *In vitro* germination rate within 9 months

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of culture period. After 3 months of storage, to achieve 76% mean *In vitro* germination rate within a month seeds need to be exposed 24 hour to 2,250mg/L $GA_3$. If they soaked 24h in sterilized distilled water, it was needed 4 months time to gain same result. Over 90% seedlings were successfully acclimatized and introduced to field conditions.

*Keywords: Acclimatized; contaminations; dormancy; filter sterilized; germination; surface sterilization.*

## 1. INTRODUCTION

*Coscinium fenestratum* (Gaertn.) Colebr. is a large dioecious woody Liana (Menispermaceae) indigenous to the Indo- Malayan region. It is found in Sri Lanka, India, Malaysia, Vietnam, Myanmar, Singapore and Thailand [1]. Its natural abundance is the forest fringes and disturbed forests in the low country (elevation <500m) wet zone (annual rain fall> 2500 mm) of Sri Lanka termed as weniwel or bangwel-geta [2].

Its stem is extensively used in Ayurvedic preparations for treating digestive disorders, chronic fevers, wounds and ulcers in South India and Sri Lanka. The bitter tonic and yellow dye extracted from the stem has found its way to Europe under the name False Calumba or Tree Turmeric [2].

The root bark is used for dressing wounds, ulcers and in cutaneous leishmaniasis. The paste prepared from stem or root with turmeric is applied to snakebites [1].

The medicinally active compound of *C. fenestratum* is berberine, an isoquinoline alkaloid with numerous bioactivities. The drug is useful in inflammations, wounds, ulcers, jaundice, burns, skin diseases, abdominal disorders, diabetes, fever, general debility and as a blood purifier. *C. fenestratum* is used in several ayurvedic preparations, cosmetic industry (facial masks, fairness creams, body lotions, etc.) and other ayurvedic products as soap, bath gels, face wash and bath oil, etc. [1].

The threat status of this species has been assessed as highly endangered in India, vulnerable in Vietnam, rare in Singapore and indeterminate in Sri Lanka [1]. Propagation of *C. fenestratum* is naturally done by seeds. The plant takes around fifteen years to mature and flower. This species has a well-established local market [3] and as a result of relatively slow growth rate, degradation of natural habitats, habitat specificity, zero cultivation, illegal over exploitation and destructive collection natural populations are disappearing at an alarming rate.

It was hardly found the literature on characteristics of seed germination of *C. fenestratum.* Previous experiment conducted on seed germination dynamic of *C*. *fenestratum* reported that seeds having a dormancy period of 6- 9 months found to be due to a combination of exogenous factors (physical, chemical and mechanical) created by hard seed coat, inhibitory substances presence in the seed coat and the endosperm and endogenous (physiological) factors as high Abscisic acid/Gibberelic acid ($ABA/GA_3$) ratio. Temperature above 30ºC and dark conditions found to be facilitating germination and recorded 92.2% germination after pretreating the seeds by exposing to direct sun light for 6h (sun cracking) followed by dipping seeds in 2250mg/L $GA_3$ solution for 24h to break the dormancy [4]. With reference to the above finding, identification of feasible *In vitro* seed germination techniques is very important

and urgent need in order to assure the germplasm conservation and mass scale production of planting material of this valuable species.

## 2. MATERIALS AND METHODS

All the experiments were carried out at the Tissue Culture Laboratory and plant house, Department of Crop Science, Faculty of Agriculture, University of Ruhuna, Sri Lanka. Mature fruits were collected from randomly selected climbers from Sinharaja, Kanneliya and Wilpita natural forest conservation areas at low country wet zone of Sri Lanka. Fruits were bagged and collected just after shedding and subjected to alternative drying (8h) and wetting (16h) conditions until easy to remove hard pericarps. Seeds were thoroughly washed to remove all the trace pulps around the seed coats to minimize secondary fungal contaminations. Shade drying was performed by laying seeds on papers as single layer, inside the laboratory for few hours. A set of seeds after drying were mixed with Topsin (Thiophanate methyl 70% WP) powder then wrapped using wet cotton wool and stored inside a refrigerator under 4ºC to be used for the experiment of evaluating the effect of seed storage on *In vitro* seed germination. Just shade dried seeds were used for the rest of the experiments.

### 2.1 Identification of Best Surface Sterilization Procedure for Establishment of Seeds in Aseptic Cultures

Seeds were separated into two sets and both were washed thoroughly using soap water and kept under running tap water for half an hour. Seeds were initially surface sterilized using 10% Clorox for 10 min, 20% Clorox for 5 min, 75% Clorox for 5 min, 70% Ethanol for 1 and 2 min to check its potential to avoid contaminations. Based on the results of the above experiment new series of surface sterilization procedures were assigned. Seeds were separated into three groups and dipped in disinfecting solutions as follows,

1. 20% Clorox solution for 5 min followed by 70% Ethanol for 2 min
2. 70% Ethanol for 1 min followed by 75% Clorox solution for 3 min
3. 0.1% Mercuric Chloride $HgCl_2$ solution (w/v) for 2 min

Two successive washings using sterilized distilled water were practiced after exposing to each solution. After surface sterilization seeds were cultured into the sterilized sand: coir dust (1:1) containing culture bottles moistened with distilled water. Numbers of non-contaminated seeds were recorded up to a month.

### 2.2 Identification of Best Fungicidal Treatment to Minimize Fungal Contaminations

As fungal contaminations were aroused during culture establishment, effect of three different fungicides on fungal growth without affecting seed viability was tested. After subjecting to surface sterilization with 0.1% $HgCl_2$ for 2 min and sun cracking, 0.6g/L Captan 50% WP (Captan), 0.7g/L Thiram 42% WP (Thiram), combination of both and 0.7g/L Topsin, were used to dip the seeds for 15 or 30 min followed by thoroughly washed twice using sterilized distilled water. Numbers of non-contaminated seeds were recorded up to 6 months. A Tetrazolium test [5] was carried out a month later to identify adverse effects of fungicides on seed viability.

Accordingly, effect of incorporating 0.7g/L Topsin into the sterilized sand: coir dust (1:1) containing culture bottles moistened with distilled water was tested on reduction of contaminations as well. Numbers of germinated seeds were recorded up to 12 months.

## 2.3 Identification of Best Seed Pretreatment to Enhance Germination

Surface sterilized seeds using 0.1% $HgCl_2$ for 2 min were subjected to sun cracking by placing them on sterilized aluminum foils. Then dipped either in filter sterilized 2,250mg/L $GA_3$ solution or sterilized distilled water for 24h and cultured under aseptic conditions. After surface sterilization, another set of seeds were treated with $GA_3$ solution for 24h before culturing (control), another set was exposed only to sun cracking after surface sterilization prior to culturing. As the culture medium, sand: coir dust (1:1) containing culture bottles, moistened by using 0.7g/L Topsin incorporated sterilized distilled water were used. Germination percentages were recorded up to 12 months.

## 2.4 Identification of Best *In Vitro* Seed Germination Medium

Surface sterilized seeds were cultured under aseptic conditions in MS [6] medium with and without 1g/L Activated Charcoal (AC). Second set of seeds were cultured into sterilized sand: coir dust (1:1) containing culture bottles either each bottle was nourished by 20ml of liquid MS medium or distilled water. The third set was cultured into water agar medium with and without 1g/L AC.

The seeds were incubated until germination under 25±2ºC temperature in a culture room or 32±2 $^0$C temperature inside an incubator under totally dark conditions. Relative humidity inside culture vessels were around 100% and all the vessels were covered using black polythene to cut off the light effect. Numbers of germinated seeds were recorded up to twelve months.

## 2.5 Identification of Best Culture Medium for Better *In Vitro* Growth of Seedlings

Based on the results of the *In vitro* germination experiment, series of culture media were assigned to check the possibilities of enhancing *In vitro* growth of the seedlings (Fig. 9). The effect of incorporating coconut water (250ml/L) into the culture media on seedling growth was tested as well. Growths of seedlings were recorded up to 2 months.

## 2.6 *Ex vitro* Seed Germination

To compare the effect of *in vitro* environmental factors with *ex vitro* environmental factors on seed germination of *C. fenestratum*, a set of surface sterilized seeds were introduced to normal environmental conditions and time taken for initiation of seed germination and germination rates were recorded up to 6 months. Seed trays were filled with sand: Coir dust (1:1) medium, placed inside a shade house and drained using 0.7g/L Topsin fungicide solution. After 24h, surface sterilized and pretreated seeds by 2250mg/L $GA_3$ for 24h were sown in planting holes. After watering up to saturation of potting media, planting trays were covered by using gauge 200 black polythene cover. The recorded temperature in the black polythene covers was 34-36ºC and relative humidity at 84%.

## 2.7 Effect of Seed Storage on *In Vitro* Seed Germination

A set of seeds, 3 months after storage were surface sterilized by 0.1% $HgCl_2$ for 2 min and cultured into the sand: coir dust (1:1) media containing MS salts and 0.7g/L Topsin after soaking 24h either in 2250 mg/L $GA_3$ or sterilized distilled water. Seed coats were damaged slightly inside the lamina flow cabinet using sterilized secateurs. Control seeds were directly cultured without subjecting to presoaking. To evaluate the effect of incorporating $GA_3$ into culture media on enhancing seed germination, water soaked seeds for 24 h were introduced to above media, which were fortified either with 2, 20, 200 or 2000mg/L $GA_3$.

## 2.8 Acclimatization of *In Vitro* Germinated Seedlings

Just after *In vitro* germination of seedlings, culture bottles were transferred to normal laboratory conditions for a week, then to a shade house. Three days later lids of the culture vessels were opened and a mixture of 0.6g/L Captan and 0.7g/L Thiram fungicides were sprayed on plants. Then they were placed in a propagator for another 3 days. To maintain high humidity, regular spraying of water is needed at least twice a day. Then plantlets were transferred to poly bags containing sand and coir dust (1:1 ratio). Osmocot, a high nitrogen fertilizer (N:P:K- 30:10:10) was added to potting media a week before usage. After 8 weeks, plants were transferred to poly bags containing cow dung: top soil: sand (1: 1: 1) ratio and placed under plant house condition for another 4 weeks before introducing to field conditions. Survival rates were measured during the experiment period up to 14 weeks.

## 2.9 Experimental Design and Statistical Analysis

Experiments on surface sterilization, seed pretreatments, culture establishment and seedling growth and *ex vitro* germination were carried out according to completely randomized design (CRD). Fungicide application experiment was conducted by using Randomized Complete Block Design (RCBD). All the treatments under each experiment were replicated by 10 times. Numbers of non-contaminated, viable and germinated seeds were counted accordingly. Growth of the seedlings was measured by recording time taken to appear $1^{st}$ leaf. Results obtained as percentages and indexes were arcsine transformed to normalized data before statistical analysis whenever necessary. Mean separations were carried out by calculating least significant difference (LSD). Data were analyzed using SAS software. Experiments were repeated in two fruiting seasons (September- 2011 and June- 2012).

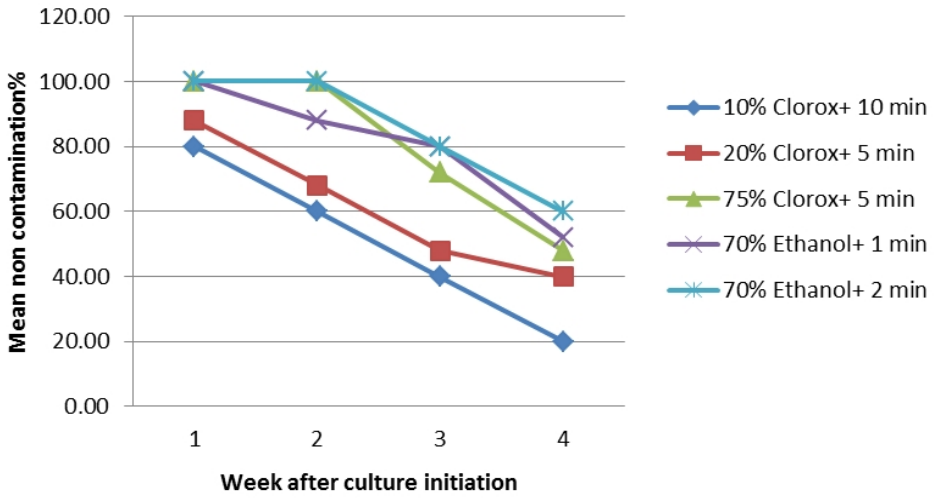
# 3 RESULTS AND DISCUSSION

## 3.1 Identification of Best Surface Sterilization Procedure for Establishment of Seeds in Aseptic Cultures

Seeds surface sterilized using Clorox and Ethanol were highly contaminated within 4 weeks (Fig. 1). When it reached to 8 weeks all the seeds were contaminated.

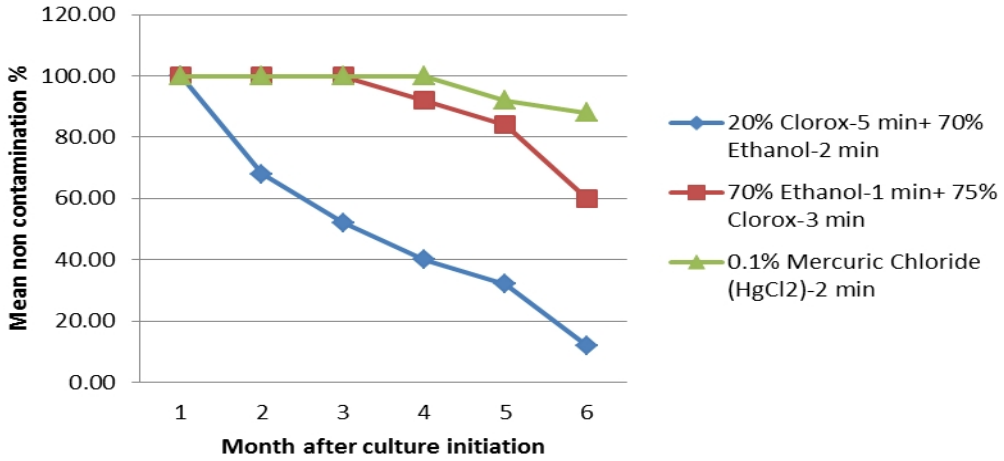
Based on the results, surface sterilization procedures were rearranged. Significantly ($P \leq 0.05$) highest non contamination rate was recorded with seeds, surface sterilized using 0.1% $HgCl_2$ for 2 min in the redesigned experiment (Fig. 2). After conducting viability tests up to 6 months it was found that even the seeds were not contaminated, the viability of seeds was reduced drastically when they were surface sterilized with Clorox and Ethanol

(Fig. 3). Significantly ($P \leq 0.05$) highest viability rates, up to 6 months were recorded with seeds surface sterilized with 0.1% $HgCl_2$ for 2 min. However, none of the seeds surface sterilized with $HgCl_2$ were germinated even they were 100% viable within 6 months observation period. Therefore, it was decided to take observations up to 12 months and to introduce some seed pretreatments to break dormancy.




| | | | | |
|---|---|---|---|---|
| CV= | 5.23 | 8.15 | 11.05 | 14.37 |
| LSD= | 6.57 | 9.09 | 9.48 | 8.48 |

**Fig. 1. Mean non contamination rates as affected by different surface sterilization procedures**




| | | | | | | |
|---|---|---|---|---|---|---|
| CV= | 0.00 | 7.08 | 7.53 | 8.18 | 14.89 | 13.69 |
| LSD= | 0.00 | 9.22 | 9.22 | 9.22 | 15.06 | 10.65 |

**Fig. 2. Mean non contamination rates as affected by different surface sterilization**

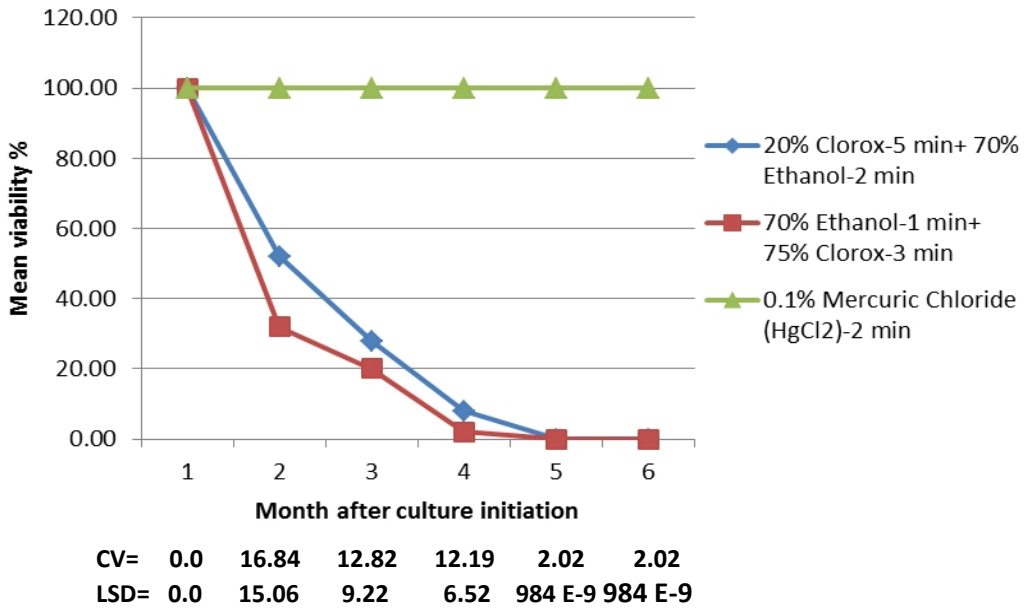



**Fig. 3. Mean viability rates as affected by improved series of surface sterilization procedures**

There were few records regarding *in vitro* germination of *C. fenestratum*. It was mentioned that seeds collected from wild populations of Sinharaja and Yagirala forest conservation areas in Sri Lanka were air-dried and stored in paper bags until the experiments were initiated [7]. However, the method of removing pod coats, maturity stage of seeds and the duration for which seeds were stored were not mentioned. The author further mentioned that prior to use, seeds were washed with 2% (v/v) Tween 20 (mild detergent) solution for 10 min, 2 successive washings with sterile distilled water followed by surface sterilization with 10% Clorox™ (0.5% Sodium hypochlorite v/v) for 10 min with thorough rinsing with sterile distilled water [6]. In another report it was recorded that seeds of *C. fenestratum* were surface sterilized by soaking in a 4% NaOCl solution for 5 min before subjected to 1500 mg/L $GA_3$ treatment resulted 60% germination rate after establishing in sterilized sand and soil mixture [8]. However, during the present study seeds surface sterilized either with Clorox or Ethanol was subjected to contamination within 4 weeks. Therefore, surface sterilization procedures were redesigned and identified 0.1 % $HgCl_2$ as the only effective surface sterilant for *C. fenestratum* seeds which was not adversely effect on viability of seeds.

## 3.2 Identification of Best Fungicidal Treatment to Minimize Fungal Contaminations

The fungal contaminations were the main problem of *in vitro* seed germination of *C. fenestratum* while bacterial contaminations were totally controlled by surface sterilizing seeds with 0.1% $HgCl_2$ for 2 min. Therefore, effects of 3 different fungicides on minimizing contaminations were evaluated. Seeds dipped in fungicides before surface sterilization was not affected on reducing fungal contaminations as the seeds exposed to open air during sun cracking. Seeds dipped in fungicides after subjecting to sun cracking, reduced fungal

contaminations up to certain extent (Table 1). Significantly highest ($P \leq 0.05$) mean non contamination rates were obtained with 0.7g/L Topsin during 6 months of observation period.

**Table 1. Mean non contamination rates achieved with different fungicide treatments**

| Fungicide treatment | Exposure time | Mean non contamination % | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 month | 2 months | 3 months | 4 months | 5 month | 6 months |
| 0.6 g/L Captan | 15 min | 100.0$^{a}$± 0.0 | 56.0$^{c}$±8.9 | 36.0$^{c}$±8.9 | 18.0$^{c}$±4.4 | 8.0$^{d}$±4.4 | 2.0$^{d}$±0.0 |
| | 30 min | 100.0$^{a}$± 0.0 | 76.0$^{b}$±8.9 | 56.0$^{b}$±8.9 | 36.0$^{b}$±8.9 | 18.0$^{c}$±4.4 | 8.0$^{cd}$±4.4 |
| 0.7 g/L Thiram | 15 min | 100.0$^{a}$± 0.0 | 72.0$^{b}$±10.9 | 52.0$^{b}$±10.9 | 32.0$^{b}$±10.9 | 16.0$^{c}$±5.4 | 6.0$^{d}$±5.4 |
| | 30 min | 100.0$^{a}$± 0.0 | 72.0$^{b}$±10.9 | 52.0$^{b}$±10.9 | 32.0$^{b}$±10.9 | 32.0$^{b}$±10.9 | 16.0$^{c}$±5.4 |
| 0.6 g/L Captan+ 0.7 g/L Thiram | 15 min | 100.0$^{a}$± 0.0 | 80.0$^{b}$±0.0 | 56.0$^{b}$±8.9 | 36.0$^{b}$±8.9 | 36.0$^{b}$±8.9 | 18.0$^{c}$±4.4 |
| | 30 min | 100.0$^{a}$± 0.0 | 96.0$^{a}$±8.9 | 96.0$^{a}$±8.9 | 96.0$^{a}$±8.9 | 92.0$^{a}$±10.9 | 56.0$^{b}$±8.9 |
| 0.7 g/L Topsin | 15 min | 100.0$^{a}$± 0.0 | 96.0$^{a}$±8.9 | 96.0$^{a}$±8.9 | 96.0$^{a}$±8.9 | 96.0$^{a}$±8.9 | 90.0$^{a}$±10.9 |
| | 30 min | 100.0$^{a}$± 0.0 | 96.0$^{a}$±8.9 | 96.0$^{a}$±8.9 | 96.0$^{a}$±8.9 | 96.0$^{a}$±8.9 | 96.0$^{a}$±8.9 |
| CV | | 0.0 | 9.7 | 12.4 | 14.4 | 14.7 | 17.5 |
| LSD | | 0.0 | 10.2 | 10.9 | 10.3 | 9.4 | 8.3 |

*Means represented by the same letter under each month are not significantly different at P≤ 0.05 probability level*

For this experiment seeds were surface sterilized using 0.1% $HgCl_2$ for 2 min which was not negatively affected on viability (Fig. 3). Result revealed that the viability of seeds reduced considerably after a month when seeds were exposed to fungicides up to 15 to 30 min either before or after the surface sterilization (Figs. 4, 5). The loss of viability may be due to the effect of fungicides used and the experiment was not continued further.

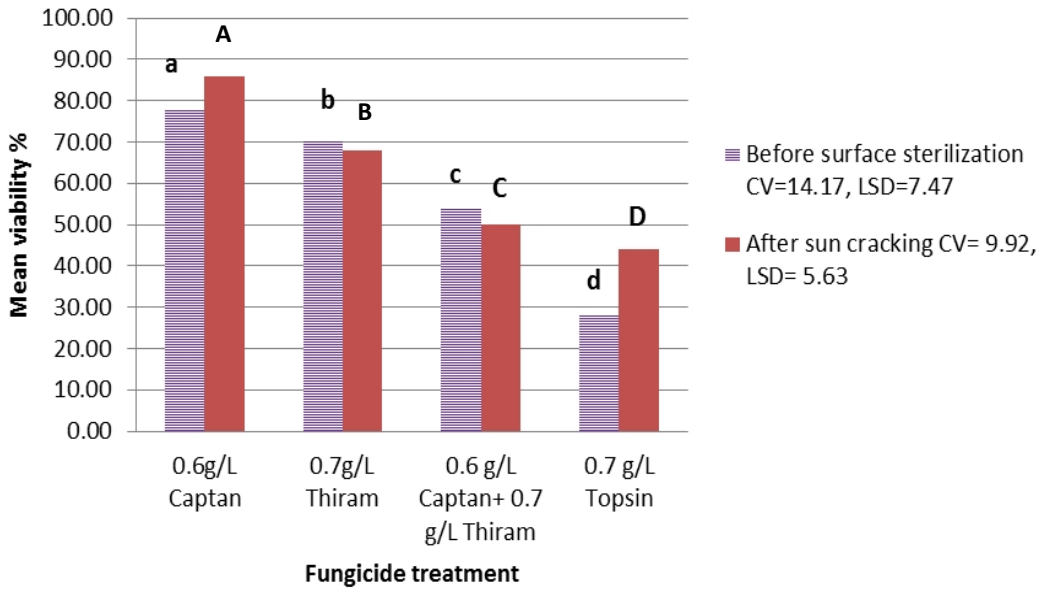



**Fig. 4. Mean viability percentages, one month after exposing to different fungicide treatments**

*Means represented by the same letter case are not significantly different at P≤0.05 probability level*

The results revealed that importance of 0.7g/L Topsin in minimizing fungal contaminations (Table 1). However, direct exposure to this fungicide may damage immature embryo where fungicide solution may absorb to the seed by imbibition or through the crack made in the

seed coat during sun cracking (Figs. 4 and 5). Therefore, incorporation of 0.7g/L Topsin into culture medium is the only option to be practiced. However, even after introducing Topsin into culture media there were minor fungal contaminations which may be due to some systemic fungal species. It was reported that isolated entophytic fungal species from *C. fenestratum* leaves and stems [9].

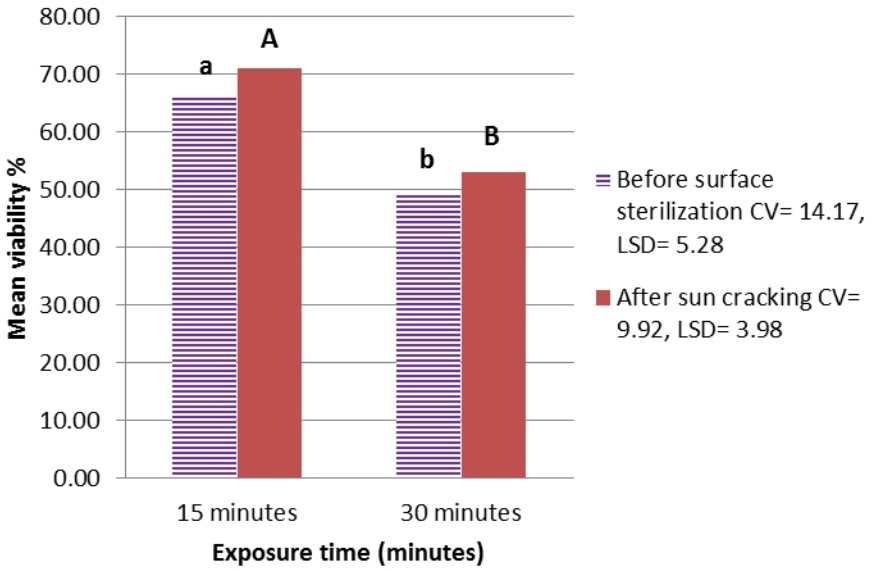



**Fig. 5. Mean viability percentages, one month after exposing to different exposure times of fungicides**

*Means represented by the same letter case are not significantly different at P≤ 0.05 probability level*

## 3.3 Identification of Best Seed Pretreatment to Enhance Germination

*C. fenestratum* seeds have a very hard seed coat which cannot be damaged aseptically inside a lamina flow. Normally it can be split using a nut cracker however, this cannot be practiced aseptically and if the seeds are separated into two halves totally the embryo will be damaged. Therefore, the only method is to expose to direct sun light up to 6h until a crack appear on the seed coat. For this also it is not possible to use a sealed container to place the surface sterilized seeds as they need to evaporate moisture to facilitate the cracking. It was recorded that *C. fenestratum* seed germination can be enhanced by dipping the seeds in 2250mg/L $GA_3$ solution for 24h [4]. Therefore, during the study 1$^{st}$ set of sun cracked seeds were dipped 24h in filter sterilized 2250mg/L $GA_3$ solution. To confirm the effect of $GA_3$ on seed germination, 2$^{nd}$ set of seeds were subjected to sun cracking only and 3$^{rd}$ set of seeds were dipped in sterilized distilled water for 24h instead to $GA_3$ after sun cracking. As the control 4$^{th}$ set of seeds were not subjected to sun cracking, but expose to $GA_3$ treatment. The results revealed that the importance of dipping seeds in 2250mg/L $GA_3$ solution for 24h after sun cracking, achieving 60% germination followed by 44% germination recorded with seeds dipped in sterilized distilled water for 24h (Fig. 6). It was recorded that presence of some inhibitory substances coupled with hard seed coat of *C. fenestratum* [4] may be the reason for germination occurred with seeds dipped in water for 24h where those substances dissolve in water. None of the other treatments facilitate germination (Fig. 6). Minor fungal contaminations were aroused 4 weeks after culture initiation even on seeds which were not

subjected to sun cracking (during sun cracking seeds were exposed to external environment). While surface sterilization all the fungal contaminants were removed from seed surfaces however, if they presence within the seed termed as systemic fungal species were survived and spread on the culture medium after couple of weeks from culture initiation.

It was recorded some sort of seed clipping before introducing seeds into culture medium [7]. However, details regarding method of seed splitting and other seed pretreatments were not mentioned. During our study it was very difficult to split seeds using a nut cracker inside the lamina flow cabinet due to the hardness of seed coats.

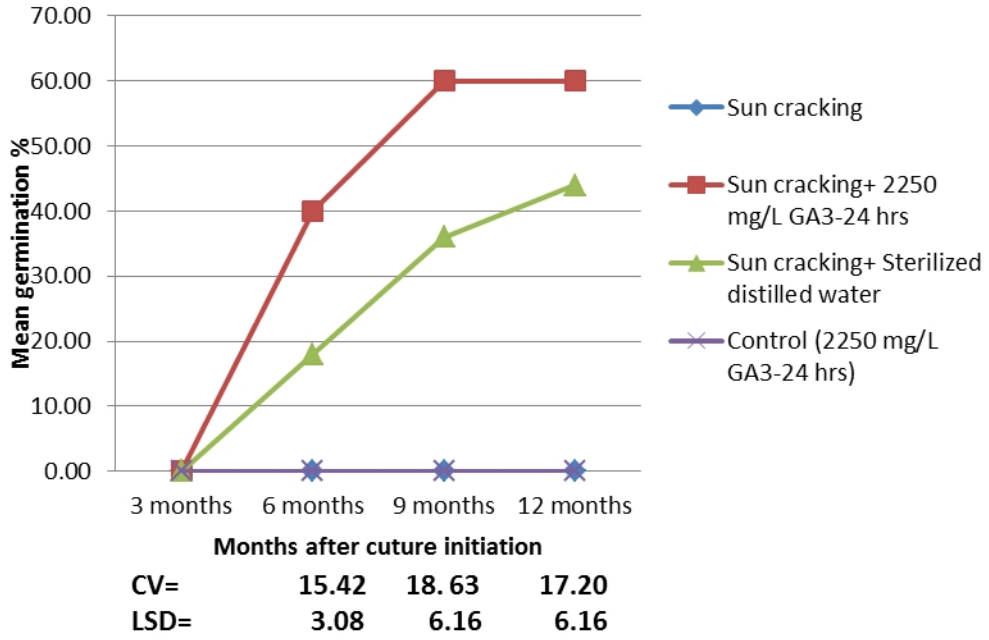



**Fig. 6. Mean germination rates of seeds after subjecting to different pretreatments**

*C. fenestratum* seed dormancy is coupled with mechanical barrier created by the hard seed coat for imbibition of water, presence of inhibitory substances and limited space created both by the seed coat and thick endosperm suppressing embryo growth and immature embryo to grow into a seedling [4]. Initial studies conducted showed that germination was facilitated up to certain extent by cracking the seed coats by exposing to direct sun light for 6 h and dipping in water for 24h [4]. Bioassay conducted using *Brassica junceae* L. to examine the presence of germination inhibitory substances in endospermic tissues and seed coats of *C. fenestratum* provide some evidence of chemical dormancy as well [4].

### 3.4 Identification of Best *In Vitro* Seed Germination Medium

Significantly different mean germination rates were resulted with *in vitro* seed establishment media at $P \leq 0.05$ probability level (Fig. 7) up to twelve months observation period. The highest seed germination (39.0%) was resulted in sand: coir dust added distilled water medium followed by 35.0% germination in 1g/L AC containing water agar medium. Due to fungal contaminations none of the seeds were survived in MS containing media. Sucrose

presence in the MS media may facilitate fungal growth and higher growth rate of fungi and huge mycelium produced by the fungi by covering the surface of the culture bottles may suppress the seedling growth.

However, after introducing 0.7g/L Topsin into culture media germination rates were enhanced drastically (Fig. 8) by minimizing the fungal contaminations up to certain extent. During 9 months culture period, the 80% highest mean germination rate was recorded with sand: coir dust medium followed by MS containing sand: coir dust medium (60%) and AC containing MS medium (58%) while water agar medium remained with 40% mean germination rate. Accordingly, it can be assumed that sand: Coir dust accelerates germination of *C. fenestratum* seeds compared to agar media. This may be due to the texture and porosity of the sand: coir dust medium which facilitates radicle growth. Further dark conditions provided and absorption of some inhibitory chemicals presence in the seeds by AC and nutrients existence in the MS medium may aid to enhance germination rates.

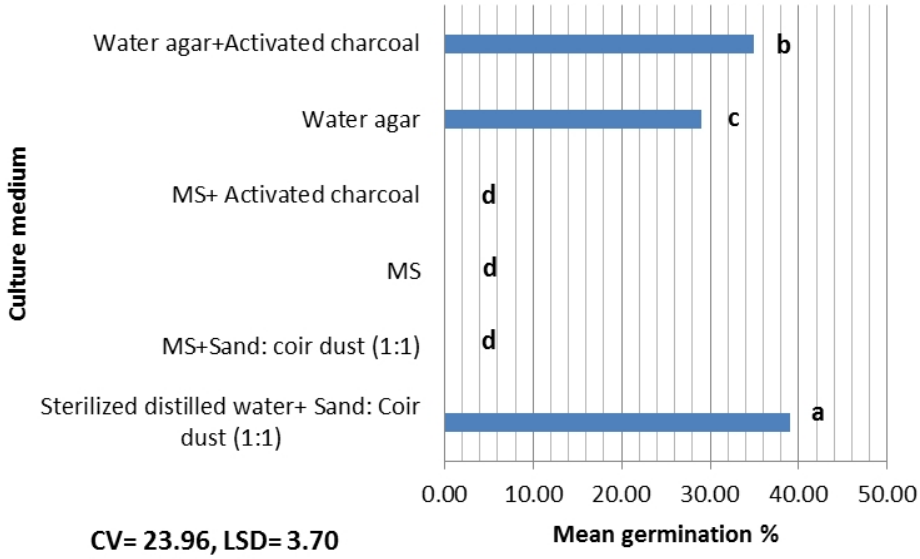



**Fig. 7. Germination rates after 12 months as affected by different culture media**
*Means represented by the same letter are not significantly different at P≤ 0.05 probability level*

Even it was recorded that clipped seeds of *C. fenesreatum* were germinated on MS basal medium supplemented with 3% sucrose and 0.8% agar with 100% germination within 4 weeks [7]; during our study without incorporating fungicide into culture media it was difficult to control contaminations. Comparatively sand: coir dust consisting media were better than agar media used where MS and water ager media fortified with 1g/L AC performed better than AC free media. However, due to presence of sucrose MS media seems to be contaminated by fungi with time even after incorporating fungicides into the media.

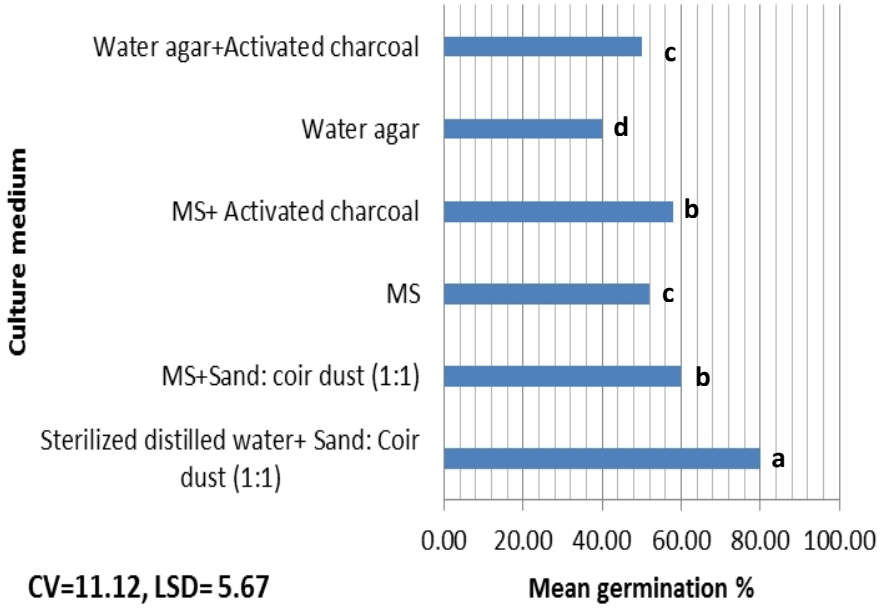



**Fig. 8. Germination rates after 12 months in 0.7g/L Topsin incorporated different culture media**

*Means represented by the same letter are not significantly different at P≤0.05 probability level*

## 3.5 Identification of Best Culture Medium for Better *In Vitro* Growth of Seedlings

It was observed that germinated seedlings remain with very slow growth rate as it takes 2- 3 months to appear first leaf after germination.

After introducing 0.7g/L Topsin into culture media, MS media gave significantly ($P \leq 0.05$) second best germination rate (Fig. 8) however, due to some secondary contaminations as we exposed seeds to external environment during sun cracking and sometimes may be due to some systemic fungal species the contaminations were failed to control 100%. The sucrose enriched MS medium may facilitate fungal growth. Therefore, instead of MS medium we used MS salts for this experiment to evaluate its effect on seedling growth while minimizing fungal contaminations. Therefore, new series of culture media were prepared using sand: coir dust (1:1) and MS salts at 20ml per each culture vessel.

It was recorded that immaturity of embryo can be overcome by plant growth regulators [10] especially using GA and cytokinins. Therefore, cytokinin enriched mature coconut water and commercially available kinetin and Benzyle Amino Purine (BAP) were used to evaluate their effect on seedling growth of *C. fenestratum*.

Incorporation of MS salts into the sand: coir dust medium facilitated growth performances of seedlings (Fig. 9) within a month after germination. However, incorporation of BAP and Kinetin individually or in combination is not effective in enhancing seedling growth of *C. fenestratum* even it was recorded the effectiveness of incorporating plant growth regulators into a culture medium to induce embryo growth and bud formation where a combination of

BAP and Kinetin performed well compared to BAP or Kinetin alone [11]. Further coconut water even it is recognized as a rich source of vitamins, amino acids and Kinetin [12], it does not facilitate growth of *C. fenestratum* seedlings (Data were not shown).

It can be recommended to introduce MS salts along with 0.7g/L Topsin into the sand: coir dust (1:1) containing culture bottles to grow *C. fenestratum* seeds *In vitro*.

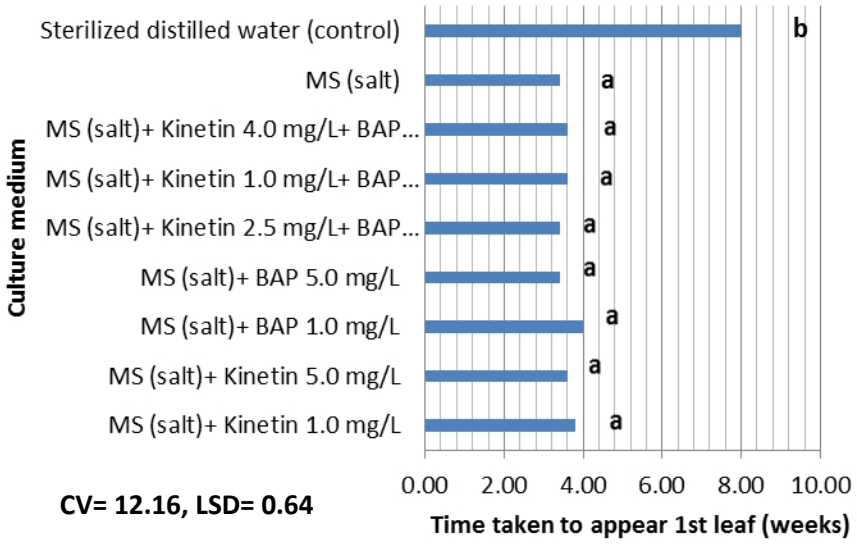



**Fig. 9. Growth of seedlings in 0.7 g/L Topsin and sand: coir dust (1:1) incorporated different culture media**

*Means represented by the same letter are not significantly different at P≤ 0.05 probability level*

## 3.6 *Ex Vitro* Seed Germination

It takes up to 6 months to begin *in vitro* germination of *C. fenestratum* seeds (Fig. 6). However, it was recorded that 2250mg/L $GA_3$ could enhance germination after 1 ½ months [4] from seed sowing inside a shade house on sand: coir dust (1:1) filled trays and achieved; over 90 % germination within 3 months. This was proofed by the seeds sown in shade house after surface sterilization by 0.1% $HgCl_2$ for 2 min with 90% germination after 3 months (Fig. 10). Therefore, *in vitro* culture conditions might play a major role in this delay in germination. Seeds under 25±2ºC temperature in a culture room were not germinated and seeds were germinated only under 32±2ºC temperature inside an incubator with total dark conditions. RH inside culture vessels were around 100%. This low temperature compared to the shade house (around 36±2ºC) may be the reason for delaying germination. *C. fenestratum* seeds are rich in food resources [9] and under high temperature and moisture the embryo begins respiration followed by burning food which facilitates germination.

After germination of seeds, culture vessels were transferred to a culture room under 25±2ºC temperature and 16h photo period with 3000 lux light intensity for further development of the seedlings.

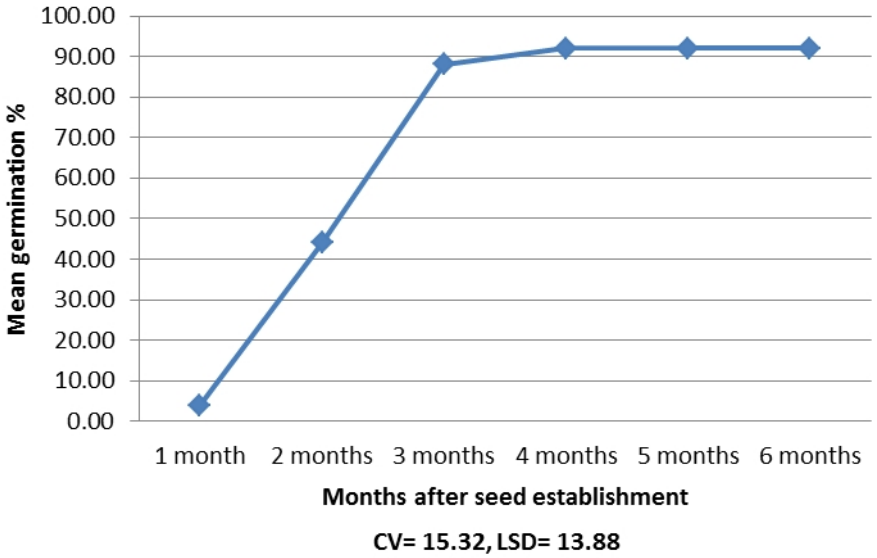



**Fig. 10. Mean germination rates of seeds established *Ex vitro* up to 6 months**

## 3.7 Effect of Seed Storage on *In Vitro* Seed Germination

It was found that seeds can be stored up to 9 months under 4ºC if seeds were wrapped using wet cotton and stored inside sealed cellophane achieving 60% germination. Due to thinning of seed coats with time [Warakagoda et al. PhD thesis, University of Ruhuna, Sri Lanka] make it easy to damage the seed coat inside a lamina air flow cabinet using a sterilized secateurs. Therefore, sun cracking is not needed for stored seeds. The seeds naturally reach the germination stage after 3 months of dormant period then continued germination up to 6-9 months [4]. Seeds treated with 2250mg/L $GA_3$ for 24h resulted 76% mean germination rate, a month after culture initiation and then up to 6 months germination rate was not increased further. Seeds soaked in sterilized distilled water for 24h reached to 76% mean germination rate with an increasing rate up to 4 months from the culture initiation (Table 2). Incorporation of 2, 20, 200 and 2000mg/L $GA_3$ into culture media were not significantly ($P \leq 0.05$) affected on mean germination rates of seeds. Seeds which were not soaked in distilled water for 24h (control) were not germinated assuring the necessity of imbibition of water to remove trace inhibitory substances and to activate enzymes towards germination.

Even the seeds were stored contact with a fungicide, were not subjected to sun cracking under open conditions prior to culturing and presence of fungicide in the culture medium; fungal contaminations can be observed after a month from culture initiation, providing some evidence of presence of some systemic fungal species inside the seeds. In addition systemic fungal contaminations are more prone to occur under high moisture and temperature conditions.

**Table 2. Mean germination rates of seeds established *in vitro* after 3 months of storage**

| Treatment | 1 month | 2 months | 3 months | 4 months | 5 months | 6 months |
|---|---|---|---|---|---|---|
| 2250 mg/L GA3- 24 hrs | 76.0$^{a}$±8.9 | 76.0$^{a}$±8.9 | 76.0$^{a}$±8.9 | 76.0$^{a}$±8.9 | 76.0$^{a}$±8.9 | 76.0$^{a}$±8.9 |
| Sterilized distilled water-24 hrs | 32.0$^{b}$±10.9 | 56.0$^{b}$±10.9 | 72.0$^{a}$±10.9 | 76.0$^{a}$±8.9 | 76.0$^{a}$±8.9 | 76.0$^{a}$±8.9 |
| Sterilized distilled water-24 hrs+ 2 mg/L GA3 | 32.0$^{b}$±10.9 | 56.0$^{b}$±10.9 | 72.0$^{a}$±10.9 | 76.0$^{a}$±8.9 | 76.0$^{a}$±8.9 | 76.0$^{a}$±8.9 |
| Sterilized distilled water-24 hrs+ 20 mg/L GA3 | 32.0$^{b}$±10.9 | 52.0$^{b}$±8.9 | 72.0$^{a}$±10.9 | 76.0$^{a}$±8.9 | 76.0$^{a}$±8.9 | 76.0$^{a}$±8.9 |
| Sterilized distilled water-24 hrs+ 200 mg/L GA3 | 36.0$^{b}$±8.9 | 52.0$^{b}$±8.9 | 76.0$^{a}$±8.9 | 76.0$^{a}$±8.9 | 76.0$^{a}$±8.9 | 76.0$^{a}$±8.9 |
| Sterilized distilled water-24 hrs+ 2000 mg/L GA3 | 36.0$^{b}$±8.9 | 52.0$^{b}$±8.9 | 76.0$^{a}$±8.9 | 76.0$^{a}$±8.9 | 76.0$^{a}$±8.9 | 76.0$^{a}$±8.9 |
| Control | 0.0$^{c}$±0.0 | 0.0$^{c}$±0.0 | 0.0$^{b}$±0.0 | 0.0$^{b}$±0.0 | 0.0$^{b}$±0.0 | 0.0$^{b}$±0.0 |
| CV | 16.0 | 11.4 | 8.3 | 5.1 | 5.1 | 5.1 |
| LSD | 7.3 | 7.3 | 6.9 | 4.4 | 4.4 | 4.4 |

*Means represented by the same letter under each month are not significantly different at P≤ 0.05 probability level*

During the study, seed germination rate was not exceeded than 76% even the seeds treated with $GA_3$ providing some evidence of loss of seed viability after 7 months of storage. Therefore, it can be assumed as it is possible to achieve 76% *in vitro* germination within one month incubation from seeds stored for 6 months as if they dipped in sterilized distilled water for 24 h prior to culture initiation. Therefore, $GA_3$ treatment is ineffective in facilitating *in vitro* germination of seeds at 6 months storage. As the seeds require high temperature (32±2 $^{0}$C) for germination and highly moist environment inside the culture vessels which are more flavored by the fungal contaminants, the reduced culture period into one month is beneficial to avoid systemic fungal contaminations.

## 3.8 Acclimatization of *In Vitro* Germinated Seedlings

Plantlets, during 14 weeks of acclimatization procedure as explained in the above were gradually adapted to normal environmental conditions with over 90% survival rate and successfully introduced to field conditions (Fig. 11).

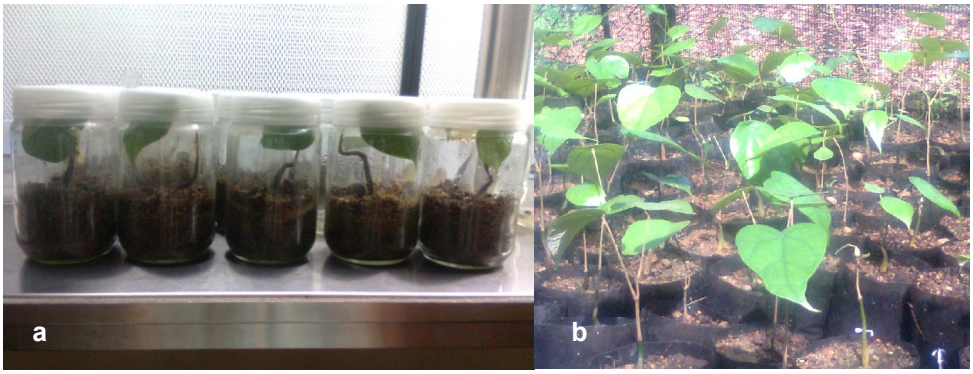



**Fig. 11a. *In vitro* germinated seedlings before acclimatization, b: Successfully acclimatized plantlets ready for field establishment**

## 4. CONCLUSIONS

Mature seeds of *Coscinium fenestratum* can be successfully surface sterilized by dipping explants in 0.1% $HgCl_2$ solution (w/v) for 2 min followed by two successive washings using sterilized distilled water. Seeds have to be washed thoroughly using soap water and kept under running tap water for half an hour prior to surface sterilization. After surface sterilization, hard seed coats of the seeds have to be damaged mechanically then dipped in filter sterilized 2,250mg/L $GA_3$ solution for 24h to break the dormancy before culturing under aseptic conditions.

Seeds have to be cultured into MS salt fortified culture media which were solidified by using sterilized sand: coir dust (1:1). To minimize fungal contaminations 0.7g/L Topsin has to be incorporated into culture medium. Incorporation of coconut water 250ml/L as a source of vitamins and Kinetin or different concentrations of BAP and Kinetin alone or in combination were not enhanced seedling growth of *C. fenestratum*.

The highest 80% germination rate can be achieved by freshly collected seeds within 9 months from culture initiation. Seeds, which were mixed with Topsin powder, wrapped using wet cotton, kept inside sealed cellophane and stored up to 3 months under $4^0$C; achieved 76% highest germination rate a month after culture initiation. The seeds needed to be treated with 2250mg/L $GA_3$ for 24h before culturing to achieve the above results. Even without the $GA_3$ treatment, seeds after 3 months storage, which were dipped 24h in sterilized distilled water achieved 76% germination within 4 months. To facilitate germination, seeds have to be exposed to total dark conditions and temperature should be increased to above 32±2ºC inside an incubator.

Incorporation of 2, 20, 200 and 2000mg/L $GA_3$ into culture media were not effective in enhancing *In vitro* germination rates.

*In vitro* germinated seedlings of *C. fenestratum* can be successfully acclimatized and introduced to field conditions.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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