Studies on Seed Germination of *Coscinium fenestratum* (Gaertn.) Colebr. – A Threatened Medicinal Plant

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Abstract

Mature seeds of *Coscinium fenestratum* recorded 92.20% germination after pre-treating the seeds by exposing to direct sun light for 6 hours (sun cracking) followed by dipping seeds in 2250 mg/L Gibberelic Acid (GA_3) solution for 24 hours. Seeds subjected to sun cracking followed by water soaking for 24 hours (59.2%) resulted significantly ($P \leq 0.05$) low germination rate. Seeds subjected sun cracking followed by water soaking for 24 hours started to germinate four months after sowing and continued up to six months while GA_3 pretreatments significantly ($P \leq 0.05$) reduce the time taken for germination from six months to three months. Germination was facilitated on sand:coir dust (1:1 ratio) medium after improving the micro climate by maintaining the temperature above 30°C, minimizing fungal contaminations, regular watering and under total dark conditions. Highest *in vitro* seed germination (39%) was achieved at 32±2°C temperature under total dark conditions where seeds were surface sterilized using 20% Clorox (5.25% NaOCl) followed by 70% Ethanol for 2 minutes and subsequently cultured into sand:coir dust medium aseptically. Seeds on water agar medium consisting 1g/L Activated Charcoal (AC) achieved 35% mean germination rate which was not significantly different ($P \leq 0.05$) with the previous. Seeds were not germinated on MS medium due to the presence of sucrose which facilitates microbial contaminations. Seeds of *C. fenestratum* coupled with exogenous (physical, chemical and mechanical) seed dormancy created by hard seed coat, inhibitory substances presence in the seed coat and the endosperm and endogenous (physiological) dormancy created by some other physiological factors as high Abscisic acid (ABA)/GA_3 ratio. Temperature above 30°C and dark conditions facilitate germination of mature seeds of *C. fenestratum* after split opening the hard seed coats by exposing to direct sun light for 6 hours followed by removing inhibitory chemicals, facilitating embryo growth to reduce the time taken for germination and reducing inherent ABA/GA_3 ratio by dipping the seeds in 2250 mg/L GA_3 solution for 24 hours.

***Keywords**: Aseptically, Dormancy, Inhibitory, Physical, Physiological*

INTRODUCTION

Coscinium fenestratum (Gaertn.) Colebr. is a large dioecious woody liana (Menispermaceae) indigenous to the Indo-Malayan region. It is found in India, Malaysia, Vietnam, Myanmar, Singapore, Thailand and Sri Lanka (Tushar *et al.*, 2008). *C. fenestratum* is naturally found mainly in the forest fringes and disturbed forests in the lowland wet zone of the Sri Lanka (Karunarathne, 2001).

It has very high economic value and its stem is extensively used in Ayurvedic preparations for treating digestive disorders, chronic fevers, wounds and ulcers in South India and Sri Lanka. This bitter tonic and yellow dye has found its way to Europe under the name False Calumba or Tree Turmeric (Karunarathne, 2001). The root bark is used for dressing wounds, ulcers and in cutaneous leishmaniasis. The paste of *Coscinium* and turmeric is applied for snake bites.

The roots and stem are reported to contain alkaloids magnoflorine, berberrubine, thalifendine, palmitine and oxyberberine *etc.* 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. It 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* (Tushar *et al.*, 2008).

The plant takes around fifteen years to mature and flower. The liana is facing a population reduction since it is overexploited from natural habitats even before they reached to reproduction stage for its medicinal importance (Tushar *et al.*, 2008) while degradation of natural habitats, habitat specificity and zero cultivation is in this account as well.

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 (Tushar *et al.*, 2008). This species has a well-established local market and as a result of illegal exploitation, destructive collection and the relatively slow growth rate natural populations are disappearing at an alarming rate (Abewardana *et al.*, 2001).

Hence, this species is now banned for exportation by the Ministry of Commerce, Govt. of India (Tushar *et al.*, 2008) identification of feasible propagation techniques is crucial to fulfill the unlimited island wide demand and for biodiversity conservation. Therefore, the present investigation was aimed to study the seed biology of *C. fenestratum* including seed moisture content, seed viability, seed storage and germinability for the sustainable growth of this endangered species in the wild.

MATERIALS AND METHODS

All the experiments were carried out at the Department of Crop Science, Faculty of Agriculture, University of Ruhuna, Mapalana, Kamburupitiya, Sri Lanka.

Collection of seeds: Mature fruits were collected from Sinharaja, Kanneliya and Wilpita forest conservation areas. Climbers were randomly selected for seed collection. Fruit bunches were bagged and fruits were collected just after shedding.

Preparation of seeds: Fruits were subjected to alternative drying (8 hours) and wetting (16 hours) conditions until it's possible to remove hard pericarps easily. Drying was performed by laying seeds on newspapers as single layers, inside the laboratory under shady conditions. Seeds were thoroughly washed to remove all the trace pulps around the seed coats to minimize secondary fungal contaminations.

Seed pretreatments: Series of pretreatments were assigned to break seed dormancy and to facilitate germination (Table 1). Based on the results obtained from this experiment, series of combined pretreatments were assigned to check the possibilities of enhancing germination rates of seeds collected from different sites.

Ex vitro seed germination: Five types of potting media were prepared using sand, coir dust and sand and coir dust (1:1, 2:1 and 1:2 ratios). Seperately sand and coir dust only medium were also used. Seed trays were filled by prepared media, placed inside a shade house and drained using Topsin M 70 (Thiophanate methyl 70% (w/w), 0.7 g/L fungicide solution. 24 hours later pretreated seeds were sawed in planting holes. After watering up to the saturation of potting media, planting trays were covered using 200 gauge black polythene sheets. The recorded temperature inside the black polythene covers was 30- 34°C.

In vitro seed germination: Seeds were surface sterilized using 20% Clorox (5.25% NaOCl) for 5 minutes followed by 70% Ethanol for 2 minutes. Immediately after dipping in each solution seeds were thoroughly washed twice using sterilized distilled water. After surface sterilization seeds were subjected to sun cracking by placing them on sterilized aluminum foils. Then dipped in filter sterilized 2250 mg/L GA_3 solution for 24 hours and cultured under aseptic conditions into MS (Murashige and Skoog, 1962) medium with and without 1g/ L AC. Second set was cultured into sterilized sand: coir dust (1:1) contained culture bottles either each bottle was nourished by 20 ml of liquid MS medium or not. Third set was cultured into water agar medium consisting or not consisting 1g/ L AC.

The seeds were incubated under 25±2 °C temperature in a culture room and 32±2 °C temperature inside an incubator under total dark conditions. Relative humidity inside the culture

vessels were maintained above 50% and all the vessels were covered using black polythene to cut off the light effect.

Experimental design and statistical analysis: Seed pretreatment experiments were conducted by using a Randomized Complete Block Design (RCBD) with 10 trees as replicates from each site. Numbers of germinated seeds were recorded weekly. Results obtained as percentages and indexes were arcsine transformed to normalized data before statistical analysis. Mean separations were carried out by applying Duncan's multiple range test (DMRT) using the SAS software. Two-way ANOVA was used to test the effects of different sites and pretreatments on seed germination. Experiments were repeated in two fruiting seasons. One-way ANOVA were used to identify the best potting medium and best *in vitro* seed germination medium by assigning treatments according to Completely Randomized Design (CRD).

RESULTS

Identification of best seed pretreatment method

The mean germination percentages resulted by series of pretreatments tested to enhance seed germination of *C. fenestratum* were significantly different to each other at $P \leq 0.05$ probability level (Figure 01). After 6 months, seeds dipped in 2000 mg/L GA_3 solution for 24 hours achieved 67.7% germination rate followed by 60.2% germination rate given by the seeds dipped in 2500 mg/L GA_3 solution for 24 hours, 56.70% germination rate from seeds subjected to sun cracking by exposing to direct sun light for 6 hours and 56.3% germination rate by seeds dipped 24 hours in water before sowing (Figure 1).

Pretreatments which achieved higher germination rates were combined and to identify the most accurate GA_3 concentration, a new series of pretreatments were allocated. Seed germination rates according to different pretreatments were significantly different ($P \leq 0.05$) to each other after 6 months (Figure 2). Higher germination rates (92.20%, 92.00%) were resulted by seeds subjected to sun cracking for 6 hours followed by dipped in 2250 mg/L and 2500 mg/L GA_3 solutions for 24 hours respectively.

The results revealed that there was an interaction effect of pretreatments and number of months after sowing on mean germination rates ($P \leq 0.05$) (Figure 3). The highest germination rate (97.90%) was achieved by seeds treated with 2250 mg/L GA_3 for 24 hours after 3 months. The germination rates resulted after 3, 4, 5 and six months were not significantly different ($P \leq 0.05$) to each other for all the pretreatments. All the GA_3 treated seeds were begun to germinate after two weeks form sowing and continued up to 3 months where the seeds subjected to water soaking for 24 hours started to germinate after 3 months and continued up to 6 months (Figure 3).

The results obtained from initial seed pretreatment methods were site specific ($P \leq 0.05$) where highest germination rate was obtained by seeds collected from Kanneliya followed by Sinharaja and Wilpita forest conservation areas (Figure 4). However with improved seed pretreatments, the germination rates at three sites were not significantly different to each other (Figure 04).

Selection of best potting medium

Potting media were significantly different to each other at $p \leq 0.05$ probability level when considering the mean germination percentages after 6 months of observation period (Figure 5).

Identification of best in vitro seed germination medium

Significantly different ($P \leq 0.05$) mean germination rates were resulted with *in vitro* seed establishment media (Figure 6) kept under total dark conditions and 32±2 °C temperature up to 12 months of observation period. Higher seed germination rates (39.0%) were resulted in sand: coir dust medium followed by 35.0% germination in 1g/L AC containing water agar medium (Figure 8). Seeds cultured on MS medium and MS media containing either AC or sand: coir dust (1:1) were not germinated due to the fungal contaminations.

DISCUSSION

Identification of best seed pretreatment method

There are two types of seed dormancy: exogenous/seed coat dormancy and endogenous dormancy. Seeds with seed coat dormancy usually have a seed coat that is impermeable to oxygen and/or water creating physical dormancy or provides mechanical barrier for embryo growth. Occasionally the dormancy is caused by an inhibiting chemical in the epidermis or adjacent interior membranes creating chemical dormancy. Methods of breaking seed coat dormancy include scarification, hot water, dry heat, fire, acid and other chemicals, water, cold and warm stratification and light (Emery, 1987). Internal dormancy is a general term encompassing a number of physiological conditions that delay germination. Not all of these conditions are fully understood or easy to counteract (Emery, 1987).

Multiple dormancy factors are also occur. In one general type there is seed coat dormancy plus internal dormancy. Seeds with this dormancy combination must be treated for the impermeable seed coat first, then for internal dormancy.

Results of chromatographic and spectroscopic investigations of the seed resources of *C. fenestratum* showed that the endosperm contained comparatively high percentage of carbohydrate (67.33%) suggesting that the seed could remain dormant for a long period (Ariyarathna *et al.*, 2001).

It was recorded that the *C. fenestratum* embryo was located deeply within the invaginations of the endospermic tissues and the thick, stony seed coat (integument) and the dry and hard endospermic tissues act as mechanical barriers to the developing embryo. The seed coat consisted of a compactly arranged lignified strongly water repellent macrosclereid layer that impedes the imbibition process (Ariyarathna *et al.*, 2001).

Bioassays conducted using *Brassica junceae* L. to examine the presence of germination inhibitory substances in the seeds of *C. fenestratum* revealed that the 5% water extracts from the seed coat and the endospermic tissues gave very low germination (2-5 %), suggesting the presence of inhibitors compared to 80-99% germination resulted with 2% extracts (Ariyarathna *et al.*, 2001).

According to these facts it is clear that *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 sub pressing embryo growth. 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 hours and dipping in water for 24 hours (Ariyarathna *et al.*, 2001).

Therefore during the study, series of pretreatments were assigning to break hard seed coat by scarification using physical methods (sun cracking, hot water and dry heat treatment) and softening the seed coat by chemical methods (H_2SO_4). To remove inhibitory substances intact with seed coat and related tissues, seeds were dipped 12- 24 hours in water.

The sun cracking is more practical than scarification practiced by making mechanical damage to seed coat by rubbing, peeling or cracking. During the study seeds prior sowing subjected to sun cracking resulted significantly ($P \leq 0.05$) higher germination rate (56.70%) compared to 34.80% germination rate resulted with 12 hours water soaked seeds (control) (Figure 1).

For hot water treatment seeds should be dropped into about six times their volume of 82°-94 °C pre-heated water (rain water is desirable if it is near neutral in pH). They should be left to cool and soak in the water for 12 to 24 hours, after which they are ready for sowing. Another and more drastic hot water treatment is sometimes used for especially thick or hard-coated seeds. For this treatment, the seeds should be placed in vigorously boiling water for a specific length of time depending on the species, then immediately removed from the boiling water and cooled in cold water (Abuakar and Muhammad, 2013). During our study we exposed seed to boiling water for 5, 15 and 30 minutes time as the seeds consisting hard seed coats where 38.7%, 41.9% and 6.5% germination rates were resulted respectively which were significantly different to each other

($P \leq 0.05$) (Figure 1). This proves that seeds exposing 30 minutes to 100 °C tempered water may damage the embryo as those seeds recorded very low germination percent.

Oven or dry heat is not often recommended, and the temperatures required are more suitable to an incubator than a kitchen oven. For this seed coat treatment, the seeds should be placed in shallow containers in a preheated incubator or oven. The specific temperature and duration depend on the species. After the treatment, the seeds should be cooled immediately and sown. The temperature suggested is between 82-100°C (Emery 1987). During the study we exposed the seeds for 85°C dry oven temperature for 6, 12 and 18 hours then dipped in cold water immediately and sown. However very low germination rates were resulted as 12.2%, 9.4% and 5.2% accordingly (Figure 1). This proves that the used temperature and exposure time were somewhat high as significantly ($P \leq 0.05$) highest germination (12.2%) was resulted with the lowest exposure time compared to other two exposure times. Acid treatments are often used to break down especially thick impermeable seed coats (Abuakar and Muhammad, 2013). Since seeds placed in concentrated sulfuric acid (H_2SO_4) will become charcoal in time, the temperature of the acid and the length of time the seeds are soaked are very important. The acid should be used at room temperature for a period of a few minutes to several hours depending on the species. The seeds should be immersed in acid in a glass, porcelain or ceramic container (Wang *et al.*, 2007). With respect to our results seeds exposed to conc. H_2SO_4 for 30 minutes achieved 20.10% germination rate which was significantly ($P \leq 0.05$) higher value compared to 6%, 9.10% and 5% germination rates resulted with 5, 15 and 60 minutes exposure times (Figure 1). These results demonstrate that dry heat with temperature above 80°C for 6, 12 and 18 hours and conc. H_2SO_4 treatments applied to *C. fenestratum* seeds are harmful to the embryo because germination is reduced considerably compared to the control (12 hours water soaked seeds prior to sowing with 34.80% germination).

For the occasional species whose seed coats contain a readily water-soluble, germination-inhibiting chemical, this substance can be removed by soaking the seeds in tap water or by leaching the seeds in slowly running tap water for various lengths of time just prior to soaking. The length of time depends on the species. During over study freshly collected mature pods were subjected to alternate wetting and drying to facilitate removal of thick pericarp this may accelerate the removal of inhibitory substances up to certain extent as well. Water soaking for 24 hours before sowing may support for the removal of inhibitory chemicals and softening of hard seed coat encouraging the embryo to grow. Compared to the control this achieved significantly higher ($P \leq 0.05$) germination rate (56.30%) (Figure 1).

Three chemicals that have proven very helpful in breaking certain types of dormancy are GA_3, Potassium Nitrate (KNO_3) and Hydrogen Peroxide (H_2O_2). The aqueous solutions of these chemicals should be used at room temperature. The concentration and length of treatment depends on the species to be treated. Seeds soaked in GA_3, KNO_3 and H_2O_2 should be stirred unless specified, but sown immediately. After this soaking they can also be air-dried and stored for short periods and then sown or given a subsequent treatment (Emery, 1987). During the study seeds treated with 2000 mg/L GA_3 for 24 hours gave 67.70% highest germination rate followed by 60.20% germination resulted with seeds dipped in 2500 mg/L GA_3 solution for 24 hours (Figure 1) even though it was recorded that the external application of 5% GA_3 did not enhance seed germination of *C. fenestratum* (Ariyarathna *et al.*, 2001). The seeds pretreated by dipping 24 hours in water achieved 56.30% germination rate comparatively at $P \leq 0.05$ significant level (Figure 1).

Low germination rates achieved during this study even after breaking the hard seed coats and after exposing to GA_3 provide clues about an under developed embryo and higher internal ABA/ GA ratio which inhibit germination. This provides evidence that seed of *C. fenestratum* having a combined exo and endogenous dormancy. The second set of pretreatments addressed all these dormancy types and achieved 92.20% germination when the seeds were dipped in 2250 mg/L Gibberelic Acid (GA_3) solution for 24 hours after split opening the hard seed coat by sun cracking (Figure 2).

There is considerable evidence that ABA is an important positive regulator of both the induction and the maintenance of dormancy. Work with the strongly dormant *A. thaliana* showed that dormancy may depend on an intrinsic balance of gibberellins (GA) and ABA biosynthesis and catabolism, which will determine the dominance of either of the hormones. Thus the net result of the dormant state is characterized by increased ABA biosynthesis and GA degradation. It appears to be the ABA/GA ratio (http://www.seedbiology.de/dormancy .asp).

Endogenous gibberellins have been widely studied in relation to the breaking of seed dormancy in various species. GA_3 has been exogenously applied as a substitute for stratification and has increased germination in many plant species, including *Leucospermum*, *Fagus sylvatica, Helianthus* and *Echinacea angustifolia.* It was suggested that GA_3 affects physiological as well as metabolic activities of seeds, resulting in early germination (Keshtkar, 2008). During the study 6 months of germination period after sowing was significantly reduced to 3 months by pre-treating the seeds with GA_3 (Figure 3).

Similar results were recorded by parallel studies carried out in India the highest germination percent (60%) was recorded with 1500 mg/L GA_3 for 12 hours where the seeds were germinated within 40 days (Goveas *et al.*, 2011). In another research the seeds pretreated with GA_3 (1000-4000 mg/L) showed 79% seed germination rate after 6 months (Ramasubbu *et al.*, 2012). It was also recorded that fresh seeds pre-treated with 2-10% KNO_3 or 3000 mg/L GA_3 were germinated with 67 to 95% germination rates respectively (Anilkumar *et al.*, 2010). However local germination studies carried out from the seeds collected from Sinharaja forest conservation area revealed that after 3 months of planting 76% germination has been recorded with 2000 mg/L GA_3 (Gunatillake, 2002).

Selection of best potting medium

Seeds that require light should not be covered when sown but need regular watering. A covering of glass or plastic over the container will help to maintain a saturated atmosphere around the seeds. A few species must be kept in darkness during the first part of the germination period (Emery, 1987). Though not really a form of dormancy, undesirable temperatures used for germination can be partially or completely inhibitory. Temperature requirements for the germination of seeds of most native California species will be met if the seeds are sown at the proper time of year. If the recommended daily high/low temperatures are not present naturally, artificial means must be used to produce them or a propagator may use to capture heat. During the study germination of *C. fenestratum* seeds was facilitated by the temperature at 30-34°C, high moisture content and total dark conditions created by the black polythene used to cover the seed trays. The sand: coir dust medium is superior to sand medium (Figure 5) in terms of facilitating seed germination as coir duct can retain water up to certain extent and supporting the growing epicotyl to merge into as a better substrate. However, when increasing the amount of coir dust in the potting media fungal contaminations were arouse due to the incensement of moisture.

Identification of best in vitro seed germination medium

The study proved the necessity of total dark conditions and 32±2 °C temperature for *in vitro* germination of *C. fenestratum* seeds. After subjecting the seeds for sun cracking followed by surface sterilization and dipped in 2250 mg/L GA_3 solution for 24 hours, seeds have to be cultured into aseptically maintained sand: coir dust (1:1) or water agar media fortified with 1g/L AC where the germination rates were not significantly different to each other ($P \leq 0.05$) (Figure 6). Both are nutrient free substrates and AC act as absorbent of toxic chemicals secreted into the culture media (Razdan, 2003) and may facilitate germination by removing inhibitory substances contact with the embryo as the seeds were totally dipped in culture media. However none of the seeds were germinated on MS related media (Figure 6) due to microbial contaminations as they consist sucrose- a good nutritional source for endophytic fungi. It was recorded that *C. fenestratum* consisting 41 endophytic fungi belonging to sixteen different taxa (Goveas *et al.*, 2011). However

during the study the highest *in vitro* germination rate resulted was 39% (Figure 6) which was from seeds cultured on sand: coir dust medium during 12 months of observation period. Further studies are needed to enhance the *in vitro* germination percentage of *C. fenestratum* seeds.

CONCLUSIONS

Seeds of *C. fenestratum* coupled with exogenous (physical, chemical and mechanical) created by hard seed coat, inhibitory substances presence in the seed coat and the endosperm and endogenous (physiological) dormancy created by some other physiological factors as high Abscisic acid (ABA)/GA_3 ratio. Temperature above 30 ^{0}C and dark conditions facilitate germination of mature seeds of *C. fenestratum* after split opening the hard seed coats by exposing to direct sun light for 6 hours followed by removing inhibitory chemicals, facilitating embryo growth to reduce the time taken for germination and reducing inherent ABA/GA_3 ratio by dipping in 2250 mg/L GA_3 solution for 24 hours. Seed germination of *C. fenestratum* is not site specific recommending the developed protocol for island wide application.

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Table

Table 1: Seed pretreatments used to enhance germination

Pretreatment	**Description**
T1	12 hr water soaking
T2	24 hr water soaking
T3	6 hr Sun cracking
T4	5 min Boiling water
T5	15 min Boiling water
T6	30 min Boiling water
T7	1 hr Boiling water
T8	6 hr dry heat
T9	12 hr dry heat
T10	18 hr dry heat
T11	5 min conc. H_2SO_4
T12	15 min conc. H_2SO_4
T13	30 min conc. H_2SO_4
T14	1 hr conc. H_2SO_4
T15	12 hr 1500 mg/L GA_3
T16	12 hr 2000 mg/L GA_3
T17	12 hr 2500 mg/L GA_3
T18	24 hr 1500 mg/L GA_3
T19	24 hr 2000 mg/L GA_3
T20	24 hr 2500 mg/L GA_3

Figures

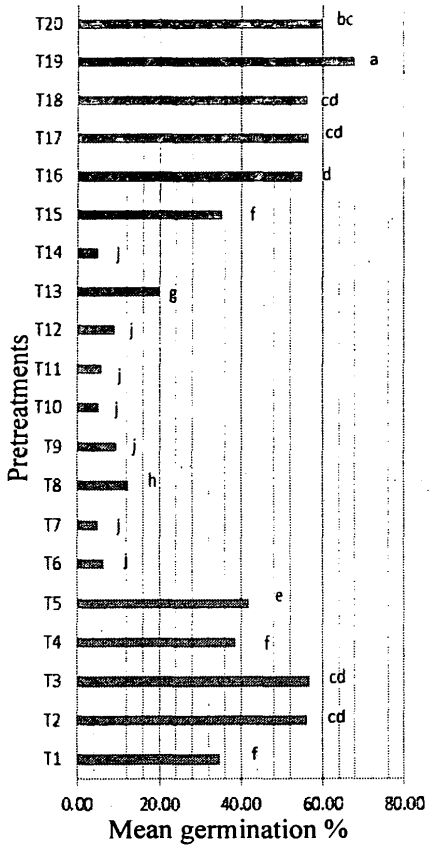

Figure 1: Germination rates after 6 months vs. initial series of seed pretreatments (CV 13.62). Means represented by the same letter are not significantly different at $P \leq 0.05$ probability level

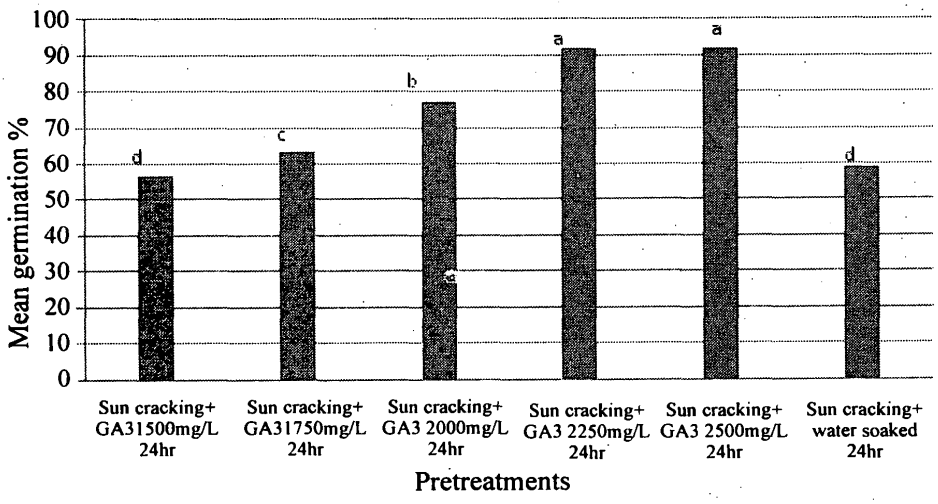

Figure 2: Germination rates after 6 months vs. improved series of seed pretreatments (CV 12.45). Means represented by the same letter are not significantly different at $P \leq 0.05$ probability level

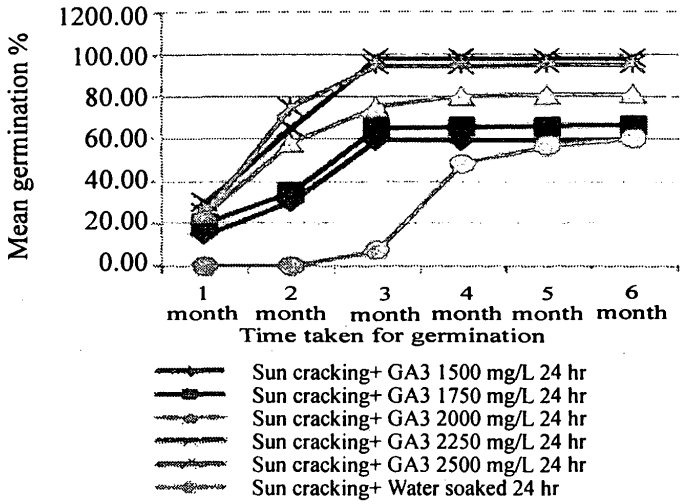

Figure 3: Germination rates vs. number of months taken to germinate (CV 15.28)

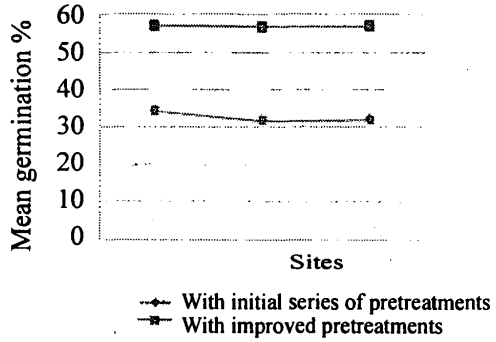

Figure 4: Germination rates obtained after series of pretreatments at three different sites

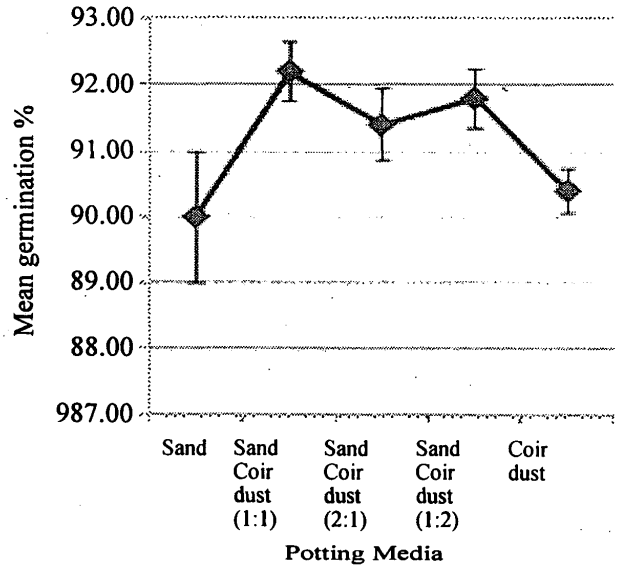

Figure 5: Germination rates after 6 months vs. different potting media. The highest germination rate (92.2%) was resulted with sand: coir dust (1:1) medium (Figure 7) compared to 90.0% germination recorded with sand medium (Figure 5).

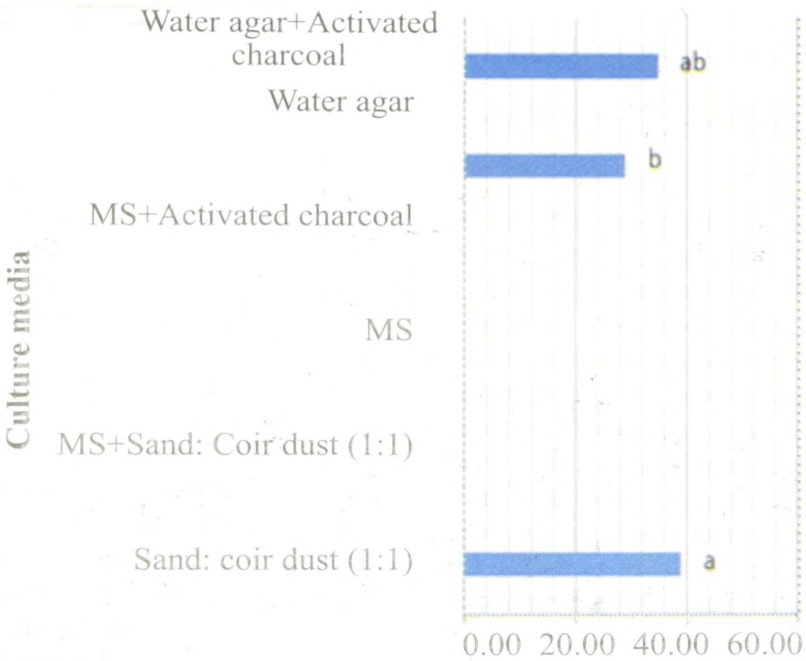

Figure 6: Germination rates after 12 months vs. different culture media. Means represented by the same letter are not significantly different at $P \leq 0.05$ probability level

Figure 7: Seeds begins to germinate and after producing the first leaf on sand: coir dust (1:1) medium

Figure 8: A seed begins to germinate *in vitro* in 1g/L Activated charcoal containing water agar medium