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Seedling Culture of *Semecarpus anacardium* L.

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ABSTRACT

Semecarpus anacardium (Anacardiaceae), a deciduous forest tree, is a potent source of medicinal compounds prescribed for a variety of ailments. Trees bear seeds in winter. Seasonal fruiting and poor viability limit the use of conventional propagation techniques. Use of *in vitro* techniques to overcome these limitations has not yet been reported. Microbe-free seedlings *of S. anacardium* could not be raised *in vitro* due to poor seed viability, acute seed-borne microbial contamination, and leaching of phenolics. Seed viability (30-35%) reduced with time and was completely lost in 6 months. Soaking seeds in concentrated sulfuric acid (H_2SO_4) helped in eliminating the pigmented layer from the surface of the seeds thereby making way for the phenolics to be released from seeds in washings which followed the H_2SO_4 treatment. Thus leaching of phenolics by the seeds in culture medium was reduced by treating them with H_2SO_4 prior to surface sterilization with NaOCl. Changes in the seed surface following acid treatments for varying periods (10, 20, 30, 40 min) were studied microscopically. Concentrated H_2SO_4 treatment for 20 min helped to increase the seed coat permeability and excretion of phenolics from the seeds. It also acted as a surface sterilant to a limited extent. Frequency of germination was increased to 63% when seeds were treated with H_2SO_4 for 20 min while exposure for 10 min was ineffective in controlling contamination whereas longer exposures (30 and 40 min) injured the embryos. Elimination of sucrose in the medium improved germination from 50 to 63%. A gradual loss of seed viability from 33 to 4% following storage for 5 months was demonstrated *in vitro*.

Keywords: contamination, growth regulator, phenolics, seed viability, sulfuric acid

INTRODUCTION

Semecarpus anacardium (Anacardiaceae), a deciduous forest tree, is a potent source of medicinal compounds prescribed for a variety of ailments like leprosy, rheumatoid arthritis, piles, asthma, cough, neurological disorders, skin diseases and sexually transmitted diseases such as syphilis and gonorrhea (Nadkarni 1976). Extract of the seeds is used in formulations against diseases such as atherosclerosis, adjuvant arthritis, hepatocellular carcinoma, antitumour activity, anticholesterol and neurological disorders (Premalatha 2000). The seed contains several alkaloids, flavonoids, bioflavonoids, and other compounds (Rao and Row 1973). The resinous liquid from the nut is suitable for lacquers, varnishes, enamels, tanning materials, and ion-exchange resins (Anonymous 1999). The kernel of the nut contains 20-25% sweet, semi-dry, non-edible oil (BSI 2004).

Two compounds, 1,2-dihydroxy-3-pentadecaenyl-8'-benzene and 1,2-dihydroxy-3- (pentadecadienyl-8', 11) benzene, isolated from seeds of *S. anacardium* demonstrated cytotoxicity against human cancer cell lines (Shin *et al.* 1999). Tetrahydroamentoflavone isolated from the seeds acts as a cyclooxygenase inhibitor having anti-inflammatory activity comparable to Ibuprofen, a well-known non-steroid anti-inflammatory drug (Selvam and Jachak 2004). Recently, acetyl cholinesterase inhibitor activity from the methanolic extract of stem bark was reported (Vinutha *et al.* 2007).

S. anacardium trees bear seeds in winter. Having poor and brief viability the seeds need to be sown soon after collection (Anonymous 1999; Sharma *et al*. 2001). This character of the seeds limits the success of conventional propagation. *In vitro* techniques are useful tools to supplement propagation of desirable trees for planting out and for research (Sansberror *et al*. 2003).

Low seed viability and poor germination frequency restrict the propagation of various plant species. Seed dormancy and the capacity of seeds to germinate are possibly associated with the seed coat. This factor needs to be checked first if seeds do not germinate (Budy *et al*. 1986; Velempini *et al*. 2003). A hard seed coat causes exogenous dormancy in seed and influences germination negatively by establishing a barrier that interferes with the water uptake required for imbibition, and gaseous exchange for respiration (Yang *et al.* 2007). To improve the germination frequency various pre-treatments for seeds are described in different plants (**Table 1**). *S. anacardium* has a hard seed coat. The germination frequency of fresh seeds was 30%, which was lost completely on storage for 6 months. Microbe-free seedlings could not be obtained *in vitro* due to leaching of phenolics followed by both bacterial and fungal growth.

The present report describes experiments conducted to overcome these limitations using various pretreatments and standardization of a protocol to achieve rapid, uniform and high frequent germination of *S. anacardium* seeds. Morphological changes that appeared on the seed surface following acid treatments are elaborated.

MATERIALS AND METHODS

Mature seeds of *S. anacardium* were collected during December from trees growing naturally in different locations around Pune, Maharashtra, India. These were washed thoroughly under running tap water and subjected to the following treatments: (i) mechanical scarification by removing manually the pericarp and mesocarp, (ii) soaking in 50% H_2SO_4 (Qualigens, Mumbai, India) for 20 min, (iii) soaking in concentrated H_2SO_4 (98.08%) for 10, 20, 30 and 40 min, (iv) cold treatment at 4°C for 7 days, (v) 2% Bavistin (50% Carbendazim, BASF India Ltd., Thane, India) treatment for 24 h, (vi) soaking in 10 N NaOH (Qualigens) for 20 min, (vii) hot water treatment at 80°C for 20 min, before surface sterilization. Other treatments applied after surface sterilization included: (viii) soaking in plant preservative mixture (PPM) (Sameer Science Lab,

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Original Research Paper

Table 1 Existing literature on various pretreatments for improving germination frequency in different plants.

Treatment	Time duration	Tissues	Reference
Mechanical		*Cassia occidentalis*	Sy *et al.* 2001
		Indigofera astragalina	
		Sesbania pachycarpa	
		Helianthemum sp.	Pérez-García and González-Benito 2006
		Parkia biglobosa	Aliero 2004
Cold (5°C)	7 days	*Ferula gummosa*	Nadjafi 2006
		Teucrium polium	
Alcohol	24 h soaking	*Ferula gummosa*	Nadjafi 2006
		Teucrium polium	
Water treatment	Boiling, 1 min	*Rhus* sp.	Li *et al.* 1999
	60°C, 30 min	*Pongamia pinnata*	Kumar *et al.* 2007
	80°C, 10 min	*Corchorus olitorius*	Velempini 2003
	80°C, 5 min	*Astragalus cyclophyllon*	Keshtkar *et al*. 2008
	100°C, 60 min	*Tamarindus indica*	Muhammad and Amusa 2003
		Tithonia diversifolia	Akinola *et al.* 2000
8-HQLS	30 sec.	*Cynara scolymus*	Vilchez *et al.* 2005
Nitric acid	10 and 30 min	*Ferula gummosa*	Nadjafi 2006
Sulfuric acid	20 min	*Tithonia diversifolia*	Akinola *et al.* 2000
(concentrated)	5 min	*Helianthemum* sp.	Pérez-García and González-Benito 2006
	15 min	*Piliostigma thonningii*	Ayisire *et al.* 2009
	15, 30, 60 min	*Cassia occidentalis*	Sy *et al.* 2001
		Indigofera astragalina	
		Sesbania pachycarpa	
		Tamarindus indica	Muhammad and Amusa 2003
	1 hr	*Rhus* sp.	Li *et al.* 1999
	3 min	*Parkia biglobosa*	Aliero 2004
Copper sulphate	Germinated in solution	*Sesbania sesbane*	Dan and Brix 2007
Sodium chloride	7 days 10°C	*Sesbania sesbane*	Dan and Brix 2007
		Spinacia oleracea	Masuda *et al.* 2005
Sodium hydroxide	20 h in cold	*Iris lactea*	Sun *et al*. 2006
PEG treatment	7 days	*Spinacia oleracea*	Masuda *et al.* 2005
		Indigofera astragalina	Sy *et al.* 2001
		Sesbania pachycarpa	
Hydrogen peroxide	10% for 5-30 min	*Corchorus olitorius*	Velempini 2003
		Areca triandra	Yang 2007
Oven drying	80-100°C for 5-30 min	*Corchorus olitorius*	Velempini 2003

Jabalpur, India) for 1 h, (ix) dipping in absolute alcohol and flaming, (x) soaking seeds overnight in 2% (w/v) 8-hydroxyquinoline sulfate (Sigma, St Louis, USA) solution, (xi) overnight soaking in 3% aqueous solution of $CuSO_4$ (Qualigens), (xii) soaking for 12 h in 3% aqueous solution of NaCl (Qualigens, Mumbai, India). WPM medium with 2% sucrose (Himedia Laboratories, Pvt Ltd., Mumbai) served as the control.

Surface sterilization

Seeds were treated with 2% Bavistin with a few drops of detergent (Exalin, Merck Specialities Pvt. Ltd., Mumbai, India) and placed on a shaker at 90 rpm for 1 h. These were washed several times in sterile distilled water and treated with 6% solution of Savlon containing chlorhexidine gluconate (1.5% v/v) and 3% cetrimide solution (w/v) (Johnson and Johnson, India) for 15 min. Savlon was eliminated by repeated washing in sterile distilled water. Finally the seeds were treated with sodium hypochlorite (NaOCl) (Merck) with 4% available chlorine for 45 min. The seeds were then rinsed 5-6 times in sterile distilled water. All operations were carried out under aseptic conditions.

Culturing of seeds

The *S. anacardium* seeds treated with concentrated H_2SO_4 for 10, 20, 30 and 40 min under non-sterile conditions were washed repeatedly with distilled water to eliminate the brown exudate produced on H_2SO_4 treatment. These were surface sterilized and cultured in various media formulations containing either woody plant mixture (WPM) (Lloyd and McCown 1980) or MS (Murashige and Skoog 1962) as basal medium. The various media formulations tested for germination of *S. anacardium* seeds are included in **Table 3**.

Plant growth regulators (PGRs), including 6-benzylaminopurine (BAP), kinetin (KIN), thidiazuron (TDZ), and gibberellic acid (GA_3) (Sigma Chemicals Co., St. Louis, USA) were tested to achieve enhanced germination frequency. An aqueous solution of GA_3 was filter sterilized using a 0.22µM membrane (Millipore India Pvt. Ltd., Mumbai) and added aseptically to sterilized medium. Other PGRs were added before autoclaving. Seeds with an emerged radical were scored for germination.

Effect of storage on *in vitro* seed germination

Acid-treated and surface-sterilized seeds were cultured in half-strength WPM medium every month for five months from the date of seed collection in December.

The pH of all the media was adjusted to 5.8 before autoclaving. Phytagel (Sigma) (0.2%) was used for gelling the medium. Media were sterilized by autoclaving at 121°C for 20 min at 1.06 kg cm^{-2}. All cultures were incubated in the dark at 25 ± 2°C for 4 weeks.

All experiments were repeated 4 times with 10 seeds in each condition. Total number of seeds germinated and the number of non-contaminated seedlings were scored. All data were subjected to analysis of variance (ANOVA). The difference among treatment means was tested using Duncan's multiple range test (DMRT) at $P<0.01$. A student's *t*-test was conducted to statistically analyze the difference in germination frequencies of the stored (5 months) seeds with or without H_2SO_4 treatment.

Morphological studies

H_2SO_4-treated and -untreated seeds were cut open horizontally and vertically through the micropylar region with the help of a pair of clippers. Photographs of seed surfaces before and after H_2SO_4 treatment and of the cut seeds were taken through a stereomicroscope.

RESULTS AND DISCUSSION

Seeds of *S. anacardium* (**Fig. 1A**) were collected in December. No significant difference was noticed in the germination frequencies of seeds in soil (25-30%) and *in vitro* (33%) using WPM medium supplemented with 2% sucrose. *In vitro* germination was noted within 10-15 days of inoculation in comparison to 25-30 days in *ex vitro* condition.

In the preliminary experiments, all seeds cultured *in vitro* were lost due to fungal and bacterial contamination. Several seeds leached phenolics causing blackening of the medium. The methods tested for enhancing germination and elimination of microbial growth in seedling culture of different species are described by several researchers (**Table 1**). The procedure used in this laboratory for surface sterilization of *Pongamia pinnata* (a hard seeded legume) seeds (Sujatha and Hazra 2006), was ineffective for *S. anacardium*. To optimize an effective method for establishment of seedling cultures, various pre-treatments were tested (**Table 2**). Manual removal of the seed coat helped in controlling microbial contamination but the seeds lost viability. This was possibly due to increased production of phenolics as the medium turned dark brown. Surface sterilization was ineffective in all other treatments except when seeds were pre-treated with concentrated H_2SO_4 and 10N NaOH. These two treatments were effective in eliminating the microbial contamination in 73 and 25% of the seeds, respectively. However, germination frequency of seeds treated with concentrated H_2SO_4 was 63% whereas it was only 29% after treatment with 10N NaOH. The liquid obtained after H_2SO_4 treatment was thick and dark brown. This could be due to phenolics and other chemicals (unidentified) leached from the seeds. Rinsate from several rinses after acid treatment was brown indicating additional leaching of the compounds. This was obvious from the difference in color of the seeds before (**Fig. 1A**) and after (**Fig. 1B**) acid treatment. The surfaces of the seeds before and after H_2SO_4 treatment were examined microscopically (**Fig. 1C, 1D**). Dark brown undulations, noted on the surface of the untreated seeds (**Fig. 1C**) were absent after 20 min acid treatment (**Fig. 1D**). Absence of the dark brown undulations and yellowish-to-light brown color of the 20 min acid-treated seeds (**Fig. 1D**) suggests removal of the brown deposits. On the surface of the acid-treated seeds (**Fig. 1D**) innumerable pores were apparent. Presumably microbes are harbored in the undulations of the seed surface and the granular dark brown deposits restrict the sterilants to access these. Thus, surface sterilization without acid treatment was ineffective. Treatment with concentrated H_2SO_4 dissolved the granular deposits and allowed the sterilants to reach the contaminants. Fungal contamination was predominant in seeds of *S. anacardium* and appeared within a few hours of culture. Bavistin, a systemic fungicide, used successfully in establishing *Pongamia* seedling culture to control fungal contamination (Sujatha and Hazra 2006) failed to restrict fungal growth in *S. anacardium*. Surface sterilization with NaOCl after 10N NaOH treatments achieved 25% microbe-free cultures but the germination frequency was 29%. Soaking seeds in hot water is an inexpensive and easy method to soften the seed coat and stimulate water uptake by seeds in tropical species (Smith and Bent 1993). Hot water treatment enhanced germination of *Tithonia diversifolia* (Akinola *et al*. 2000) and *Tamarindus indica* L. (Muhammad and Amusa 2003). Hot water, or 8-hydroxyquinoline sulfate treatment were ineffective in surface sterilization of *S. anacardium* seeds. Flaming of the seeds reduced germination frequency to 23%. Soaking the surface-sterilized seeds in PPM at 2% for 1 hr was ineffective against the contaminants. Overnight soaking of seeds in 3% $CuSO_4$ or 3% NaCl failed to control contamination although germination frequency increased to 42 and 56%, respectively (**Table 2**).

The presence of anthocyanins and phenolics and hardness of the seed coat inhibit germination (Bhattarai *et al.* 2008). H_2SO_4 has been used effectively for treatment of hard-coated seeds. The optimum time for H_2SO_4 treatment depends on the hardness of the coat. It varied from 3 min in *Parkia biglobosa* (Aliero 2004) to 90 min in *Sesbania rummondii* (Elastin 1984). Soaking in 50% H_2SO_4 for 20 min (**Table 2**) failed to reduce contamination in *S. anacardium* seeds, but the germination frequency was higher (40%) than the untreated seeds (33%). Highest germination frequency (63%) with reduced contamination was achieved in seeds treated with concentrated H_2SO_4 for 20 min. Induction of germination due to hydrolysis of seed tissues has been reported (Budy *et al.* 1986; Velempini *et al*. 2003). Contamination was eliminated in *S. anacardium* seeds by increasing the period of H_2SO_4 treatment to 30 and 40 min but the germination percentage decreased to 27 and 0%, respectively (**Table 2**). Surfaces of the seeds treated with H_2SO_4 for various time periods were examined microscopically (**Fig. 1E-**

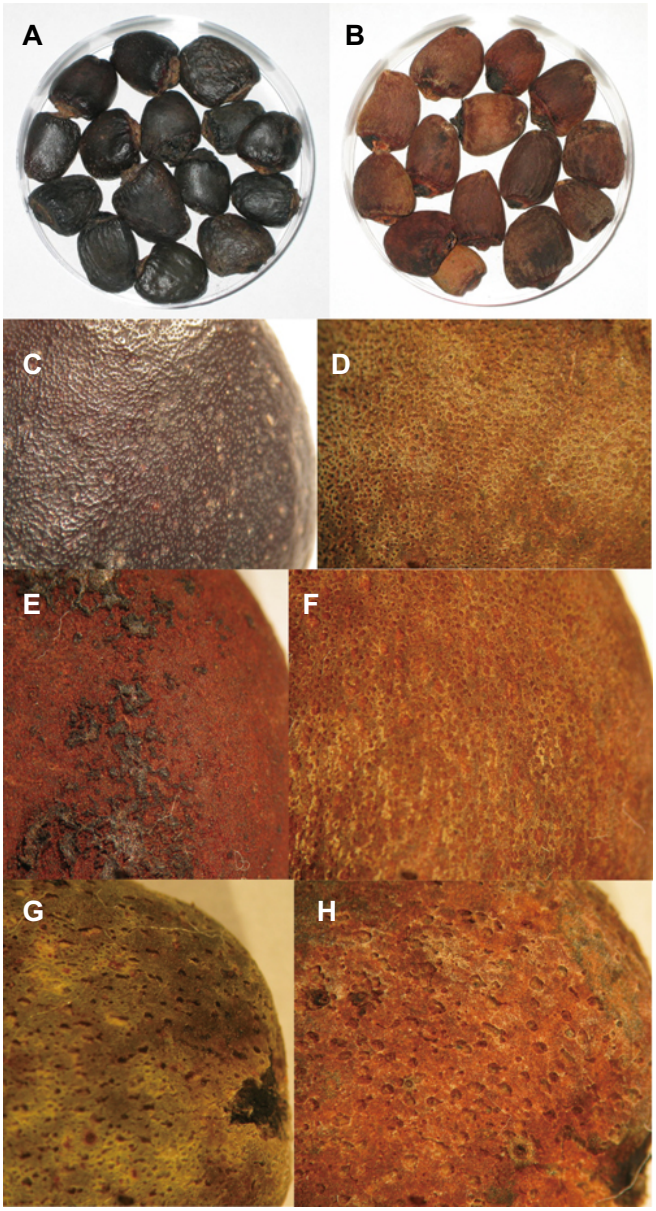

Fig. 1 (**A**) Seeds of *Semecarpus anacardium.* (**B**) Change in surface color of *S. anacardium* seeds due to elimination of the dark-brown pigments after concentrated sulfuric acid treatment. (**C**) Surface of the seed with granular undulation before sulfuric acid treatment. (**D**) Absence of granular undulation and presence of innumerable pores on the surface of the seed after concentrated sulfuric acid treatment for 20 min. (**E**) Surface of seed treated with sulfuric acid for 10 min. Presence of residual pigment indicates incomplete removal of the pigment. (**F**) Surface of seed after 20 min sulfuric acid treatment. Absence of pigment and presence of numerous small pores suggests complete removal of the pigment layer. (**G, H**) Surface of seeds after 30 and 40 min sulfuric acid treatment. Appearance of larger pores indicates erosion of the seed coat.

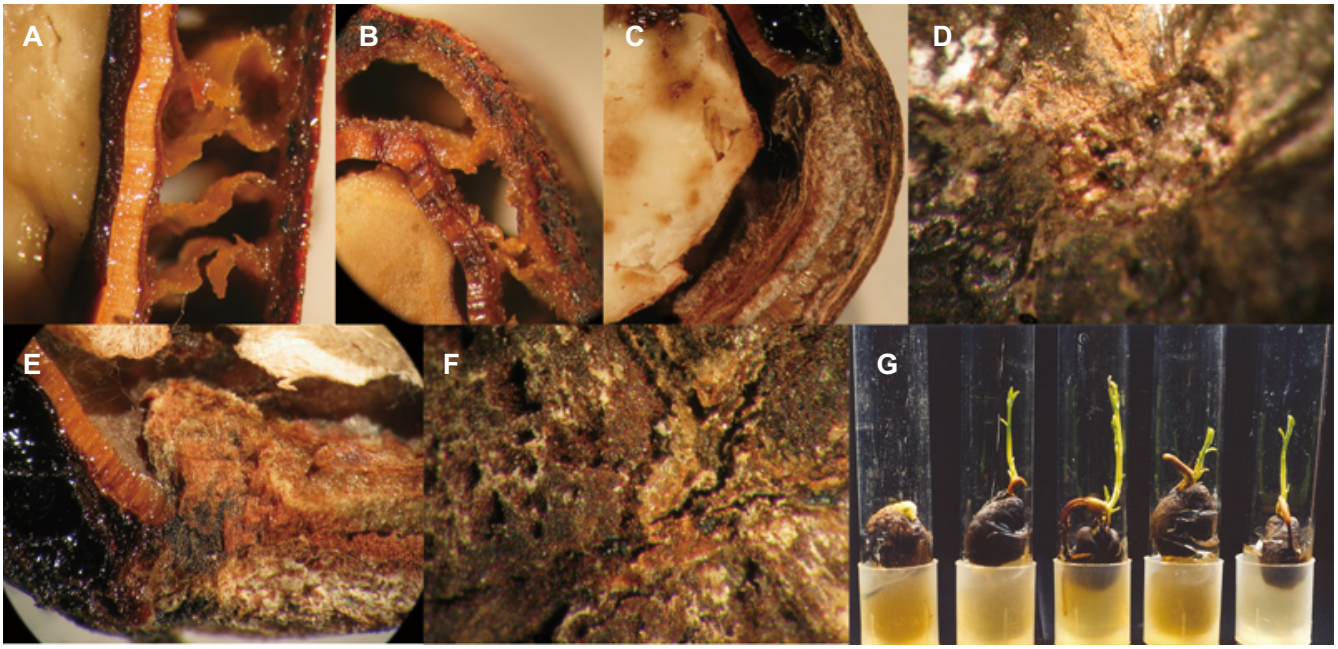

Fig. 2 (**A**) Horizontally cut seed showing, epicarp (Pc), mesocarp (Mc), endocarp (Ec). (**B**) Horizontally cut acid treated (20 min) seed showing, eroded epicarp (Pc), mesocarp (Mc) and endocarp (Ec) are unaffected. (**C**) Seed section cut vertically. Intact micropylar region before acid treatment is apparent. (**D**) Surface appearance of micropylar (Mp) region of intact seed. (**E**) Section of acid-treated (20 min) seed cut through Mp region vertically. The Mp region appears distorted but no cracks were seen. (**F**) Mp region of seed after acid treatment for 40 min. Appearance of cracks suggests damage in this region due to over exposure in sulfuric acid. (**G**) *In vitro*-raised seedlings of *S. anacardium* obtained from seeds treated with concentrated sulfuric acid for 20 min before surface sterilization.

Table 2 Effect of different pretreatment on *Semecarpus anacardium* seed germination.

Treatments	Condition of treatment	Non-contaminated seeds (Mean % ± SD)	Germination (Mean % ± SD)
Control	Surface-sterilized seed	0 ± 0 e	33 ± 9.57 gh
Mechanical treatment	Seed coat removed mechanically before surface sterilization	10 ± 0 a	0 ± 0 j
50% H_2SO_4	20 min	0 ± 0 e	40 ± 8.16 cd
Conc. H_2SO_4	10 min	20 ± 8.16 cd	35 ± 12.91 efg
Conc. H_2SO_4	20 min	73 ± 9.57 b	63 ± 5.0 a
Conc. H_2SO_4	30 min	10 ± 0 a	27 ± 9.57 h
Conc. H_2SO_4	40 min	10 ± 0 a	0 ± 0 j
Cold treatment	Seeds pretreated at 4°C for a week	0 ± 0 e	38 ± 5.0 cdef
24 Hr bavistin	On shaker 90 rpm	0 ± 0 e	39 ± 16.07 cde
NaOH 10 N	20 min	25 ± 7.5 c	29 ±13.14 h
Hot water (80°C)	Hot water treatment for 20 min	0 ± 0 e	35 ± 5.77 efg
8-HQLS 2%	Over night soaking (12 h)	0 ± 0 e	35 ±12.90 efg
Flame	Surface sterilized seeds dipped in alcohol and flamed	0 ± 0 e	23 ± 5.0 i
PPM 2%	1 h after surface sterilization	0 ± 0 e	38 ± 26.29 cdef
$CuSO_4$ 3%	Overnight soaking (12 h)	0 ± 0 e	43 ±23.12 c
NaCl 3%	Overnight soaking (12 h)	0 ± 0 e	56 ± 20.46 b
ANOVA		S 1%	S 1%

Mean followed by the same letters within a column do not differ significantly at $P \leq 0.01$ according to DMRT.

H). The presence of residual granular brown deposits on the surface of the seeds treated for 10 min (**Fig. 1E**) indicated incomplete removal of the deposits. Seeds treated for 20 min did not show any deposit and the distribution of the pores was obvious (**Fig. 1F**). Longer treatment (30 and 40 min) in acid created larger pores (**Fig. 1G, 1H**). Reduction in the germination frequency in seeds treated for longer period in acid could be due to injury to the embryos (Sy *et al.* 2001).

Microscopic examination of the dissected seeds of *S. anacardium* revealed three layers around the seed (**Fig. 2A**). The outer layer is a compact epicarp with a smooth surface. The middle layer was spongy with channels, which were filled with phenolics. The innermost one was a hard, stony endocarp. The outer surface of the epicarp was absent in the seeds treated with concentrated H_2SO_4 (**Fig. 2B**). In mature seeds of *S. anacardium* the micropylar end is devoid of endocarp (**Fig. 2C**). Acid treatment caused erosion of the epicarp (**Fig. 2B**), which possibly allowed excretion of inhibitory phenolics and entry of water into the seed to facilitate germination of the embryo. In *Rhus* species (Li *et al.* 1999) anatomical changes were noted in the endocarp after 1 hr of H_2SO_4 treatment. In *S. anacardium* a clearly outlined micropylar region (**Fig. 2C, 2D**) was seen in the untreated seed. After treatment for 20 min with concentrated H_2SO_4 the erosion of the epicarp was obvious (**Fig. 2E**). When acid treatment was increased to 40 min, cracks were noted in the micropylar region (**Fig. 2F**) and in surroundings areas. Failure in germination of these seeds could be due to injury of the embryos caused by entry of acid through these cracks. The use of concentrated H_2SO_4 for longer duration had lethal effects on the embryos of the seeds in *Piliostigma thonningii* (Ayisire *et al*. 2009)

Failure of a seed to germinate in favorable environmental conditions is defined as seed dormancy (UC Davis). This phenomenon is attributed to three mechanisms

including hard seed coverings, chemical inhibitors including growth regulators, phenolics, etc. and morphological aspects such as undeveloped embryos. Exogenous dormancy was associated with (i) impermeability to water, (ii) hardness of the seed coat which does not allow the embryo to expand during germination and (iii) presence of inhibitors in the outer coverings. *S. anacardium* seed have a hard seed coat that leaches enormous amount of phenolics in culture. It is presumed that scarification of seeds with concentrated H_2SO_4 not only made the seeds imbibe water but also eliminated some of the inhibitory phenolics to reduce the dormancy, resulting in enhanced germination frequency.

Seed dormancy and the capacity of seeds to germinate were associated with a hard seed coat in *Corchorus olitorius* (Velempini *et al*. 2003). A hard seed coat and presence of phenolics in *S. anacardium* influenced germination negatively by establishing a barrier that interfered with the water uptake required for imbibition, and for gaseous exchange for respiration. In *Anacardiaceae* the cause of dormancy was physical due to water impermeability of the pericarp (Baskin and Baskin 1998). In *S. anacardium*, germination increased from 33 to 63% in seeds treated with concentrated H_2SO_4 for 20 min. Soaking in acid partially digested the outer surface of the pericarp and rendered it permeable to water. Erosion of the seed surface due to acid treatment was obvious (**Fig. 2B**). Thus, a passage for the release of phenolics and water imbibition was created. Production of a thick, dark brown liquid after acid treatment indicated rapid release of phenolics, which was eliminated by repeated washing with water. These phenolics could be growth inhibitory. Increase in germination frequency to 63% (**Tables 2, 3**) in acid-treated seeds supports this observation. On opening of the pores on the seed surface, the inhibitory compounds were released and water entered to initiate the process of germination. Acid treatment was also effective in controlling contamination in 73% of the cultures. This could be due to destruction of the harboring microbes, which came in contact with the concentrated H_2SO_4.

Several media combinations with or without PGRs (**Table 3**) were tested to enhance the germination frequency. Half-strength WPM without sucrose was more suitable for germination of *S. anacardium* seeds. It was 63% in this medium compared to 56% in half-strength MS medium without sucrose. Between the two basal media, WPM has more sulphate and calcium. Moreover, NaOCl, which was used as surface sterilant following the pretreatments, could have increased the germination frequency as in *Podophyllum hexandrum* Royle (Nadeem *et al.* 2000).

Uniform and consistent germination in seeds has been reported with the use of PGRs (Nadeem *et al.* 2000; Nikolic *et al.* 2006; Sujatha and Hazra 2006). However, none of the media formulations containing PGRs (**Table 3**) was effective in enhancing the germination frequency of *S. anacardium* seeds *in vitro*. On the contrary, the frequency was reduced on incorporation of PGRs in half-strength WPM medium without sucrose in which 63% germination was achieved. This suggests that failure of the *S. anacardium* seeds to germinate is due to exogenous dormancy associated with the hard seed coat and the phenolics and is not due to lack of any of the PGRs tested (**Table 3**).

Results of the experiment conducted to assess seed viability of *S. anacardium* seeds over the period of storage demonstrated that germination frequencies of the seeds *in vitro* and in soil were similar in freshly collected seeds and after one month of storage (**Table 4**). Seeds exhibited a gradual drop in viability after two (55%), three (32%) and four months (22%) of storage. The viability of the seeds was lost completely after 6 months. When the seeds with or without H_2SO_4 treatment were planted in soil five months after collection (April) the germination frequency was 4 and 21%, respectively (**Table 5**). This result corroborates with the data (22%) generated by culturing H_2SO_4-treated seeds *in vitro* in April (**Table 5**). Length of storage period influence seed germination in plants like *Avicennia marina* (Farrant *et al.* 1986) and *Annona cherimola* Mill (Padilla and Encina 2003).

This report describes for the first time the germination behavior of *S. anacardium in vitro* and in soil. The protocol standardized for seed germination may find application in obtaining an increased number of uniform seedlings from freshly collected seeds to supplement conventional propagation of this species. The information generated from this study may be extended to other seeds having a hard seed coat and high phenolic contents. The experiment demonstrated that concentrated H_2SO_4 not only helped in increasing the permeability of the hard seed coat and excretion of phenolics but also acted as a surface sterilant to a limited extent. Improved germination in the absence of sucrose supplement is also an important observation in this study. Since establishment of seedling culture is a major constraint in propagation of several tropical tree species this protocol may find application in increasing germination frequency *in vitro*, to provide microbe-free explants (**Fig. 2G**) for further studies on micropropagation and genetic transformation.

Table 3 Effect of media composition and growth regulators on germination of *Semecarpus anacardium* seeds.

Media	Germination (Mean % ± SD)
MS	17 ± 15.3 cd
MS charcoal	23 ± 05.8 bcd
1/2 MS	37 ± 15.3 abcd
1/2 MS with out sucrose	57 ± 15.3 ab
WPM	34 ± 11.5 abcd
WPM charcoal	50 ± 26.5 abc
1/2 WPM	50 ± 10.0 abc
½ WPM with out sucrose	63 ± 11.5 a
WPM BAP 2.22 µM	13 ± 05.8 d
WPM BAP 4.44 µM	20 ± 17.2 cd
WPM BAP 8.88 µM	17 ± 11.5 cd
WPM KIN 2.32 µM	30 ± 17.3 bcd
WPM KIN 4.64 µM	17 ± 15.3 cd
WPM KIN 9.28 µM	27 ± 05.8 bcd
WPM BAP 2.22 µM + 2.32 KIN µM	10 ± 17.3 d
WPM BAP 4.44 µM + 4.64 KIN µM	13 ± 05.8 d
WPM TDZ 0.227 µM	20 ± 10.0 cd
WPM TDZ 2.27 µM	7 ± 05.8 d
WPM TDZ 4.54 µM	3 ± 05.8 d
WPM TDZ 9.08 µM	3 ± 05.8 d
WPM GA_3 0.29 µM	30 ± 17.3 bcd
WPM GA_3 1.44 µM	30 ± 26.5 bcd
WPM GA_3 2.88 µM	27 ± 23.1 bcd
WPM GA_3 5.76 µM	23 ± 32.1 cbd
ANOVA	S1%

Mean followed by the same letters within a column do not differ significantly at P ≤ 0.01 according to DMRT.

Table 4 Reduction in seed viability on storage.

Time of culture	Germination (Mean%± SD)*
December	64 ± 0.58 a
January	63 ± 2.52 ab
February	55 ± 7.64 abc
March	32 ± 7.65 d
April	22 ± 7.46 d
ANOVA	S 1%

Mean followed by the same letters within a column do not differ significantly at P ≤ 0.01 according to DMRT.
* H_2SO_4-treated seeds

Table 5 Seed germination in soil after storage for 4 months*.

Treatment	Percentage of germination
Control	4
Concentrated H_2SO_4 for 20 min	21
t-test	S 5%

*Seeds collected in December and after storing tested in soil in April.

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REFERENCES

Aliero BL (2004) Effects of sulfuric acid, mechanical scarification and wet heat treatments on germination of seeds of African locust bean tree *Parkia biglobosa*. *African Journal of Biotechnology* **3**, 179-181

Akinola JO, Larbi A, Farinu GO, Odunasi AA (2000) Seed treatment methods and duration effects on germination of wild sunflower. *Experimental Agriculture* **36**, 63-69

Anonymous (1999) *Wealth of India* (Vol IX), CSIR Publication, pp 271-275

Ayisire BE, Akinro LA, Amoo SO (2009) Seed germination and *in vitro* propagation of *Piliostigma thonningii* – an important medicinal plant. *African Journal of Biotechnology* **8**, 401-401

Baskin CC, Baskin JM (1998) *Seeds: Ecology, Biogeography and Evolution of Dormancy and Germination*, Academic Press, San Diego, CA, 666 pp

Bhattarai SP, Fox J, Gyasi-Agyei Y (2008) Enhancing buffel grass seed germination by acid treatment for rapid vegetation establishment on railway batters. *Journal of Arid Environments* **72**, 225-262

Botanical Survey of India (2004) Database of oil yielding plants. Available online: http://mnes.nic.in/list/oil-plants.pdf

Budy J, Evans R, Young J (1986) *Understanding Seed Handling for Germination*, Volunteers in Technical Assistance, Arlington, VA. Available online: http://www.cd3wd.com/CD3WD_40/VITA/SEEDGERM/EN/SEEDGERM.HTM

Dan TH, Brix H (2007) The influence of temperature, light, salinity, and seed pre treatment on the germination of *Sesbania sesban* seeds. *African Journal of Biotechnology* **6**, 2231-2235

Elastin FE (1984) Drummond rattlebox (*Sesbania rummondii*) germination as influenced by scarification, temperature and seedling depth. *Weed Science* **32**, 223-225

Farrant JM, Pammenter NW, Berjak P (1986) The increasing sensitivity of recalcitrant *Avicennia marina* seeds with storage time. *Physiologia Plantarum* **67**, 291-298

Keshtkar AR, Keshtkar HR, Razavi SM, Dalfardi S (2008) Method to break dormancy of *Astragalus cyclophyllon. African Journal of Biotechnology* **7**, 3874-3877

Kumar S, Radhamani J, Singh AK, Varaprasad KS (2007) Germination and seed storage behaviour in *Pongamia pinnata* L. *Current Science* **93**, 910-911

Li X, Baskin JM, Baskin CC (1999) Anatomy of two mechanism of breaking physical dormancy by experimental treatments in seeds of two North American *Rhus* species (*Anacardiaceae*). *American Journal of Botany* **86**, 1505-1511

Lloyd G, McCown B (1980) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia. International Plant Propagation Society Proceedings* **30**, 421-427

Masuda M, Hata N, Ombwara FK, Agong SG (2005) Effect of acid scarification, priming with PEG, NaCl or sea water as osmoticum and dehydration on spinach seed germination at 30°C. *Journal of the Japanese Society for Horticultural Science* **74**, 134-138

Muhammad S, Amusa NA (2003) Effect of sulphuric acid and hot water treatments on seed germination of tamarind (*Tamarindus indica* L). *African Journal of Biotechnology* **2**, 276-279

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiologia Plantarum* **15**, 473-479

Nadkarni AK (1976) *K.M. Nadkarni's Indian Materia Medica*, Popular Prakashan Pvt. Ltd., Bombay, Volume I, pp 1119-1125

Nadeem M, Palni LMS, Purohit AN, Pandey H, Nandi SK (2000) Propagation and conservation of *Podophyllum hexandrum* Royle: an important medicinal herb. *Biological Conservation* **92**, 121-129

Nadjafi F, Bannayan M, Tabrizi L, Rastgoo M (2006) Seed germination and dormancy breaking technique in *Ferula gummosa* and *Teucrium polium*. *Journal of Arid Environments* **64**, 542-547

Nikolic R, Mitic N, Miletic R, Neskovic M (2006) Effect of cytokinins on *in vitro* seed germination and early seedling morphogenesis in *Lotus corniulatus* L. *Journal of Plant Growth Regulation* **25**, 187-194

Padilla IMG, Encina CL (2003) *In vitro* germination of cherimoya (*Annona cherimola* Mill.) seeds. *Scientia Horticulturae* **97**, 219-227

Pérez-García F, González-Benito ME (2006) Seed germination of five *Helianthemum* species: Effect of temperature and presowing treatments. *Journal of Arid Environments* **68**, 688-693

Premalatha B (2000) *Semecarpus anacardium* Linn. nut – A boom in alternative medicine. *Indian Journal of Experimental Biology* **38**, 1177-118

Rao NSP, Row LR (1973) Phenolic constituents of *Semecarpus anacardium*. *Phytochemistry* **12**, 671-681

Sansberro P, Hebe R, Mroginski L, Luna C (2003) *In vitro* plant regeneration of *Schinopsis balansae* (Anacardiaceae). *Trees* **17**, 542-546

Selvam C, Jachak SM (2004) A cyclooxygenase (COX) inhibitory biflavonoid from the seeds of *Semecarpus anacardium*. *Journal of Ethnopharmacology* **95**, 209-212

Sharma PC, Yelne MB, Dennis TJ (2001) Bhalltaka (*Semecarpus anacardium*). In: *Data Base on Medicinal Plants Used in Ayurveda* (Vol 5), Central Council for Research in Ayurveda and Siddha, Govt of India, New Delhi, pp 9-28

Shin GY, Cordell GA, Dong Y, Pezzuto JM, Rao AVNA, Ramesh M, Kumar RB, Radhakrishnan M (1999) Rapid identification of cytotoxic alkenyl catechols in *Semecarpus anacardium* using bioassay linked high performance liquid chromatography–electrospray/mass spectrometric analysis. *Phytochemical Analysis* **10**, 208-212

Smith JM, Bent SP (1993) Dormancy and germination. *Annual Review of Plant Physiology* **30**, 130-150

Sujatha K, Hazra S (2006) *In vitro* regeneration of *Pongamia pinnata* Pierre. *Journal of Plant Biotechnology* **33**, 263-270

Sun YC, Zhang YJ, Wang K (2006) NaOH scarification and stratification improve germination of *Iris lactea* var. *chinensis* seed. *HortScience* **41**, 373-374

Sy A, Grouzis M, Danthu P (2001) Seed germination of seven Sahelian legume species. *Journal of Arid Environments* **49**, 875-882

UC Davis: Seed dormancy. Available online: http://trc.ucdavis.edu/egsutter/plb171/lecturespdf4/5-Seed%20dormancy02.pdf

Vilchez M, Paulus AO, Mayberry KS (2005) Globe artichoke seed treatment to control seed-borne fungi and seed quality evalution. *Acta Horticulturae* **681**, 581-585

Velempini P, Riddoch I, Batisani N (2003) Seed treatments for enhancing germination of wild okra (*Corchorus olitorius*). *Experimental Agriculture* **39**, 441-447

Vinutha B, Prashanth D, Salma K, Sreeja SL, Pratiti D, Padmaja R, Radhika S, Amit A, Venkateshwarlu K, Deepak M (2007) Screening of selected Indian medicinal plants for acetylcholinesterase inhibitor activity. *Journal of Ethnopharmacology* **109**, 359-363

Yang Q-H, Ye W-H, Yin X-J (2007) Dormancy and germination of *Areca triandra* seeds. *Scientia Horticulturae* **113**, 107-111