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Responses of Pretreatment and Nutrient Media on Seed Germination in *Gymnocladus assamicus*, a Critically Endangered Legume Tree Species from North-East India

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ABSTRACT

The present work revealed the effect of different pretreatment under *in vivo* and *in vitro* seed germination of *Gymnocladus assamicus*, a critically endangered and endemic legume tree species from Northeast India. Of the various treatments 24 hours Sulphuric acid treatment was the optimum for breaking seed dormancy *in vitro* and *in vivo* seed germination. Present work also showed that nutrient medium was not effective on seed germination. The highest germination (98.7%) was achieved on distilled water moist germination paper under *in vitro* condition while full MS medium was found to be least effective. The present protocols offered an efficient axenic seedling production. The seed can be stored at ambient temperature for one year. In addition SEM studied revealed the dense compact cuticle architecture on the surface of seed is responsible for water impermeability. Thus the present study revealed that the main obstruction to natural recruitment of seedling is the physical dormancy which could be overcome through optimal acid treatment.

Key words:

Critically endangered, Gymnocladus assamicus, Scanning electron microscopy (SEM), Seed germination, Sulphuric acid, Tetrazolium chloride (TTZ).

INTRODUCTION

Seed is an ultimate sexual propagator which copes with various evolutionary events in a dynamic ecosystem like biotic, abiotic stresses and has been leading to survive for adaptation in nature. Seed containing dormant miniature embryonic plant enable the plant to be stored for longer period and germinate under favorable condition in their life cycle. For successful seed germination, a viable seed that have vigorous embryo is crucial factor. Moreover seed morphology, anatomy and water permeability of seed coat are crucial factors known to play an important role in the dormancy behavior of seed. Thus germination process is an active metabolite and dynamic mechanism involving complex communication among biochemical, nutrient and hormonal signal of endosperm and embryo. But they are not always in a linear co-relationship with species status in the sense of plant demographics and seed production, because of several crucial factors that are involved in seed germination process. This complex process referred to rehydration, resumption of metabolic activity, consumption of nutrient reserves, and gradual

development of artificial system which enables the embryonic stage to young plant and becoming an autotrophic existence. However, several constraints control dormancy of seed which might be experimentally manipulated through different pretreatments like scarifications, change in moisture and temperature in order to enhance seed germination. Although all seeds are not capable to germinate in high frequency, it needs proper studies to characterize the pattern of dormancy in seed. Thus it is imperative to study seed viability and dormancy in order to obtain high number of seedlings for nursery industries as well as *in situ* and *ex situ* conservation.

Gymnocladus assamicus is recently reported as critically endangered and endemic species confined to a few isolated area in West Kameng district in Arunachal Pradesh and West KhasiHills in Meghalaya, India (Choudhury et al. 2009). *Gymnocladus assamicus is* recently comprised in the IUCN Red List of Threatened species as Critically Endangered under Red List category & Criteria due to its shrinking population status (Saha et al. 2015) and also prioritized plant list for national recovery program under endangered and threatened species in India (Ganeshaiah 2005). Mature seed pods are used as substitute of detergent, soap, anthelminthic for domestic livestock and in socio-rituals activate by tribal people and also roasted seed used as substitute of coffee or ground nut (Choudhury et al. 2007; Gupta et al. 2013a). The extract of different parts of *Gymnocladus assamicus* reported as various degree of antioxidant potential (Gupta et al. 2013b). The seed dormancy imposed by hard seed coat of this species act as major obstacle for seedling recruitment in natural habitat (Choudhury et al. 2009). Limited reports are available only for *in vivo* seed germination studies of *G. assamicus* (Choudhuryet al. 2009) whilst *in vitro* seed germination has not been reported yet. Our present investigation was undertaken extensively to study for improvement of in vivo and in vitro seed germination which was not previously addressed elsewhere.

MATERIALS AND METHODS

The mature seed pods of *G. assamicus* were collected two times during field trips in April-May, 2009-2010 from the Dirang Valley, West Kameng district in Arunachal Pradesh, India at an altitude of 1700-2000 m. Tetrazolium chloride test was conducted for seed viability at every six month interval during one year storage period at room temperature. The sections of seeds were incubated in the dark in a 0.1% aqueous solution of tetrazolium chloride (pH-6.5) for 24 hrs. Seed topography of *G. assamicus* was observed through scanning electron microscopy (SEM). Only small section of treated and untreated seed coat of *G. assamicus* was excised carefully and all specimens were gold coated by Sputter-coater apparatus with a thin layer of approximately 20 nm to 30 nm and images were taken under different magnifications with SEM, JSM-6360 (JEOL; Magnification 8x WD 48 mm to 300000X).

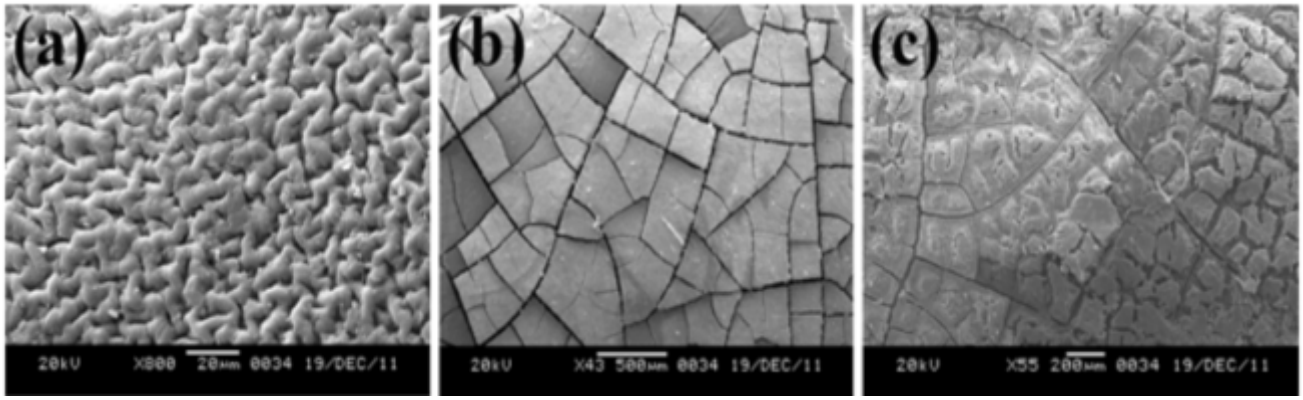
To examine the presence of physical dormancy, seeds were given mechanical, chemical scarification and dry heat treatment. The effect of mechanical scarification was investigated by carefully rubbing the outer stony seed coat with Knife-saw until it slightly cracks the outer layer of seed coat. For chemical scarification the seeds were immersed in concentrated sulphuric acid (36N H_2SO_4) for 1, 12, 24 hrs (1440 min.) and 36 hrs (2160 min.). Following acid treatment seed was washed under running tap water until the pH become neutral and finally rinsed in distilled water. For dry heat treatment, seeds were treated with dry heat under oven at a range of different temperatures (60, 80 and 100˚C) and duration (5, 15, 30, and 45 minutes). The treated seeds were sown in soil and kept in green house condition (i.e. 24˚C, RH. 85%) for germination studies. The above chemical scarified seeds were also set up for in vitro seed germination. The Basal medium of full MS, half MS (Murashige and Skoog 1962), WPM (Lloyd and McCown 1980), and sterilized damp germination paper as control (without pretreated seed) were used for in vitro seed germination studies (Table 1). About 80 ml to 100

ml of media was dispended in 250 ml or 500 ml flask and autoclaved along with all other equipments needed for the culture at 121˚C and 15lb pressure for 20 minutes. Seeds were disinfected using 10% (v/v) sodium hypochlorite solution with 2-3 drops of Tween-20 for 30 min. followed by washing with sterile distilled water 3-4 times. 2-5 seeds were inoculated per flask. All cultures were maintained under warm white fluorescent light for 16 hrs photoperiod and 25 ± 2˚C temperature. The day of emergence of radical was considered as the day of germination of seed .

All treatments were having three replicates and repeated four times with each replicate of 10 seeds. The germination percentage, mean germination time and germination value were subjected to analysis of variance (ANOVA) and further all means of treatments were compared by Tukey HSD multiple comparisons at the 5% level of significance The 3D surface graphical depiction were performed using Origin Pro 9.1 version (Origin Lab, Northampton, MA, USA).Mean germination time (MGT) was evaluated from Ellis and Roberts (1981) and Germination value (GV) was evaluated from Djavanshir and Pourbeik (1976).

RESULT AND DISCUSSION

All seed pods were in dehiscent condition (partial opening of seed pod) with light brown color (Fig. 1d). Each pod contains 6 to 8 oval black stony seeds. The present SEM observation revealed very compact test a of seeds with dense packed palisade cells layer (Fig. 1a). On different treatments, the test a was observed with more frequent cracking on heat treatment (Fig. 1b) while 24hrs acid (36N H_2SO_4) treatment removed or ruptured the densely cuticulated layer of seed coat (Fig. 1c). This stony seed coat at mature state had compact galactomannan or mannan polymers on the wall of the endosperm cells which becomes slimy mucilaginous during the imbibitions and hydrolysis (Fig. 1e) (Reid and Bewley 1979) and it may be helped to emerging of radical through mechanical force or pressure developing during increasing volume of galactomannan or mannan polymers. Similar imbibition was observed during present seed germination study of *G. assamicus* which could be presumed similar activity by galactomannan or mannan polymers (Choudhury et al. 2009). The major barrier observed was outer stony seed coat during moisture intake. The principal behind hard seed coat helps against mechanical damage, to survive in soil during drought or allows natural dispersal and recolonization after fire. The fresh seed tested with TTZ showed viability with deep red color on the most active site of endosperm and embryo visible by naked eye (Fig. 1f).The viability of orthodox seeds was reported even below 12% moisture content (Connor et al. 1998) which is similar to our result of moisture retention recorded as 12.20% and 63.33% germination response observed after one year storage (data not shown). Choudhury et al. (2009) also reported similar findings, therefore the seeds of *G. assamicus* can be classified as a true orthodox in nature. Hence, by nature seed can be stored for long period in soil bank.



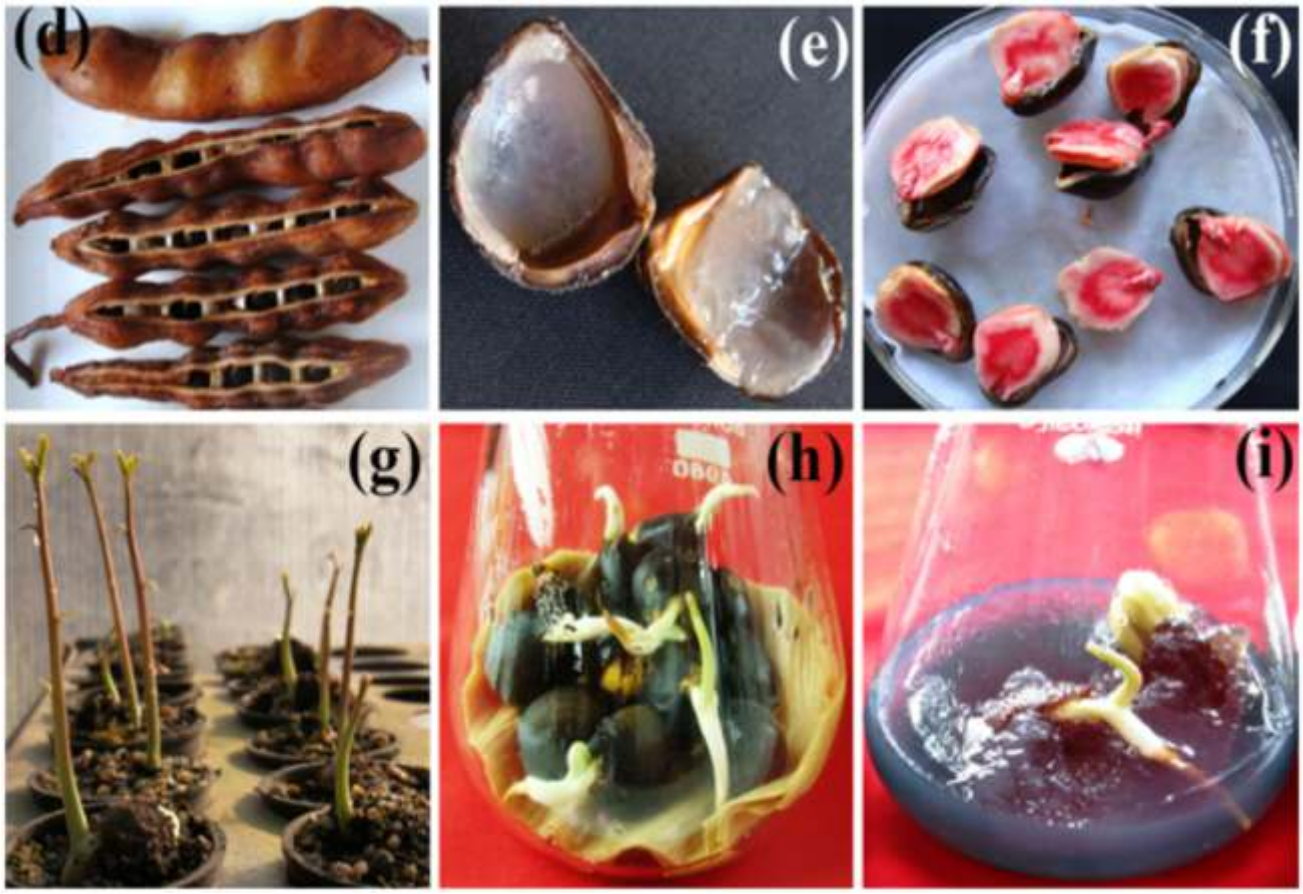


Fig. 1 Scanning electron microscopic (SEM) and seed germination of G. assamicus. **(a)** SEM of untreated seed surface. **(b)** SEM of seed surface after treatment at 80°C temperature. **(c)** SEM of seed surface after treatment of 24 hrs sulfuric acid. **(d)** Matured seed pods showing partial opening of seed pod. **(e)** The longitudinal section of seed shown as whitish mucilaginous content after imbibition. **(f)** Fresh seed TTZ test shown maximum viability with deep red colors spot on embryo. **(g)** Two week old seedling of pretreated seed with H_2SO_4 for 24 hrs treatment. **(h)** Showing 10 days old germination on seed germination paper (SGP2). **(i)** Showing 17 days old germination in semisolid WPM medium.

All the pre treatments of seed improved *in vivo* germination responses as shown in 3D surface plot (Fig. 2 a,b,c) in comparison to untreated seed; the most significant (Tukey HSD, $P<0.05$) seed germination percentage was recorded 93.4% with 24 hours acid (36N H_2SO_4) treatment (T6), followed by mechanical (T5) and heat treatment (T3) with 15 minutes at 80ºC temperature in Fig 1. While acid treatment (Conc. H2SO4) beyond 24 hours and dry heat treatment with 15 minutes at 80ºC drastically reduce germination in *G. assamicus*. This result might be discussed as prolong treatment cause fetal effect on embryo. Similar studies were reported by several authors during improvement of germination responses in different legumes species (Aliero 2004). However, the present investigation reveals minor deviation in germination percentage from study conducted by Choudhury et al. (2009). In nature, several other factors play role while developing crack of the tegumentary barrier in legumes eg. temperature variation and alternate dry and wet periods (Roslton 1978), microorganism surface activity and the chemical scarification induced by the herbivore digestive system (Al Sherif 2007).

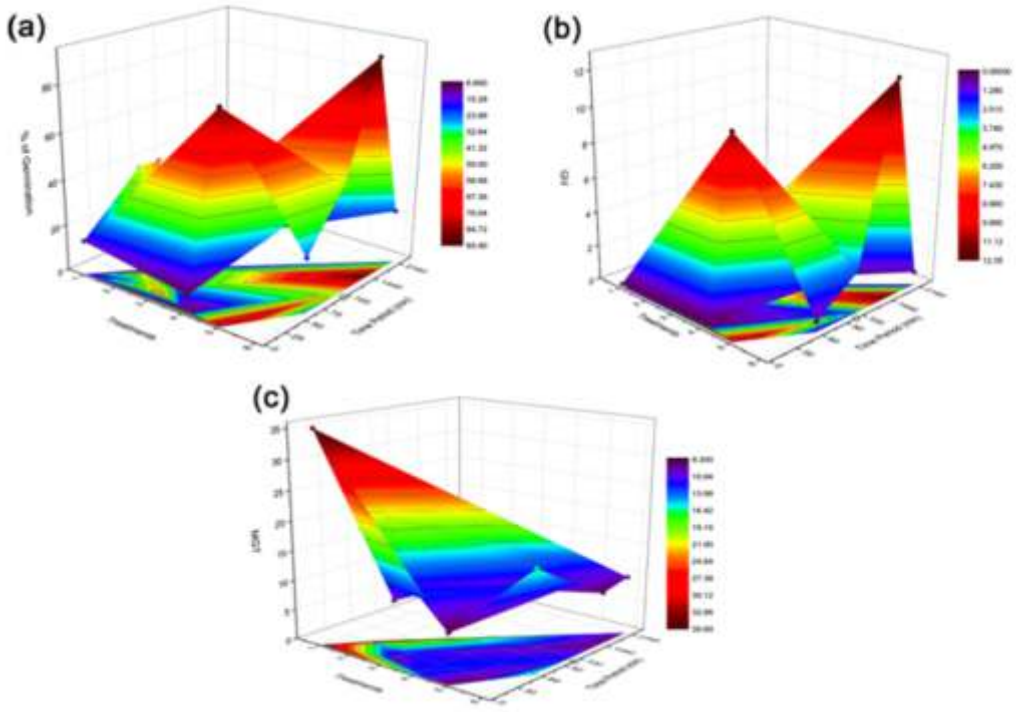


Fig. 2 The 3D surface graph showing the effect of physical and chemical scarification; in graph Treatment 1=control; Treatment 2=heat treatment at 60°C, Treatment 3=heat treatment at 80°C; Treatment 4= heat treatment at 100°C; Treatment 5=mechanical treatment; Treatment 6=chemical treatment (H_2SO_4). **(a)** Percentage of germination with effect of treatments and time exposure. **(b)** Germination value with effect of treatments and time exposure. **(c)** Mean germination time with effect of treatments and time exposure. The gradient of color intensity representing effects of treatment.

According to our present investigation, the highest response in germination percentage, highest GV and germination rate (lowest MGT) was obtained at 24 hours acid (Conc. H_2SO_4) treatment while Choudhury et al. (2009) reported that within the short time regime of 2 minutes to 2 hours soaked in Conc. H_2SO_4, germination varies from 40% to 50%. This deviation might be interpreted as harvesting time differ from previous studies of *G. assamicus* where the seed pods were collected during the month of January. Several authors have reported in their work the relationship between time of harvesting and germination responses influence the quality of seed (De Pauw and Remphrey 1993). It was also reported that hardness of seed coat depends on maturity of seed which come through right harvesting period and all of these factors ultimately have effects on nature of treatment and length of exposure in Okra seeds (*Abelmoschus esculentus* L. Moench) (Mohammadi et al. 2012). The percentage of germination enhanced significantly as harvest time was delayed i.e. towards full maturity of seed (Olasojiet al. 2012). Effects of maturity on germination responses in legume species of *Albizia lebbek* (L.) was reported in the study Singh et al. 2008 and Bhardwaj et al. 2002. Hence it might have influenced the pattern of treatment or intensity of treatment which is also reflected in our present result and might be the cause for deviation from previous studied of *G. assamicus.* Thus, overall result provides rigorous data in favor of physical dormancy which remains the major obstacle in *G. assamicus* which could be overcome through physical or chemical treatments, hence, our presented results are in concurrence with the findings of Choudhuryet al. (2009) as true orthodox type seed.

In vitro germination showed that all the three formulated media was not feasible to get adequate

seed germination, although WPM semi solid medium showed better results in contrast to MS and ½MS medium. The semisolid WPM medium gave moderate (36.67%) seed germination in comparison to other treatments. The increasing trend in percent germination response was observedby reducing the nutrient content, thus low salt concentration content is best for *G. assamicus* seed (Table 1); this can be interpreted as the size of endosperm as well as legume species have sufficient amount of nutrient to take over germination events after optimal scarification under favorable environment. The highest germination of 96.95% (Table 1) was recorded in SGP_2(Tukey HSD, $P<0.05$) which differed significantly from all other treatments (Fig. 2h),without any supplemented media, followed by seed germination paper supplemented with WPM basal salt medium, hence seed germination paper soaked with sterile distilled water was the most effective medium for seed germination. Similar result was reported for *in vitro* seed germination in *Vacciniumm eridionale* (Castro et al. 2012).Thus present method can be implemented at nursery level practice, as mass culture of sterile seedlings and large scale seedlings recruitment in natural habitat.

Table 1 Effect of optimal chemical (36N H_2SO_4) scarification on different media in vitro seed germination

Treatments		% of Germination Response	Mean Germination Time (MGT)	Germination Value (GV)
MS	Control	10.26±1.19 e f g	30.52±0.44 h i j	0.12±0.01 c d e f g h
	24hrs H_2SO_4	16.68±1.16 d e	15.01±0.75 a b c d e	0.30±0.13 c d e
½ MS	Control	16.65±1.0 d e f	23.01±1.45 f g	0.24±0.06 c d e f
	24hrs H_2SO_4	23.61±1.90 d	12.08±0.50 a b c d	0.68±0.04 c d
WPM	Control	16.65±1.23 d e f	21.33±0.90 e f	0.24±0.06 c d e f
	24hrs H_2SO_4	36.67±1.88 c	10.63±0.33 a b c	1.72±0.11 c
SGP_1	Control	16.65±1.23 d e f	26.55±0.77 f g h i	0.14±0.02 c d e f g h
	24hrs H_2SO_4	73.52±1.79 b	8.96±0.30 a b	6.47±0.41 b
SGP_2	Control	16.65±1.23 d e f	26.27±1.29 f g h	0.15±0.03 c d e f g
	24hrs H_2SO_4	96.95±0.76 a	8.17±0.34 a	13.8±0.85 a

Means followed by similar letters within a column are not significantly different (Tukey HSD multiple comparisons; P<0.05)

CONCLUSION

G. assamicus seed germination studies revealed all the pretreatment improved *in vivo* and *in vitro* seed germination in compare to untreated seed. The highest germination of 96.95% was obtained in autoclave distilled water soaked germination paper (SGP_2) under in vitro condition and it has shown significant differences when compared to all other treatments.. The second highest percentage (93.34%) of germination was obtained in vivo from the 24 hrs acid treatment (Conc. H_2SO_4) in comparison to all mechanical and heat treatment. While nutrient media did not improve in vitroseed germination. Thus present finding concludes that *Gymnocladus assamicus* have true orthodox type seed and need optimal scarification for breaking physical dormancy of seed.

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