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Original Contribution

FACTORS INFLUENCING THE SEED GERMINATION OF *SOYMIDA FEBRIFUGA* (ROXB.)A.JUSS. (MELIACEAE)

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ABSTRACT

Soymida febrifuga (Roxb.)A.Juss. (Meliaceae) is an indigenous lofty deciduous medicinal tree endemic to India. Traditional multiplication of this plant is limited by difficulty in rooting of stem cuttings, high seedling mortality rates and low seed viability. Recently we published an alternative mode of shoot multiplication by *in vitro* culture to propagate and conserve genetic stock of this medicinal plant. In the present study, prior to *in vitro* shoot multiplication, attempts were made to understand the seed germination capabilities of *S. febrifuga* under *in vivo and in vitro* conditions. An emphasis has been laid on various factors such as sterilants, type of media, hormones, sucrose concentration and seed viability influencing *in vitro* seed germination which is used as a starting material for *in vitro* morphogenic studies. Our results clearly demonstrate 95% of *in vitro* seed germination but not *in vivo* led to the raising of healthy seedlings with appropriate root system. Strikingly we observed duration and rate of seed germination were high on MS full strength compared to MS half and quarter strength medium. Nonetheless, seed germination and its growth was found effective on MS full strength medium containing 1% and 1.5% sucrose medium unfortified with growth hormones. These findings have implications for the conservation of recalcitrant seed germplasm.

Key words: *Soymida febrifuga*, phenology, seed germination, seed viability

INTRODUCTION

Soymida febrifuga (Roxb.) A. Juss. (Meliaceae) is an indigenous lofty deciduous medicinal tree and monotypic genus endemic to India (1). The plant is lofty deciduous tall tree, grows up to 22-25 mtrs in height, 2.5-3.0 mtrs in girth (2). The plant is found on dry stony hills and on laterite soils. Direct sowing on ridges on 30 cm height, is more successful than transplanting the nursery raised plants. Propagated by seeds and germination is best in porous, well-drained soil but are liable to damp off in badly drained damp soils. Seedling growth is slow and sensitive to frost and growth ceases during the cold season. Tree is subjected to defoliation by insect (1).

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Bark contains a resinous bitter principle well adapted for gargles, vaginal infections, enemata, rheumatic swellings, and stomach pain. The bark is said to be used as an anti-cancer remedy, for blood coagulation, wounds, dental diseases, uterine bleeding and haemorrhage and acrid, refrigerant, antihelminthic, aphrodisiac, laxative; good for sore throat; cures 'tridosha' fevers, cough, asthma and is anti-inflammatory in action. Secondary metabolites such as methyl angolensate was isolated from callus of *Soymida febrifuga* (3), found to possess antimicrobial (3) and anticancer properties (4, 5). We recently developed a protocol for the *in vitro* propagation of *Soymida febrifuga* using mature nodal explants (6). Conventionally this plant is propagated through seeds, have low germination capacity and seedlings are more prone to insect attack. Propagation through stem cuttings is

cumbersome that restricts propagation due to difficulty in rooting.

MATERIALS AND METHODS

Phenological Studies. Phenological studies were under taken on *S.febrifuga* growing at Divyaramam Nursery, Tirupathi, Andhra Pradesh, India to understand the physiological changes and to note the cycle of blossom and fruit set.

***In vivo* Seed Germination.** Direct seed germination test *in vivo*, was performed to determine germination percent of the species, before going for *in vitro* propagation studies. Mature fruits of *S. febrifuga* were collected from Divyaramam nursery, Tirupathi, Chitoor District, Andhra Pradesh in the month of May and shade dried. After dehiscence of the fruits the healthy seeds were separated, dried and stored for the experimental work. The selected seeds were counted and stored both at room temperature and at 7^0C. Percentage of seed germination was determined by sowing in different pots. Seeds were germinated both under *in vivo* and *in vitro* conditions at 15 day intervals after harvest up to 150 days.

Seed Viability Test. The period of seed viability was ascertained by subjecting the seeds to germination at 15 day intervals and by Tetrazolium test (TTC). Ten seeds were taken for experiment with three replicates and conducted thrice at monthly intervals. The main principle of TTC test lies in the conversion of colourless 2,3,5-Triphenyl Tetrazolium Chloride to pink coloured Triphenyl formazone by respiratory enzymes of viable seeds (7). Seeds of *S. febrifuga* in three replicates of 25 each, were soaked overnight (16-18h) in distilled water. Vertical incisions were made on cotyledons of seeds and soaked them in phosphate buffer (0.06M) of 2, 3, 5-Triphenyl Tetrazolium Chloride (0.1% w/v). Phosphate buffer was prepared by mixing 8.16g of dried anhydrous KH_2PO_4 and 5.68g of dried anhydrous Na_2HPO_4 with pH 7 in distilled water. The seeds were incubated overnight at room temperature and were washed thoroughly under running tap water to remove traces of TTC solution. Evaluation of seed viability was done based on the development of colour in the cotyledon and embryonal axis. The percentage of viability was calculated by the following formula.

$$\text{Percentage of viability} = \frac{\text{No. of seeds developing colour}}{\text{Total no. of seeds}} \times 100$$

***In vitro* Seed Germination.** Seeds were collected from the fruits of 15-20 years old mature tree growing in Divyaramam nursery, Tirupathi, Chitoor District, Andhra Pradesh, India. Seeds were washed with 5% teepol for 15 min and washed thoroughly in running tap water. They were surface sterilized with 70% alcohol for 30 sec and followed by 5-6 rinses in sterile distilled water. Finally the seeds were treated with different sterilizing agents followed by rinsing 3 times in sterile distilled water. Then they were inoculated aseptically in test tubes, containing 10-15 ml of MS solid and liquid medium separately. The seed germination was observed within 12-15 days on MS solid and liquid medium.

Inoculation. Prior to inoculation, the laminar airflow cabinet was smeared with ethyl alcohol. All the required paraphernalia for inoculation were transferred to the inoculation chamber. Sterilization of the chamber was done by switching on the ultraviolet lamp for half an hour before inoculation. During inoculation, the instruments like scissors, forceps, needles etc., were dipped in ethyl alcohol and flamed both before and after their use to maintain aseptic conditions. Hands were smeared with ethanol from time to time to avoid contamination. Inoculations were done in front of a spirit lamp kept inside the chamber.

Culture Conditions. All cultures were incubated in a culture room at 25 ± 2°C with a relative humidity of 50-60% and 16 h photoperiod at a photon flux density of 15-20μE m^2/s^{-1} from white cool fluorescent tubes.

RESULTS AND DISCUSSION

Medicinal plants are of great interest to pharmaceutical industries for the production of secondary compounds (8) and are

unscrupulously harvested from the wild populations resulting in depletion of species, causing loss of biodiversity and leading to the possibility of adulteration of medical formulations. Deforestation and changing environmental conditions have been causing a threat to many species, thereby some species have already become extinct and some others are threatened with extinction. Application of plant tissue culture techniques would be useful for large-scale micropropagation of medicinal plants, the production of phytochemicals (9) and also to serve as vehicles for in depth investigation of physiological and biochemical processes (10). Hence, *ex situ* conservation and the availability of the medicinally important plants throughout the year has become indispensable. *S. febrifuga* selected in the present study has immense importance and potential in natural medicine (1, 2).

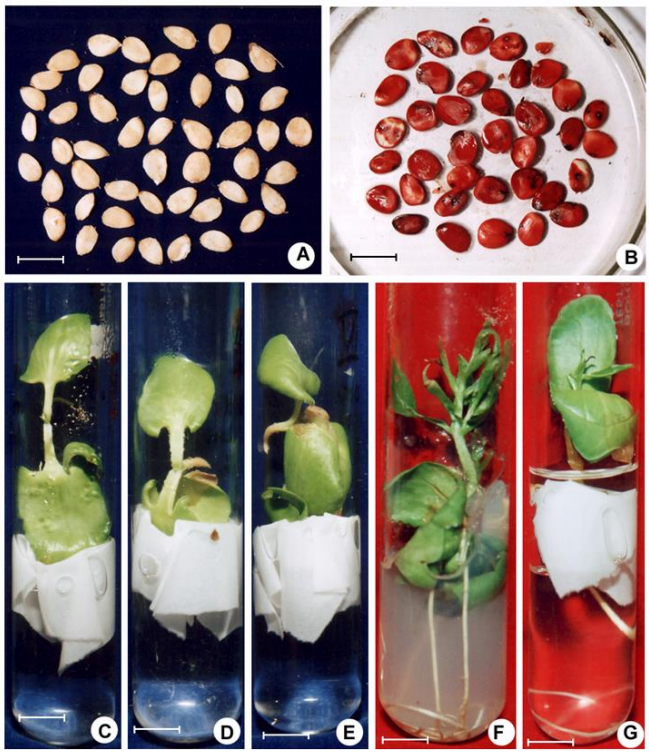


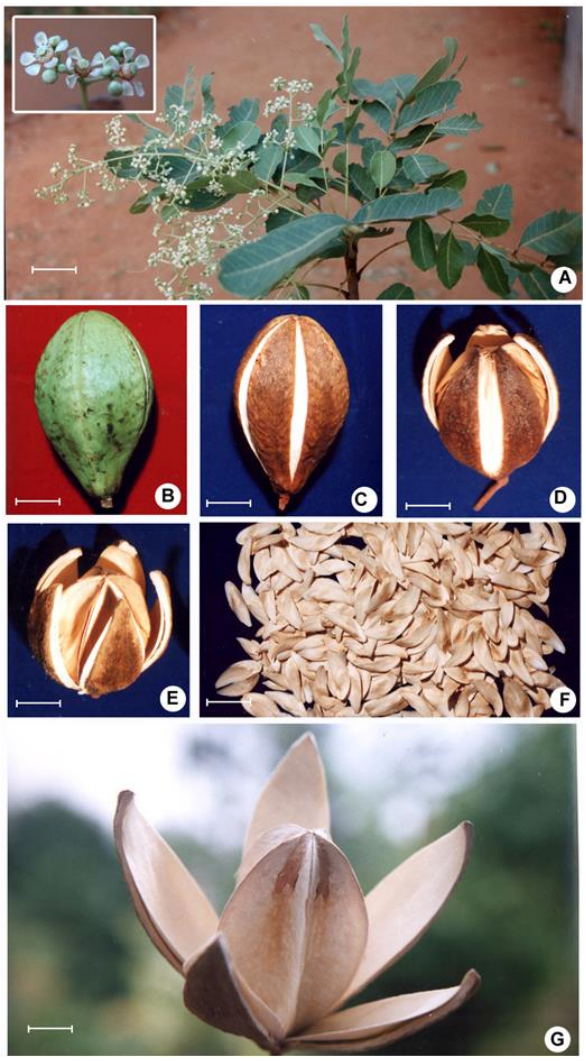
Figure 1. Seed viability and germination of *Soymida febrifuga*: Seeds treated with 1% TTC **(A)** Non viable seeds not showing colour **(B)** Viable seeds showing red colour (Bar = 2.0, 2.0 cm) **(C)** Aseptic seedling on B5 medium after 4 weeks (Bar = 8.5 mm) **(D-E)** Aseptic seedling on woody plant medium after 4 weeks (Bar = 8.0, 8.5 mm) (**F)** Raising of aseptic seedling on Murashige and Skoog (MS) basal solid medium (Bar = 7.5 mm) (**G)** Raising of aseptic seedling on MS basal liquid medium (Bar = 8.0 mm)

Supplementary Table 1. *Geographical conditions of the study area*

Longitude	Latitude	Mean annual Rainfall (mm)	Mean relative humidity (%)	Mean Temp.		Sun shine (h)
				Max.	Min.	
79^0 20' to 79^0 26' E	13^0 36' to 13^0 42' N	817 m.m.	74.7	25^0C	17^0C	7.6

Supplementary Table 2. *Phenological study of S. febrifuga*

Leaf shedding	Flowering	Fruiting	Mean fruit length	Fruit dehiscence	Seed dispersal	Seed collection
January-February	March- April	May - July	7.0 – 8.0 cm	July	Wind	July-August



Supplementary Figure 1. Morphology of *Soymida febrifuga* **(A)** A twig with flowering (Bar = 2.0 cm) (Inset: Flowers in close up) (**B)** Immature fruit (Bar = 3.0 cm) **(C)** Mature fruit about to dehisce (Bar = 3.1 cm) **(D-E)** Fruits showing dehiscence (Bar = 3.4, 3.7 cm) **(F)** Winged seeds (Bar = 2.6 cm) **(G)** Woody 5-celled septifragal capsule after complete seed dispersal (Bar = 1.0 cm)

Phenological Studies. Phenological studies were undertaken on *S. febrifuga* growing at Divyaramam nursery, Tirupati to note the cycle of flowering and fruit set **(Supplementary Figure 1)**. The geographical conditions of the studied area are represented in **Supplementary Table 1**. Information on phenology is important for plant reproduction (11) and for the study of plant–animal interactions that affect pollination and dispersal. In *S. febrifuga*, initiation of flowering was noticed in mid March and the peak flowering was noticed in April. The fruiting was observed between early May–late July (**Supplementary Table 2**).

Seed Germination Studies. In the natural environment, sexual and asexual reproduction have their appropriate selective advantages according to the stage of evolution of different kinds of plants. Plants selected and exploited by man also have different propensities for propagation by seed or by vegetative means. Seed germination is the most common method of multiplication of species in flowering plants. Plants grown from seed are without most of the pest and diseases which may have afflicted their parents (12).

The percentage of germination and average seed output of a species are the factors which determine its reproductive capacity (13). In the present study attempts were made to know the seed germination capabilities of *S. febrifuga* under *in vivo and in vitro* conditions. An emphasis has been laid on various factors such as sterilants, type of media, hormones, sucrose concentration and seed viability influencing *in vitro* seed germination which is used as a starting material for *in vitro* morphogenic studies.

Soymida febrifuga is naturally propagated through seed **(Supplementary Figure 1F**). One of the constraints associated with the conventional propagation is very short span of seed viability. No alternative mode of multiplication is available to propagate and to conserve genetic stock of this medicinal plant. Tissue culture offers an effective alternative method for rapid multiplication of *S. febrifuga*.

***In Vivo* Seed Germination.** Germination test, *in vivo*, was performed to determine seed viability before going to *in vitro* seed culture. Seeds stored both at 7^0C and at room temperature were germinated *in vivo*. The seeds were picked at random and divided into six lots of 100 each. Seed germination test was conducted at normal room temperature. The seeds were sown in pots filled with soil and compost manure and tested for percentage of germination at 15 days interval upto 5 months after collection.

The seedlings raised in pots filled with soil and compost were found to be unhealthy and suffered from root rot. The germination percentage decreased gradually from 15 days (50%) to 5 months (0%) and there was no germination after 5 months period **(Table 1).** To identify the reason behind this, viability test was conducted, so as to test whether the delayed germination is due to dormancy or due to non-viability of the seeds.

Table 1. *Seed germination (%) of S. febrifuga. Data represents 20 replicates and the values recorded after 2 weeks of culture initiation*

Time (days)	Percentage of Seed germination	
	Seeds stored at 7^{O}C	Seeds stored at room temperature
15	100	100
30	90.0	80.5
45	82.5	68.3
60	75.0	56.6
75	62.5	42.3
90	50.0	30.8
120	39.3	22.8
150	17.6	10.0
After 5 months	No seed germination	No seed germination

Biochemical Test for Seed Viability. Before going for *in vitro* seed culture studies, biochemical seed viability test was conducted (TTC test). Following TTC test, 100% of the seeds of *S. febrifuga* showed viability by developing red coloration **(Supplementary Figure 1B)** all around cotyledonary cut surface up to 20 days from date of harvest. The percent of seed viability after 20 days decreased gradually and showed negative reaction with TTC solution after 5 months of storage **(Supplementary Figure 1A)**. Seed viability decreases with storage of time. Percentage of germination falls down after storage due to loss of viability. The loss of viability during prolonged storage of seeds may be due to denaturation and inactivation of proteins and enzymes over drying. Seed vigour as a quality attribute has gained significance as the germination potential does not reflect field performance potential of seed lot under varied environmental conditions. The loss of seed vigour and viability have been associated with the deterioration of membrane integrity and reduced dehydrogenase activities, and the reduction in seedling growth has also been reported to be a measure of loss in vigour (14). Similar results were observed in the current study. Abideen (15) reported that the proper growth of the seedlings and subsequent survival in the field depended mainly on the vigour of seedlings.

***In vitro* Seed Germination.** Seeds were germinated under *in vitro* conditions in all kinds of media tested **(Table 2)** at 15 day intervals up to 150 days after harvest. Comparison of percentage germination under *in vivo* and *in vitro* conditions was made. Germination percentage under *in vivo* and *in vitro* conditions represented in the **Table 3** clearly indicates that percentage of germination was poor in *in vivo* conditions and it is better in *in vitro* condition. The reason might be loss of moisture content in the seeds of *in vivo*. When stored at 4^{o}C they retained moisture content and exhibited good viability.

Table 2. *Comparison of in vitro and in vivo seed germination (%) of S. febrifuga. Data represents 20 replicates and the values recorded after 2 weeks of culture initiation*

Time (days)	% of seed germination	
	In vitro	***In vivo***
15	100	100
30	92.5	50.5
45	87.6	38.3
60	78.7	26.6
75	71.0	12.3
90	67.0	5.8
120	60.5	2.8
150	50.0	0.0
After 5 months	35-40	0.00

Table 3. *Effect of various sterilizing agents on in vitro seed germination of S. febrifuga. Data represents 20 replicates and the values recorded after 2 weeks of culture initiation*

Sterilants	Conc %	Time in minutes	% of germination
$HgCl_2$	0.01	15	70.0
	0.05	15	100.0
	0.1	5	0.00
NaOCl	1.0	5	22.0
	2.0	5	35.0
	5.0	5	15.0
	10.0	5	7.0
C_2H_5OH	25	5	30.0
	50	3	47.0
	70	1	60.0
	100	1	12.0
H_2O_2	5.0	2	15.0
	10.0	1	8.0

The effect of different sterilents in preventing contamination, media type in inducing germination and subsequent growth of seedlings was studied and the results of which are given below (**Table 4**). Due to severe and uncontrollable microbial contamination, excessive phenolics (one of the inhibitory agent for organogenesis) and genetically less regenerating potentiality, the mature explants of some species exhibit difficulty for organogenesis than the *in vitro* grown seedling explants (16-18). Since plant regeneration was achieved from the *in vitro* grown seedling explants, it is very essential to raise healthy seedlings. Various types of disinfectants like mercuric chloride ($HgCl_2$), sodium hypochlorite (NaOCl) and hydrogen peroxide (H_2O_2) were used for sterilizing the seeds. As high concentrations of disinfectant damage plant tissues and mild dosage do not control the microbial

contamination, it is essential to determine the best concentration and the duration of exposure to sterilant. Among various sterilents employed to prevent contamination, 0.1% $HgCl_2$ inhibited seed germination even when the duration of time was reduced from 5 min to 2 min. The high toxicity of mercuric chloride on soft tissues may be the causative factor for inhibition of seed germination as said by Kumar (19). A similar result with $HgCl_2$ has been reported (20,21). When 0.01% $HgCl_2$ for 5 to 10 min was employed, 50% of seeds got contaminated **(Table 4).** In the present study, surface sterilisation of seeds with 70% alcohol for 60 sec followed by 0.05 % $HgCl_2$ for 15 min was effective in raising stocks of aseptic seedlings. The results obtained with NaOCl are in contradictory to the reports obtained (22,23).

Table 4. *Effect of different media on aseptic seed germination in S. febrifuga Data represents 20 replicates and the values recorded after 2 weeks of culture initiation*

Medium	**Germination rate (%)**	**Time taken for germination (days)**	**Nature of response**
Water agar	10	25-30	Weak, poor with delayed germination.
MS full strength + 1.5% sucrose	100	8-12	Healthy seedlings with well developed shoot/root ratio Adventitious shoots produced from the intact seedling
MS full strength + 1% sucrose	85	10-12	Healthy seedlings with elongated shoot and enlarged cotyledons
MS half strength	30	20-30	Poor and delayed germination
MS quarter strength	15	20-30	Short, poor root system developed, delayed germination
WPM full strength	55	10-15	Short, poor root system developed, delayed germination
WPM half strength	40	15-20	Poor and delayed germination
WPM quarter strength	45	20-25	Poor and delayed germination
B5 full strength	50	18-25	Short, poor root system developed, delayed germination
B5 half strength	45	20-25	Poor and delayed germination
B5 quarter strength	25	25-30	Poor and delayed germination

To identify the best medium for seed germination various media fortified with or without hormones were tested. The germination of seeds commenced on the 4th day after soaking on all media and the germination of *in vitro* sown seeds on various media did not differ much from each other. Among the chemically defined media (MS, WPM and B_5) with and without growth regulators healthy seedlings with appropriate root system was observed on full strength MS medium **(Table 4; Figure 1C-G**). The duration and rate of germination were poor when seeds were germinated on MS half and quarter strength medium **(Table 4).** Seed germination and growth of seedlings was found effective and best on MS full strength medium containing 1% and 1.5% sucrose medium unfortified with growth hormones. Seeds cultured on water agar media showed only 10% germination with seedlings of poor shoot and root development. These plants were weak, slender and pale yellow in color having weak and branched root system. Weak seedlings with no root formation and only with cotyledons were developed on B5 and WP medium **(Figure 1C-E)**. The improvement of seed germination by growth regulators has been reported in studies of number of woody plants (Singh, 1990). But in the present target taxa studied, the application of growth regulators did not promote any germination. Seeds rich in hydrolyzing enzymes and stored food materials maintained the balance

of endogenous hormones and sprouted easily on MS basal medium (25).

Successful *in vitro* plant regeneration in various medicinal plants has been reported using aseptic seedling explants (20, 22, 26-29). The advantages of using seedling derived material is due to the fact that it is essentially less affected by phenolic compounds (generally the accumulation of which inhibits organogenesis).

Selection of appropriate nutrient medium is also essential for the success of all experimental systems in plant tissue culture. Different culture media have been used for *in vitro* cultures (30). Bhojwani and Razdan (31) suggested that in order to formulate a suitable medium for a new system, it is best to start with a well known basal medium such as MS. Accordingly MS medium was initially used in the present study. Two other basal media, namely, B5 and WPM, were also tested for their effect on morphogenic ability. Mature tree explants are relatively poor in regeneration (32). Seedling explants are, in general, more responsive than explants derived from mature trees and many tree species have been successfully propagated from seedlings. Many researchers used explants derived from aseptic seedlings for micropropagation (33, 34). In many woody species adventitious shoots were produced directly on explants derived from newly germinated seedlings (35, 36).

The use of intact seedlings for regeneration studies offers many advantages. It by passes the step of explant preparation due to the fact that these are free of endophytic microbes. The additional advantage of using seedling derived tissues for micropropagation is essentially due to the fact that these are free from phenolic compounds whose accumulation inhibit organogenesis in more mature trees (12). Because the differentiation occurs in intact seedlings, the number of manipulations required to induce regeneration is reduced to one in comparison to several procedures involving the culture of various explants. Thus the method of using intact seedlings in culture for direct shoot proliferation could simplify the micropropagation procedure (37).

Seedling explants in general are more responsive than explants derived from mature trees and many tree species have been successfully propagated from seedlings. Most of the studies carried out by others on tree species have utilised seeds and juvenile tissue that are more amenable to *in vitro* manipulation than the tissue from mature plants (38). For this reason juvenile explants have been extensively employed for the clonal propagation of woody plants (39). In the present study like in many research studies (40) seeds were germinated aseptically on MS basal medium to develop protocols for micropropagation from seedling explants.

In many woody plants, it is only juvenile explants which can be established in culture (41). Adult explants often produce more phenolic substances, than juvenile ones. In the present investigation on *S. febrifuga*, juvenile explants were used, as phenolic substances are one of the hindrance in this species. There are numerous examples in the literature where explants derived from the juvenile phase are most effective in culture. These include *Cassia torosa* (42), *Tamarindus indica* (43), *Pterocarpus santalinus* (44), *Peltophorum pterocarpum* (45), *Samanea saman* (46) and *Soymida febrifuga* (47,48). The superiority of MS medium over other salt formulations have been demonstrated for many other plant species such as *Hybanthus enneaspermum* (49), *Guizotia abyssinica* (50) and *Cunila galioides* (51) and it has been frequently used successfully in tissue culture studies of tree species (52).

CONCLUSION

It is of great interest to develop biotechnological methods to improve the cultivation of *S.febrifuga* due to its medicinal importance. Our study have substantial implications as the conventional propagation of *S.febrifuga* through stem cuttings is cumbersome that restricts propagation due to difficulty in rooting. Hence the present study establishes first successful attempts to establish rapid method for ex situ conservation of this endemic plant by in vitro seed germination. These plants can be grown in fields as a part of domestication strategy inorder to minimize the pressure on wild plants. More importantly, it could be potentially useful for the production of propagules throughout the season constantly compared to wild species.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: KKC,AM,GRG. Performed the experiments: KKC. Analyzed the data: KKC,AM,GRG. Wrote the paper: KKC, AM,GRG.

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