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Journal of Threatened Taxa

10.11609/jott.2023.15.8.23631-23826
www.threatenedtaxa.org

26 August 2023 (Online & Print)
15(8): 23631-23826
ISSN 0974-7907 (Online)
ISSN 0974-7893 (Print)

Open Access

ISSN 0974-7907 (Online); ISSN 0974-7893 (Print)

Publisher
Wildlife Information Liaison Development Society
www.wild.zooreach.org

Host
Zoo Outreach Organization
www.zooreach.org

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Cover: Coromandal Sacred Langur *Semnopithecus priam* - made with acrylic paint. © P. Kritika.

Journal of Threatened Taxa | www.threatenedtaxa.org | 26 August 2023 | 15(8): 23805–23811

ISSN 0974-7907 (Online) | ISSN 0974-7893 (Print)

https://doi.org/10.11609/jott.8493.15.8.23805-23811

#8493 | Received 27 April 2023 | Final received 16 June 2023 | Finally accepted 17 July 2023

OPEN ACCESS

COMMUNICATION

Seed germination and storage conditions of *Ilex embelioides* Hook.f. (Magnoliopsida: Aquifoliales: Aquifoliaceae), a threatened northeastern Indian species

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Abstract: *Ilex embelioides* Hook.f. is a threatened tree species endemic to northeastern India. The species propagates naturally through seeds but shows a brief dormancy period. The present study was carried out to assess the germination behaviour, and identify the suitable storage conditions of *I. embelioides* seeds. Results revealed that warm stratification for 30 days could effectively break dormancy. Seeds pre-treated with gibberellic acid (GA_3, 2,000 mg L^{-1}) showed highest germination (63.89 ± 0.91%) as compared to other concentrations of GA_3 as well as potassium nitrate (KNO_3). Highest in vitro seed germination percentage (65.56 ± 2.92%) was recorded on (Murashige and Skoog) MS medium containing 10 mg L^{-1} GA_3. The viability of the seeds declined with storage period irrespective of its storage condition. After 30 days, highest viability (54.72 %) was observed in seeds stored in moist sand at 25°C. Therefore, application of GA_3 and warm stratification (25 ± 1°C) along with a substrate (moist sand) for 30 days may be considered as ideal conditions for effective germination and storage of *I. embelioides* seeds. This study can also be used for mass propagation of the species for reintroduction in the wild.

Keywords: Dormancy, endemic, growth regulators, moisture content, seed storage, viability.

Editor: K. Haridasan, Palakkad, Kerala, India.

Date of publication: 26 August 2023 (online & print)

Citation: Malngiang, L., K. Upadhaya & H. Choudhury (2023). Seed germination and storage conditions of *Ilex embelioides* Hook.f. (Magnoliopsida: Aquifoliales: Aquifoliaceae), a threatened northeastern Indian species. *Journal of Threatened Taxa* 15(8): 23805–23811. https://doi.org/10.11609/jott.8493.15.8.23805-23811

Funding: None.

Competing interests: The authors declare no competing interests.

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Author contributions: LM carried out field and laboratory experiments, data analyses and draft manuscript preparation. KU and HC planned the experiments and prepared the final manuscript.

Acknowledgements: The first author is thankful to Rajiv Gandhi National Fellowship for providing with financial assistance (Award No. 201920-NFST-MEG-00185 dated 10.04.2020). The help and support received from the headmen and the local village people during the field study is also acknowledged.

INTRODUCTION

Ilex embelioides Hook.f. is an endemic (Haridasan & Rao 1985; Lasushe et al. 2022) and 'Vulnerable' species of northeastern India (Mir & Upadhaya 2019). It belongs to Aquifoliaceae (holly) family which comprises of more than 500 species distributed throughout temperate and tropical regions of the world (Galle 1997; Cuenoud et al. 2000). In India, the genus *Ilex* consists of 26 species (Das & Mukherjee 2017). *Ilex embelioides* is a small tree attaining a height of 10 m and is restricted to primary subtropical and tropical forests. The flowering period in the species was observed during rainy season (April–October) and the fruiting occurs during winter season (mid November–January). The seeds remain dormant for about three months (January–March). Seed germination occurs during March–April with the onset of rain (Upadhaya et al. 2017).

The genus *Ilex* has small seeds, globular to oval-oblong rudimentary (underdeveloped but differentiated) embryos that grow inside the seed before the emergence of radicle (Martin 1946; Hu 1975; Baskin & Baskin 2004). The embryo in *Ilex* seeds has low growth potential (physiological dormancy), and warm and/or cold stratification treatments are required to overcome dormancy (Tsang & Corlett 2005; Tezuka et al. 2013). A number of studies have been carried out to understand seed germination and dormancy in other species of *Ilex* such as *I. paraguariensis* (Sansberro et al. 2001; Souza et al. 2022), *I. uaranine*, *I. brasiliensis*, *I. brevicuspis*, *I. uaran* var. *uaranine*, *I. paraguariensis* var. *paraguariensis*, and *I. theezans* (Galindez et al. 2018). However, there is no information on seed germination of *I. embelioides*, which is now facing the threat of extinction due to habitat destruction, overexploitation for use as poles and fuel wood, poor regeneration and possibly climate-related physiological stress (Singh et al. 2023). In order to prevent the species from extinction, mass propagation of the species through seed germination is required (Iralu et al. 2019). Therefore, the present study was carried out to assess the germination behaviour, and identify the suitable storage conditions of *I. embeloides* seeds. Also in vitro seed germination was conducted to identify optimal conditions for germination.

MATERIALS AND METHODS

Matured fruits of *I. embelioides* (Image 1A) were collected from two matured individuals (>5 cm diameter at breast height) from Umtong (25.4124^{0}N & 92.0034^{0}E) and five individuals from Laitryngew (25.2255^{0}N & 91.5475^{0}E) during the month of December 2021. Seeds were separated from the berries and thoroughly washed with water for 10–20 min to remove the pulp. Healthy seeds were separated from damaged or dead seeds using floatation methods (Pipinis et al. 2011). The seeds were disinfected with 75% ethanol for 30 s, and immediately rinsed with sterile water and dried with absorbent paper. The cleaned seeds were stored at room temperature (24 ± 2^{o}C) and later used for various experiments.

Moisture content

To estimate the moisture content, three replicates of 50 seeds each were oven dried at 80°C for 24 h and the final mass was noted. Moisture content was determined following ISTA (2008) as follows:

$$M_c\ (\%) = (W_2 – W_3/W_2 – W_1)\ x100$$

Where, M_c is the moisture content, W_1 is the weight of the container, W_2 is the weight of the container with seeds before oven drying and W_3 is the weight of the container with seeds after oven drying.

Effect of gibberellic acid (GA_3) and potassium nitrate (KNO_3) on seed germination

Plant growth regulator viz., GA_3, and KNO_3 were used in different concentrations to enhance the germination percentage and reduce the germination time following the protocol as adopted by Iralu & Upadhaya (2016, 2018) and Borah et al. (2023). The different treatments include: (i) control (without GA_3 or KNO_3), (ii) seeds soaked in different concentrations of GA_3 i.e., 200, 500, 1,000, 2,000, 4,000 mg L^{-1} for 48 h, (iii) seeds soaked in different concentrations of KNO_3 i.e., 0.5%, 1%, 1.5% and 2% for 48 h and (iv) seeds soaked in different concentration of GA_3 for initial 48 h and transferred to KNO_3 and kept for another 48 h.

The treated seeds were placed in plastic petri dishes over moist filter paper and kept for germination at 25 ± 1°C. Germination was monitored regularly for a period of 60 days and a seed was considered as germinated with the emergence of a radicle of 1 mm size (Vera et al. 2010). For each replicate in each treatment, the germination percentage was calculated as G = (n/N) × 100, where n is the number of germinated seeds and N is the total number of seeds. Also, the mean germination days were calculated using the formula:

$$\frac{n1+n2+n3+\cdots}{N}$$

Where, n1, n2, n3…. are the number of days taken by individual seeds to germinate and N is the total number

Image 1. A—twig showing *Ilex embelioides* fruit | B—Germination of seeds treated with 2,000 mg L^{-1} GA_3 (C) Seed germination on MS medium containing 10 mg L^{-1} GA_3 (D) Seedling after two months of transplantation in soil. © Leoris Malngiang.

of seeds (Ellis & Roberts 1981).

In vitro seed germination

For in vitro seed germination, the seeds were scarified and soaked in distilled water for 24 h, then washed with 2% sodium hypochlorite for 5 min under aseptic condition, and placed on MS medium (Murashige & Skoog 1962) containing 3% sucrose, vitamins and different concentrations of GA_3 (1 mg L^{-1}, 5 mg L^{-1}, 10 mg L^{-1} and 20 mg L^{-1}). The cultures were maintained at 25 ± 2°C under a 16/8 h photoperiod (flux of 50 µmol $m^{-2}s^{-1}$) of cool white fluorescent lights and in the dark. Germination was monitored for a period of 60 days and the germination percentage was calculated.

Effect of stratification on seed germination

To evaluate the effect of warm and cold stratification on seed germination, three replicates of 30 seeds each were packed in sealed polythene bag containing moist sand and stored at 5 ± 1°C in darkness and in an incubator at a constant temperature of 25 ± 1°C for 0 (control), 10, 20, 30, 40, and 50 days. After each treatment, the seeds were thoroughly washed under tap water and incubated in light at 25 ± 2°C and germination was monitored regularly for a period of 60 days.

Seed viability and storage

To understand the seed viability characteristics, three tests were conducted, viz., seeds (approximately 200 seeds/bag) were stored in airtight containers at room temperature (24 ± 2°C and 65% humidity); seeds were packed in airtight polybags and stored at 5 ± 1°C. In the third and fourth test, moist sand was added and the seeds were stored at a constant temperature of 25 ± 1°C and 5 ± 1°C, respectively. The seeds stored in each of the above conditions were retrieved after 10, 20, 30, and 40 days and the viability test was carried out using the Tetrazolium assay (Enescu 1991).

Data analysis

To compare the effect of different concentration of GA_3 and KNO_3 on seed germination, and different storage conditions on viability of seeds, analysis of variance (ANOVA) followed by Tukey's least significant difference test ($p < 0.05$) was done using Origin Pro 2016.

RESULTS AND DISCUSSION

The moisture content of fresh seeds when dried for 24 h in oven at 80°C decreased by 46.99 ± 0.64%.

A decline in moisture content over a short period of time revealed intermediate nature of seeds (Hong & Ellis 1996; De Vitis et al. 2020). Seed moisture of 10–40% is often considered as desirable for retaining seed longevity in many species (Hampton & Hill 2002; Dadlani et al. 2023).

Plant growth regulator played a major role in breaking seed dormancy in *I. embelioides*. Seeds treated with 2,000 mg L^{-1} GA_3 showed highest germination percentage (63.89 ± 0.91%) and the mean number of days required for germination (16.22 ± 0.87) was also reduced (Image 1B). The use of GA_3 increased the germination percentage of *I. embelioides* during the first four–eight weeks. This result was similar to that observed in *Ilex maximowicziana* (Chien et al. 2011), *I. brasiliensis*, and *I. theezans* (Galindez et al. 2018) where the seeds treated in GA_3 germinated during the first 12 weeks. The decrease in the germination percentage (26.98 ± 1.46%) of *I. embelioides* seeds with the increase of GA_3 concentration (4,000 mg L^{-1}) could be due to the surplus hormone that led to the toxicity of GA_3 (Akbari et al. 2008; Borah et al. 2023).

Though KNO_3 is widely used chemical for enhancing seed germination (Agrawal & Dadlani 1995), it was not very effective in breaking seed dormancy of *I. embelioides* (Table 1). However, when KNO_3 was used in combination with GA_3, the germination percentage increased significantly. This may be attributed to the fact that nitrate reduces abscisic acid concentration in seeds which is responsible for dormancy, whereas GA_3 promotes germination (Hilhorst & Karssen 1992). The germination percentage (46.19 ± 3.59%) of seeds under control condition (soaked in distilled water) took the maximum number of days (39.11 ± 0.40) for germination ($p < 0.05$) as compared to other treatments (Table 1).

Table 1. Effect of GA_3 and KNO_3 on seed germination of *Ilex embelioides*.

Treatments	Mean Germination (Days)	Germination (%)
200 mg L^{-1} GA_3	33.00 ± 0.83 ^{bc}	18.09 ± 1.26 ^{e}
500 mg L^{-1} GA_3	22.89 ± 1.37 ^{d}	42.38 ± 0.48 ^{bc}
1,000 mg L^{-1} GA_3	20.33 ± 0.69 ^{de}	50.98 ± 2.05 ^{b}
2,000 mg L^{-1} GA_3	16.22 ± 0.87 ^{e}	63.89 ± 0.91 ^{a}
3,000 mg L^{-1} GA_3	21.78 ± 0.59 ^{d}	56.04 ± 1.98 ^{b}
4,000 mg L^{-1} GA_3	25.00 ± 1.35 ^{d}	26.98 ± 0.84 ^{d}
0.5% KNO_3	29.78 ± 0.89 ^{c}	36.82 ± 1.37 ^{c}
1% KNO_3	31.22 ± 0.89 ^{c}	39.39 ± 1.16 ^{bc}
1.5% KNO_3	30.89 ± 0.29 ^{c}	36.40 ± 0.41 ^{c}
2% KNO_3	35.78 ± 0.48 ^{ab}	39.01 ± 0.49 ^{c}
200 mg L^{-1} GA_3 + 1.5% KNO_3	24.00 ± 0.69 ^{d}	52.78 ± 0.83 ^{b}
500 mg L^{-1} GA_3 +2 % KNO_3	23.89 ± 0.40 ^{d}	55.84 ± 0.83 ^{b}
Distilled water (Control)	39.11 ± 0.40 ^{a}	46.19 ± 3.59 ^{b}

For each treatment, means followed by the same letter in each column do not differ significantly at $p < 0.05$ (Tukey test).

In vitro seed germination of *I. embelioides* was observed after 40 days of culture both under light and dark conditions (Image 1C). ANOVA of the result revealed that the germination percentage sharply decreased in seeds kept under the dark– as compared to light– conditions (Table 2). The germination percentage (65.56 ± 2.92%) was significantly higher ($p < 0.05$) in seeds cultured in media containing 10 mg L^{-1} GA_3 under light condition. This result was in agreement to the in vitro seed germination observed in *Ilex brasiliensis*, *I. pseudoboxus* and *I. theezans* (Dolce et al. 2015).

Both warm and cold stratification helped in breaking dormancy of *I. embelioides* seeds. Seeds warm stratified (25 ± 1°C) for 30 days showed the highest germination percentage (96.67 ± 1.93%) after 40 days of incubation (24 ± 2°C). However, cold stratification was less effective compared to warm stratification and no germination was observed in seeds stratified for 40 days. Seed viability declined after 50 days of incubation irrespective of the stratification periods (Figure 1). Since embryos of the seeds require relatively high (non–cold stratifying) temperatures for growth, the species showed simple morphophysiological dormancy.

Underdeveloped embryos were observed in cold stratified seeds incubated at 24 ± 2°C for 10 to 40 days. Further, germinated seeds of *I. embelioides* did not exhibit a long (several-week) delay between emergence time of radicle and cotyledon and is a characteristic feature of seeds having non deep simple morphophysiological dormancy. This finding was similar to that reported in *Ilex amara* (Zamith & Scarano 2004), and *I. nitida* (Marrero 1949). The high germination of *I. embelioides* (>80%) when stratified at 25 ± 1°C, was similar to that observed in *Ilex aquifolium*, *I. glabra, I. montana* (Nikolaeva et al. 1985), *I. opaca* (Ives 1923; Barton & Thornton 1947), *I. verticillata* and *I. vomitoria* (Nikolaeva et al. 1985) and *I. maximowicziana* (Chien et al. 2011). Thus, warm stratification could break non deep simple morphophysiological dormancy.

The viability of the seeds declined with the storage period irrespective of how they were stored. However, seeds stored at 25°C with moist sand showed the highest viability percentage (54.72 ± 1.67%) even after 30 days. Whereas, seeds stored in airtight container

Table 2. Seed germination of *I. embelioides* on MS medium with different concentration of GA_3.

Concentration of GA_3 (mg L^{-1})	Light		Dark	
	Mean Germination (Days)	Germination %	Mean Germination (Days)	Germination %
1	45.44 ± 0.56 [a]	40.00 ± 1.92 [b]	46.67 ± 0.88 [a]	14.81 ± 0.98 [ab]
5	41.78 ± 0.80 [ab]	43.33 ± 1.93 [b]	44.11 ± 1.37 [ab]	12.22 ± 1.61 [b]
10	39.78 ± 1.24 [b]	65.56 ± 2.94 [a]	41.78 ± 0.62 [b]	18.15 ± 0.98 [a]
20	44.56 ± 0.78 [a]	35.57 ± 2.22 [b]	46.11 ± 0.97 [ab]	10.74 ± 0.37 [b]

For each treatment, means followed by the same letter in each column do not differ significantly at p <0.05 (Tukey test).

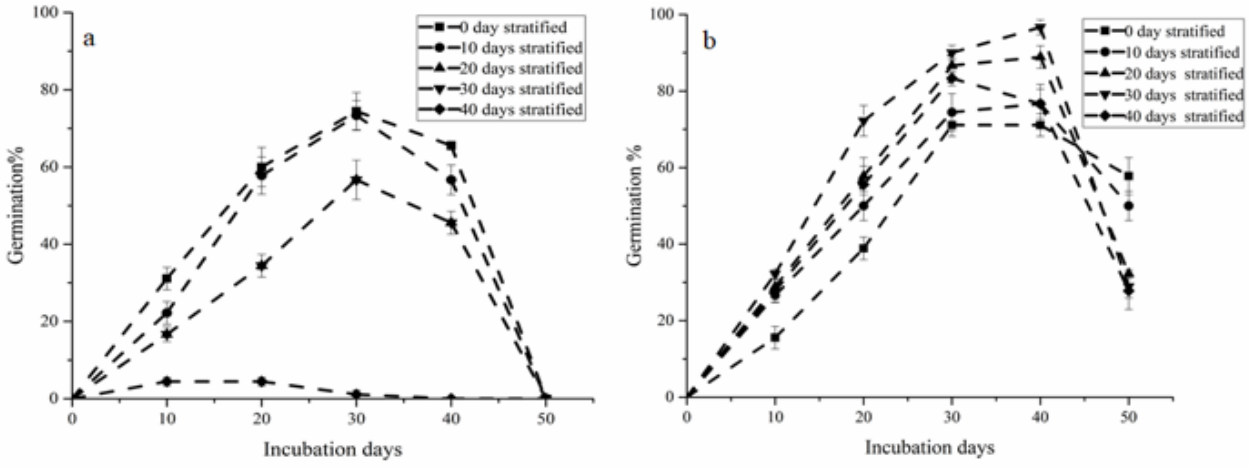

Figure 1. The effect of stratification on germination of *Ilex embelioides* seeds incubated at 25 ± 2°C for 50 days: a—cold (5 ± 1°C) | b—warm (25 ± 1°C).

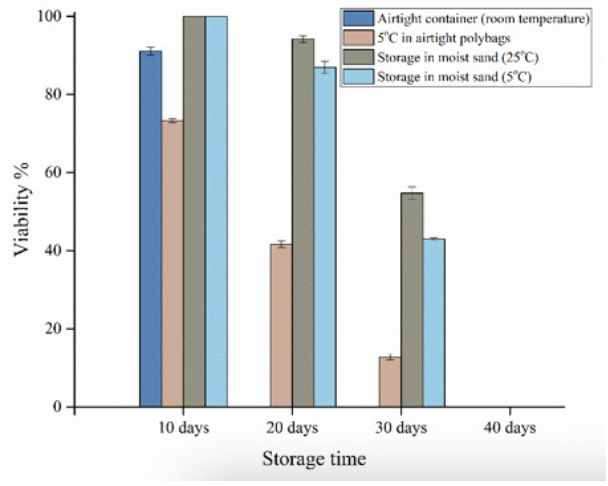

Figure 2. Viability percentage of seeds subjected to different storage conditions.

declined their viability to 91.11 ± 1.00% after 10 days and completely lost viability after 20 days. Viability of the seeds stored in air tight polybag at 5°C declined to 41.67 ± 0.83% and 12.78 ± 0.74% after the 20th and 30th day, respectively. Seeds stored in moist sand at 5°C remained 100% viable after 10 days but the viability declined to 86.94 ± 1.55% and 43.05 ± 0.28% after 20th and 30th days of storage. The comparison of viability percentage of *I. embelioides* seeds subjected to different storage conditions is presented in Figure 2. The low viability of *I. embelioides* revealed that the seeds should be germinated as early as possible.

CONCLUSION

Under natural conditions the low seed germination and high exploitation of *Ilex embelioides* poses a serious threat for its very existence. The seeds of *I. embelioides* exhibit simple morphophysiological dormancy which means the seeds have underdeveloped embryo and physiological dormancy. Application of GA_3 and warm stratification (25 ± 1°C) of seeds for 30 days in moist sand are recommended for germination. The in vitro

seed germination experiment has demonstrated that seed cultured on MS medium with 10 mg L^{-1} GA_3 is also effective for mass propagation. Though viability was high in seeds stored at 25°C in moist sand, the viability declined with the storage period. This indicates that the seeds should be germinated as early as possible. The suitable protocols for mass multiplication of the species developed (Image 1D) in the present study would help not only in reducing the germination time but also to obtain large number of seedlings for reintroduction of the species in its natural habitat.

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Journal of Threatened Taxa is indexed/abstracted in Bibliography of Systematic Mycology, Biological Abstracts, BIOSIS Previews, CAB Abstracts, EBSCO, Google Scholar, Index Copernicus, Index Fungorum, JournalSeek, National Academy of Agricultural Sciences, NewJour, OCLC WorldCat, SCOPUS, Stanford University Libraries, Virtual Library of Biology, Zoological Records.

NAAS rating (India) 5.64

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Journal of Threatened Taxa

www.threatenedtaxa.org

ISSN 0974-7907 (Online) | ISSN 0974-7893 (Print)

August 2023 | Vol. 15 | No. 8 | Pages: 23631–23826
Date of Publication: 26 August 2023 (Online & Print)
DOI: 10.11609/jott.2023.15.8.23631-23836

Publisher & Host

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