Eco. Env. & Cons. 30 (February Suppl. Issue) : 2024; pp. (S101-S108)

ISSN 0971–765X

DOI No.: http://doi.org/10.53550/EEC.2024.v30i02s.020

Effect of different pretreatments of *Aquilaria malaccensis* seeds for enhancement of germination rate

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(Received 1 July, 2023; Accepted 8 September, 2023)

ABSTRACT

A few plant species of the Thymelaeceae family are highly valuable and demanding because they contain resinous oil in their stems, branches, and roots. From this plant, resinous oil is extracted through the distillation process. Traditionally and from the religious point of view, the oil has a higher potential in the manufacturing of perfume, incense sticks, smoke, and many other medicinal and religious purposes. It is a highly economic plant, so its conservation is very important. Aquilaria plants are propagated through seeds and the seeds are recalcitrant, i.e., they are chilling sensitive and also cannot be stored at room temperature because of their high moisture content. They are also called desiccation sensitive. Below a certain level of moisture content, their embryos become death or inviable. Conservation of germplasm in the case of recalcitrant seeds is a major issue to be solved in the current situation. Our main focus was to enhance the germination of the *Aquilaria malaccensis* seeds for the early development of the seedlings within a short time. Here we are applying different pretreatments of Aquilaria seeds, such as Acetone for 1 min, 2 min, and 3 min, likewise Methanol, Ethanol, hot water, acetic acid, etc for enhancing seeds germination and it was found that highest germination percentage in the treatment where seeds are soaked in 1 min acetone (70%) in comparison to the control, which shows only a 56.7% germination rate. Their germination rate was determined by regular observation for up to 2 months, and the growth curve was also prepared by calculating the weight of the seeds. The findings of the current study will help to develop a protocol for early germination as well as mass propagation within a short period of time. The results of the Aquilaria species are significantly different from each other.

***Key words**: Aquilaria seeds, Conservation, Germination, Pretreatment, Recalcitrant*

Introduction

Aquilaria malaccensis, a member of Thymelaeceae family, widely grown and highly prized tree. In Aquilaria till now 21 species are found (The Plant List. 2013). International Union for Conservation of Nature (IUCN) classifies among these seven species are vulnerable (IUCN Red List). All of the species in the genus fall within this category, per Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (UNEP-WCMC 2015). Oleoresin is obtained from the tree's infected wood part using distillation process. Since ancient times, many people in Asia and the Middle East have utilized agarwood for the development and manufacturing of the perfume, for the creation of incense sticks, for medical purposes, and to produce aromatic smoke in various other cultural and

religious activities (Chaudhari, 1993). Since ancient times, agarwood oil has been extensively used in Arabian nations. According to some reports, agarwood oil is used to cure condition like paralysis, gout, rheumatoid arthritis, diarrhea and dysentery. This oil historically been used as antiasthmatic, aphrodisiac, astringent, cardiotonic and carminative agents to treat a verity of illness. Traditionally, the oil has been used for the treatment of several ailments like antiasthmatic, aphrodisiac, astringent, cardiotonic, and carminative agents. Additionally, A*quilaria malaccensis* possessed cytotoxic, antitrypanosomal, antibacterial, and antiallergic properties (Wagh 2017). North Eastern Indian states including Assam's Golaghat, Jorhat and Sivsagar District are home to the agarwood plants. The mature plant begins to bloom in April or May, and the fruits ripen in June or July then ready for dispersal. Aquilaria plants grew up to 20–40 m tall and had an overall diameter of 60 cm. The mature plant, *Aquilaria malaccensis*, has smooth bark with some white spots, as opposed to the young plant's light brown color with small hairs. Wood without resin has an internal structure that is white in color, light in weight, and soft, whereas resin-containing wood is hard, dark, and heavy. Aquilaria leaves have Alternate, elliptic or lanceolate, 3-3.5 cm broad, 6-8 cm long, and with 12–16 pairs of vein. Inflorescence can be found in axillary or terminal positions. Umbels can grow up to 5 mm long, are hermaphrodite, and are either white or yellowish-green in appearance. The Aquilaria tree produces green fruits with an oval-shaped capsule, a leathery exocarp that is 4 cm long and 2.5 cm wide respectively and also contain some short hairs. One fruit typically has two seeds, although occasionally there is only one. According to previous reports, 70.5% of fruits had two seeds, the other ones only had one, and different trees produced variable numbers of seeds. A solitary tree can yield roughly 5,092 to 16,660 seeds every year, depending on the age of the plant and the region in which it grows (Saikia and Khan, 2012). The seeds are ovoid in shape, light in weight, blackish-brown in color, and densely covered in microscopic red-brown hairs, depending on the tree's age. We received about 1500 seeds from one kilogram of Aquilaria seeds. Aquilaria is spread via seeds; however cuttings can also be used for mass propagation. Other vegetative propagation methods, such as tissue culture, occultation, and marcotting (air-layering), are also frequently used. When fruits of *Aquilaria malaccensis* reach maturity, they explode, releasing seeds that either fall to the ground or may be carried some distance by the wind due to their small size and light weight (Joshi, 1993). According to their physiological activity, orthodox and recalcitrant seeds, which are produce by angiosperm plants. The majority of angiospermic plants roughly 75-80% produce orthodox seeds and remaining 20-25% recalcitrant seeds. Aquilaria seeds are hardy, sensitive to cooling and cannot live in dried or frozen circumstances. Recalcitrant seeds continuously metabolize, lowering their moisture content below a threshold at which they quickly lose viability. Fresh seeds are vulnerable to microbial contamination due to their high moisture content and those recalcitrant seeds are drying sensitive, making it impossible to dry them below the essential moisture level. As a result, maintaining these seeds over the long term a very challenging undertaking. The preservation of recalcitrant seeds depends greatly on the moisture content of the seeds. Its germination is also impacted by variations in light and temperature. Once a year, typically during the monsoon season when it is hot and muggy, Aquilaria produces seeds. *Aquilaria malaccensis* has fruits with one or two seeds. Fruits split open and release seeds when they are fully developed and dry. If exposed to appropriate conditions, dehiscence seeds germinate right away, otherwise they lose their viability. The colour of the *Aquilaria crassna* fruit changes from green to brown as it ripen, allowing the seeds to fall to the ground and may be carried by wind (Jensen, 2002). The mature fruit contain oval, brownish black seeds that are between 0.5 to 0.8 cm in diameter (Koskela, 2002). A mature seed can remain on a tree for upto two weeks before losing viability (Jensen, 2002). Additionally, moisture content fluctuates, for example new seeds have a recalcitrant moisture content of 49 % and will desiccate to a maximum of 25 % with a 33 % germination rate. Even if there is a growing demand for agarwood on the global market our current supply is insufficient, so it is critical to expand mass cultivation of the plants (Rassol and Mohamed, 2016). In this case, facilitating seed germination is a crucial requirement for agarwood nurseries.

Materials and Methods

Seed collection

Mature fruits were collected from the CSIR-North

East Institute of Science and Technology (CSIR NEIST), Jorhat Assam NEIST Experimental Farm in the months of July and August 2019-2020 having geographical location 26.7378° N, 94.1570° E, and they were transferred to the laboratory in a poly bag for further studies. Seeds were recovered from the fruits and labeled separately. Fruits, as well as seed weight, length, breadth, and seeds per fruit, were measured and counted. Measuring the length of the fruit and breadth separately of each seed by using an Absolute Super caliper IP 67 (Made in Japan). Similarly, seed weights were measured by electronic balance (Sartorius-Reltec calibration Pvt. Ltd). The seeds were pre treated with different chemical and physical agents to observe germination percentage.

Pretreatments of Aquilaria seeds with different chemical reagents such as acetone, Methanol, ethanol, hot water, and acetic acid before sowing

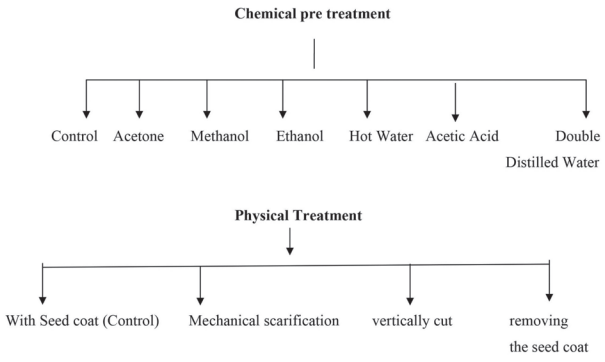
Fresh seeds were separated from fruits in room temperature (25 ºC at 60% relative humidity) and air dried at room temperature. These Seeds were properly washed in deionized water, dried between sheets of filter paper and then used in the current study. 25 numbers of seeds per replicate and 4 repetitions per treatment were used in all germination assays. On Whatman No. 1 filter paper circles moistened with 5ml of distilled water, then seeds were placed in 10 cm petri dishes. For germination in the incubator petri dishes were kept at 20±1 ° C. Radicle emergence through testa were recognized as germination. The seed germinated were recorded every 24 hrs for 60 days. After completion of the experiment the ungerminated seeds were tested for viability at 25°C by tetrazolium staining. All of the experiments were repeated at least twice and the averages were presented. Germination Index was calculated as germination per cent multiplied with root length after 17 days (GI = Germination per cent X Root length in mm).

Chemical treatment: Total 700 number of seeds was taken for chemical treatment. Seeds were immersed in acetone (Qualigen fine chemicals, Mumbai, India), Methanol (Merck India Ltd, Mumbai), ethanol (99%, Bengal Chemicals and Pharmaceutical Ltd., Calcutta, India), acetic acid (Spectrochem Pvt Ltd, Kalbadevi, Mumbai) and hot water (80°C) in a glass-stopper flask for 30, 60, 120 and 180 sec and double distilled water. Seeds were air dried and tested for viability at 25 °C by tetrazolium staining as per ISTA (1985) standard procedure.

Physical treatment

Mechanical scarification: For physical treatment, a total of 400 mature seeds were taken. For each treatment, four replications were considered, with each replication containing 25 seeds. Among them, 100 seeds were taken and broke the seed coat mechanically, then kept inside Petri plates containing moist filter paper for germination. All the mechanically scarified seeds were placed at room temperature and kept watered to make them moist and to help in germination. They were observed for up to 60 days and calculated their germination percentage. In other experiments, 100 seeds were taken and washed with distilled water, and the seed coats were cut vertically with the help of a sharp blade. Another 100 seeds were taken to remove the seed coat completely, leaving only an embryo, while the remaining 100 seeds served as controls. All the Petri plates containing seeds were kept for germination at room temperature. Seeds were planted in 8.5 cm diameter Petri dishes lined with moist Whatman No.1 filter paper and grown at room temperature (30± 3 ° C). In each plate, only 8–10 seeds are placed for germination. The plates were kept at room temperature and day-to-day observations were made for up to 60 days. All the seeds were placed at room temperature and kept watered to make them moist and to help in germination. They were observed for up to 60 days and calculated their germination percentage.

Diagrammatic representations of the different chemicals and physicals pretreatment of seeds



Determining seed germination percentage: Germination percentage of the seeds was calculated by the following formula

$$\text{Seed germination (\%)} = \frac{\text{Seed germinated}}{\text{Total number of seeds}} \times 100$$

Moisture content of the Seed: Seeds moisture content was determined by oven drying at 103±2° C for 17 hours. Each single seeds weight was taken separately for moisture content determination. The moisture content was determined by a percentage by weight (on fresh weight basis) is determined to one decimal place, by using following the formula

$$\%\text{age seed moisture content (mc)} = \frac{\text{M2-M3}}{\text{M2-M1}} \times 100$$

Where

M1 = Weight of the weighing bottle/container with cover in gm

M2 = Weight of the weighing bottle/container with cover and seeds before drying

M3 = Weight of the weighing bottle/container with cover and seeds after drying

{Note: The seed moisture determination must be done in two replicates, with precise weighing (i.e. up to three decimal places) using lightweight weighing bottles/containers.}

Seed growth curve: Seed growth curve measured with the help of taking a day to day weight of the seeds which were placed in the Petri plates above a moist filter paper placed for germination. 15 seeds were placed for this experiment for germination and the growth record was taken from day 1 up to day 10 days i.e. before the development of Plumule and radicle.

Statistical Analysis: Sine transformation was used for analyzing the data. It is an angular transformation and is appropriated for data on proportions, data obtain from a count, and data expressed as decimal functions or percentages. The mechanism of arcsine transformation is greatly facilitated by using a table of the arcsine transformation. Calculating means are significant at the 5% level. All statistical analysis was done by using ANOVA.

Results

The fruits of Aquilaria malaccensis were collected and measured its length and breadth. Two distinct fruit size were recorded with 26.82x17.6 mm and 30.30x19.09 mm length and breadth respectively (Table 1). After removing the pericarp from fruits, it was recorded that large fruits bears two seed and small one with one seed. Size and weight of the seeds were found almost similar in single and double seeded conditions. It was recorded that among the fruits, 70.5% of produces two seeds, while the rest produce one seed and is often considered as abnormal for the plant (Saikia and Khan 2012). However, we did not observe any differences in their germination rates, so both the seeds were considered healthy seeds for plant propagation.

Seed treatments were done with different chemical reagents at different time intervals shown in the Table 2. Treatment of seeds with acetone for 1 minute was found to increase seed germination up to 70% and was significantly higher than the control (56%). Two and three minute's exposure of seeds with acetone found significant decrease of seeds germination. However, seed treatment with ethanol for 1 minute showed slight increase of seed germination (60%) than control. Similar result of seed germination was obtained, when seeds were treated with methanol and acetic acid. Hot water treatment did not exhibit any superior result than control, but seeds treated with double distilled water show slight increase of germination percentage. But mechanical and physical seed treatment exhibited better germination of Aquilaria seeds. When seeds are scarified mechanically, the seeds germination was noted 65%, which is almost 10% more than the control. Seed coat creates problem in many seeds during germination. It seems seed coat is the problem of germination in Aquilaria seeds. It was found that when seed coat of Aquilaria seeds were cut vertically, the seed germination percentage rise to 64% (Fig. 1). It is easy for root primordia to come out without any barrier through the cut portion. The result of germination was recorded maximum (76%), when the seed coat is completely removed from the

Table 1. Measurements (±SEM) of the *Aquilaria malaccensis s* fruits and seeds. (N=100 Seeds)

Fruits of	Fruit		Seed		
A. malaccensis	Length (mm)	Breadth (mm)	Length (mm)	Breadth (mm)	Weight (gm)
One seeded	26.82±0.5104	17.60±0.219	15.26±0.208	5.34±0.079	0.113±0.001
	(23.49-30.45)	(13.76-17.04)	(14.9- 18.3)	(4.85-5.89)	(0.111-0.118)
Two seeded	30.30±0.9886	19.09±0.525	16.15±0.162	5.53±0.064	0.112±0.004
	(26.95-31.82)	(16.76-19.97)	(14.90-17.30)	(5.20-5.94)	(0.049-0.138)

seed (Fig. 2). Therefore, from the different treatment of seed germination it was found that acetone treated seeds for 1 minutes and physical removing of seed coat give better germination result of Aquilaria seeds.

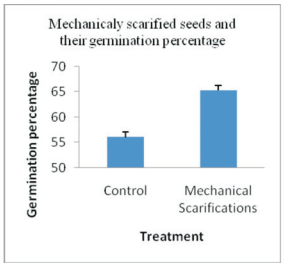


Fig. 1. Mechanically scarified seeds and their germination percentage.

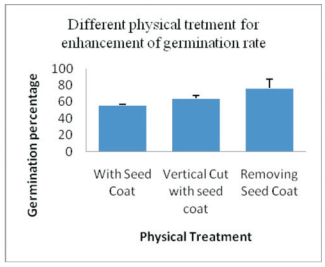


Fig. 2. Physical treatment in the seed coat

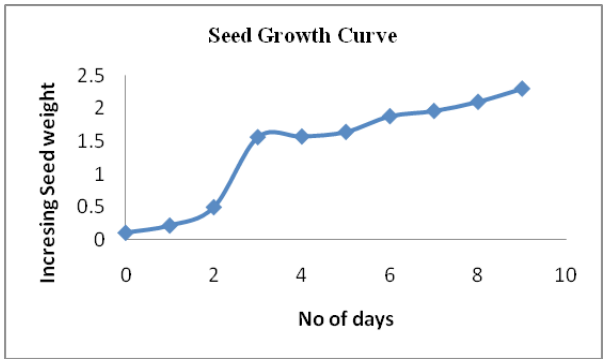


Fig. 3. Seed Growth curve

Seed growth curve was prepared by measuring the weight of the seed on a day-to-day basis up to the development of the radicle and Plumule. In the initial few days, seeds were in the dormant state and they started imbibitions. Some biochemical changes occur within the seeds at that time, and sudden increases in weight are found. Growth increased gradually, which is shown in figure 9. The process of seed germination involves several steps; the entire germination process can be divided into three stages: activation or awakening, translocation, and seedling growth. The plumule is the growing point of the shoot that emerges from the higher end of the embryo axis, on top of the cotyledons, and the radicle is the rising tip of the root that emerges from the bottom of the embryo axis. The epicotyls are the higher parts of the seedling stems and the area below the cotyledons are termed the hypocotyl. Two different types of germination are usually seen in plants as well as in the uncultivated plants among them. Epigeal germination is characterized by the seed germination in dicot plants and cotyledons that come above the soil surface, such as in beans, gourds, castor, tamarind, and onions, etc. For example, hypogeal germination is characterized by the seed germination in which dicot plants' cotyledons stay underneath the soil. Mango, custard apple, pea, gram, lotus and maize among other plants are example of where we saw hypogeal germination (Sharma and Srivastav, 2004). The fruits, seeds of *Aquilaria malaccensis* along with its emergence of radicle and plumule during germination are shown in Fig 10.

Discussion

The shelf life of the Aquilaria seed is very low; it was reported to be only 5–14 days (Devi *et al.* 2018) . On a fresh weight basis, the average seed moisture content was found to be 56.3%. In the earlier report, it was found that the germination rate of heavy seeds is faster in comparison to light seeds. In many tropical species, superior and quicker germination of heavier seeds has been reported (Devi *et al.*, 2018; Howe *et al.*, 1982; Dunlop *et al.*, 1983; Tripathi *et al.* 1990, Barik *et al.* 1996, Bhuyan *et al.*, 2000; Khan and Shankar, 2001; Shankar, 2006). Although it has been reported that some other species the seed weight may not be dependent on germination (Gross, 1986).

Table 2. Different soaking times and their germination rate (Germination percentage ±SEM)

Sl No.	Time Point	Control	Solvent					
			Acetone	Methanol	Ethanol	Acetic acid	Hot water	Double distilled water
1	30 Sec	56.6±3.8	-	-	-	-	63±1.92	-
2	1 Min	56.6±3.8	70±10	60±3.34	60±5.8	3.33±3	30±7.07	64±8.81
3	2 min	56.6±3.8	36±13.3	36±5.09	46±8.81	0	10±0	60±15.2
4	3 min	56.6±3.8	16±3.33	20±3.4	36±8.81	0	-	63±12.01

Before sowing the seeds, a sterilization technique is applied to reduce the contamination. Here we used different chemical treatments to enhance the germination rate. Seed germination was reported after 30 days of sowing (Tabin and Shrivastava, 2014). We found the highest germination percentage in the treatment where seeds are soaked in 1 min acetone (70%) in comparison to the control, which shows only a 56.7% germination rate. The germination percentage was found slight higher than the control when the seeds were treated with ethanol for 1 minute, followed by methanol for 1 minute, and the seeds soaking in hot water for 30 seconds. From earlier report it was observed that seed treatment of *Aquilaria agallocha* with normal water and hot water produced higher germination rate (90.18%), when the seeds soaked in hot water for 3 minutes and followed by keeping them in normal tap water for 24 hours (Das, 2015). We found zero percentage of germination in the treatment with acetic acid. Mechanically cut seeds show the highest and early germination rate in comparison to that of control. In our studies it was also observed that removing seed coat completely provide highest germination percentage, followed by vertical cutting and control (Figure 2). An Aquilaria seed has a hard seed coat, so it delays the germination and shows seed dormancy. Seeds soaked in double distilled water for 24 hours at room temperature showed 65% germination rate. Previous report showed that seeds soaked in concentrated sulphuric acid exhibit 45% germination rate (El-Juhany, 2009). The plant that is similar to Aquilaria is *Gyrinops walla*, which is an agarwood-containing species, and it is usually grown in Sri Lanka, confined to low country wet zone. Germination of seeds shows similarity with Aquilaria and its seeds start to germinate within 1 to 2 weeks after sowing. The seed viability declines drastically within a short period like Aquilaria. Similarly, germination percentage of juniper seeds was reported

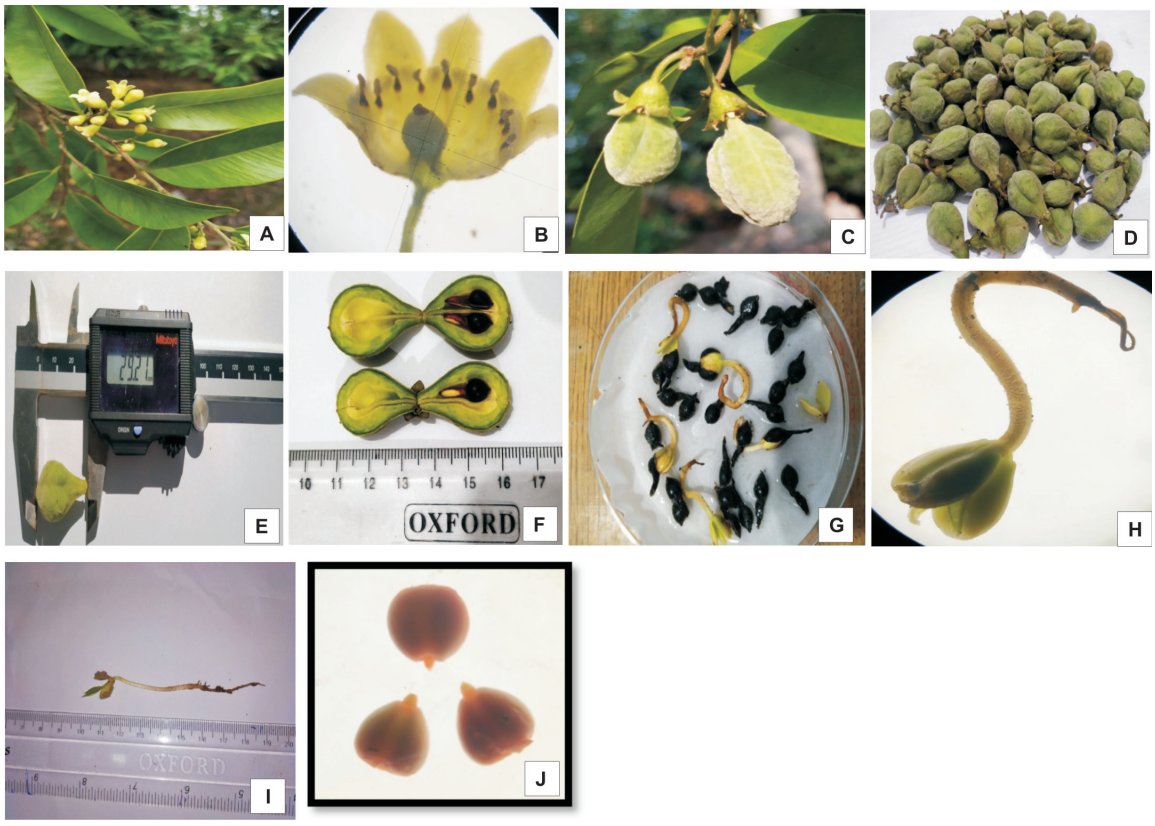


Fig. 4. A) Flower with twig; B) Internal structure of flower seen under microscope; C) Fruits of *Aquilaria malaccensis*; D) Fruits of *Aquilaria malaccensis* after plugging from the plant; E) Measuring length breadth of the fruits with vernier scale; F) Fruits showing one and two seeded taking their measurements; G) Seeds placed for germination in the petri plates with moist filter paper; H) Plumule and radical elongation; I) measurement the seedlings; J) Viable seeds after treatment with TTC solution seen under microscope

as 42.4 % in control and 85.7 % in scarified seeds (El-Juhany 2009). Short term preservation of plant germplasm is refractory seeds; artificial seed technology has been created. Recalcitrant seeds are found on the plant Aquilaria malaccensis so it is difficult to preserve the seeds. Recalcitrant seeds are seasonal and extremely drying sensitive loses viability with even a small decrease in the moisture content. Storage of the seeds was possible for up to 60 days at 4°C showing revival rate of 8.3% and for 50 days at 23- 24°C with germination rate is 16.7% (Devi *et al.*, 2018). Although it is a desiccation sensitive seed, storage may result in failure of viability and germination as well as slowing down the growth of saplings. Somehow, Seed storage and time have also influenced the seedling growth, so it was reported that freshly sown seeds show a better germination rate as compared to those sown after 5 days of storage (Alwis, 2016). When we measured the seed growth curve, after 0–2 days, slight changes are found in their weight. This phase is called the lag phase or establishment phase. After 2-4 days, they abruptly change their weight where we find a fast growth rate. This is considered the log phase. After 6–8 days, the rate of growth starts decreasing, so it is called the stationary phase. After that phase, seeds germinated and the emergence of radicle and plumule were seen. From radicals, roots will be developed and from plumule, shoots will be developed. When moisture content decreases, seed viability also decreases, the endosperm starts to shrink, and both the embryo and endosperm lose their water content and become non-viable (Shankar, 2012).

Conclusion

Agar is a promising economic tree for diversifying the agro forestry system in Assam, as climate of Assam, particularly upper Assam is favorable for its growth and development. One of the major challenges of its promotion is the scarcity of high-quality seedlings and mass propagation techniques. From our investigation, we can suggest that pretreatment of the seeds i.e. soaking with 1 min acetone, scarified seeds mechanically, soaking with 1 min ethanol, 1 min methanol, and seeds soaking with hot water for 30 min show the highest and early germination rate in comparison to those of control. As *Aquilaria malaccensis* is a highly economic and endangered plant, early germination technique within a short period is important and thus present investigation is relevant. Many households in upper Assam are engaged in commercial agarwood cultivation, so seed treatment with selected solvent, as described in the present investigation, will encourage people to cultivate and regenerate more plants for regaining agarwood industry and strengthening rural economy.

Funding

Authors are highly thankful to the Council of Scientific & Industrial Research (CSIR) and Government of India for sponsoring the project 'CSIR-Aroma Mission' (Project Code: HCP0007) and provide financial assistant to carry out this research work.

Acknowledgement

The authors acknowledge the help and cooperation received from the Director, CSIR- North East Institute of Science & Techenology, Jorhat Assam, India during conducting the research work.

Conflicts of Interest

Author declared no competing interest.

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