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Studies on the reproductive biology and seed biology of *Aconitum nagarum* Stapf: a threatened medicinal plant of North East India

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ABSTRACT:

Present study was undertaken to study the reproductive behaviours and seed biology of *Aconitum nagarum*. As per the present study, the species starts flowering from october first week onwards. The flowers are blue in colour, arranged as slender raceme, petals and filaments glabrous, carpel five and bisexual. The flowers bloom acropetally and anthesis was observed between 6.00 - 6.30 AM. Anther dehisced longitudinally from 7.00 AM till 9.30 AM. The number of anthers were found to be 49 per flower. It was observed that flower colour changes as the plant goes on fully dehisced. The flowering duration per flower varied from 4-6 days followed by fruit formations and matures within 10-15 days. The average flowers per plant varied from 8-28 and common pollinator was found to be bees. Mean seeds per plant was ~270-540 and pollen per anther was approximately 1000 - 2000. The seeds exhibited ~38% germination from seeds stratified at 4°C for 96 h.

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Aconitum nagarum, Floral biology, Medicinal plant, Reproductive biology.

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INTRODUCTION

Among 34 biodiversity hotspots of the world, India is a home for four of them extending to the neighboring countries – the Western Ghats/Sri Lanka, the Himalaya, the North-Eastern region and the Nicobar Island (MoEF, 2014). India is also one of the 17 mega biodiversity countries and has 26 recognized endemic centres that account for about one third of the flowering plants. North East India is a centre of mega-biodiversity and is equally rich in flora and fauna and contain more than one-third of the country's total biodiversity. The region is the home for number of plant species which are endemic to the region. But the populations of these economically important plant species are down sized in their natural habitats due to various factors including reproductive bottlenecks. Reproductive bottleneck includes failure of pollination, pre and post fertilization barriers leading to no or poor seed set, poor reproductive vigour due to inbreeding depression and low germination rate imposed constraints on the multiplication and survival of the species. Therefore, any conservation approach has to be based on an in-depth study of plant reproductive biology which provides information on the ability of seed germination, seedling viability, age of plant at which they reaches reproductive phase, reproductive longevity, seed setting ability etc., Such studies would provide fruitful insights in planning various programmes specific to different habitats (Silva and Silingardi, 2001, Abera *et al.*, 2008; Singh *et al.,* 2010). Studies on the phenology of medicinal plants are the basic knowledge to be obtained for the right season for collecting plants and propagules and for establishing the appropriate growth environment for propagation purpose (Moza and Bhatnagar, 2007, Abera *et al.*, 2008).

Aconitum nagarum Stapf. is an endemic medicinal plant of North-Eastern part of India and grows in grassy sloppy mountain (Figure 1a). The species is of great medicinal importance for its tuber (Figure 1b). The alkaloid produced from the tubers are used in curing wide range of diseases and also used as arrow poison. The diterpenoid alkaloids from *A. nagarum* have been isolated by different workers (Dong *et al.*, 2000, Zhang *et al.*, 2005, Ji and Wang, 2006). The alkaloids are used in the treatment of antipyretic, anti-rheumatic, paralysis and snake bite (Srivastava *et al.*, 2010). The species has also an antibacterial activity against several bacteria (Sinam *et al.*, 2012). Due to habitat destruction, over collection for herbal drugs etc., the species has entered into threatened category in their natural habitat. Besides different anthropogenic activities, decrease in the population size is also because of their abnormal reproductive behaviors. The present study was undertaken to study the reproductive behaviors and seed biology of this species.

MATERIALS AND METHODS

Floral biology

The study was conducted in Dzukou valley at Khonoma village, Nagaland, at an elevation of 2684 m above sea level (ASL) 25º36'44.8" N and 94º00'03.4" E and Shirui hills of Ukhrul district, Manipur at an elevation of 2427 m ASL, 25º 06' 39. 6"N and 094º 27' 13.3"E among the grassy bamboo slope. The reproductive phenology and floral morphology viz, time of budding, time of anthesis and stigma receptivity, different stages of anther development, anther dehiscence, fruit and seed setting etc., were studied. Floral phenology of *A. nagarum* at Dzukou valley, Nagaland and Shirui hills, Manipur were comparatively studied. In order to estimate flower production, total number of flowers per plant was counted manually in the selected plants. Seeds per pod were counted to quantify production of pods. Anther counts were done on randomly selected flowers. Pollen counts were made on 20 anthers from different flowers. The anthers were collected during the onset of blooming season successively for three years. The anthers were collected from the flowers and kept in moist cotton pad and

maintained in the polybag till they were brought in to the laboratory. The anther lobe was removed from the style with the help of forceps and blade and the anther lobe was put on a slide. The anther was smashed uniformly by adding a drop of glycerine and spread evenly. The slide was covered with the cover slip. The slide was kept under the microscope and pollen present in 10 microscopic fields were counted. Total number of pollen was determined by multiplying the pollen present in the mean microscopic field and total number of microscopic field per slide.

Distribution pattern of the plant and its associated species

The distribution of *Aconitum nagarum* in North-Eastern parts of India (Nagaland and Manipur) was taken into account for the comparative study of plant distribution pattern. A study was conducted to understand the rule of associated species on the growth, reproduction and survival of *A. nagarum*.

Seed biology

The plant and mature fruits were collected from the forest of Dzukou valley, Khonoma, Nagaland, India at an elevation of 2648 m ASL from the grassy bamboo slope. *Aconitum nagarum* reached peak flowering from the first week of October and seed setting starts from the second week of October. Therefore harvesting of seeds can be done from the third week of October. On contrary, in Shirui hills, Manipur, at an elevation of 2427m asl, peak flowering starts from the first week of november and seed setting was from the second week of november. During the second week of november few flowers were seen but are very rare. Matured seeds could be collected from the third week of november.

Seed collection and processing

For any study of this nature may be affected by various factors like seed collection technique, processing of seeds and post harvest method etc. In the present study, seed collection and processing protocol developed

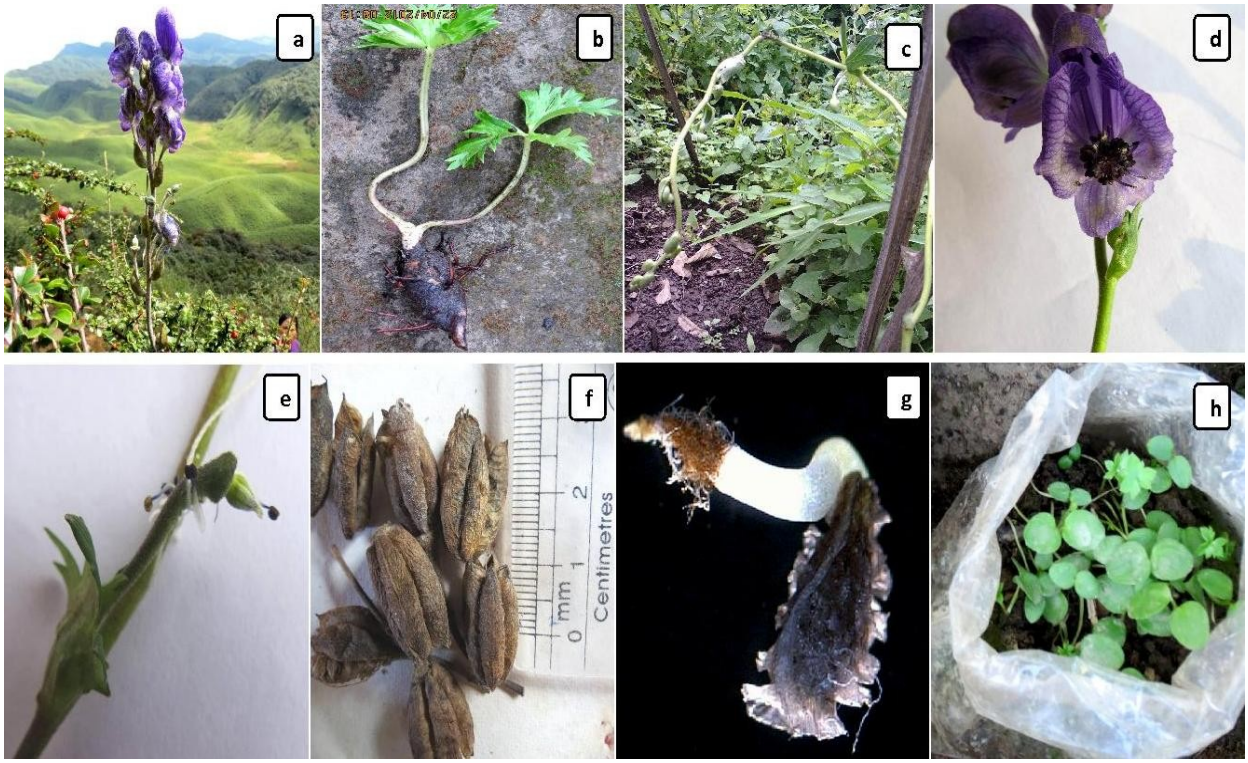

Figure 1. a. *Aconitum nagarum* plant growing in the hilly slope; b. Tuber of *A. nagarum*; c. Floral bud of *A. nagarum*; d. *A. nagarum* flower at full bloom; e. Immature fruits; f. Mature dry fruits; g. Germinated seed showing the radical and h. Rooted seedlings formed from the germinated seeds in the poly bag.

by Deb *et al.,* (2012) was followed with suitable modifications as per laboratory condition and experiment. In the present study, mature seeds were harvested randomly from the natural habitat during 2011-2013 along with the plant stalk. The stalks were wrapped in newspapers and covered with polythene bag and transported to the laboratory within 1-2 days. The collected fruits were dried by spreading uniformly over the old newspaper for 1-2 days in the laboratory at 25ºC. The dried fruits were removed from the stalk and seeds are taken out of the carpel. The seeds were then stored in poly bag in the laboratory for further experiments. The processed seeds were washed with 'Labolene' (1:100, v/v) (a commercial laboratory detergent) and rinsed under running tap water and finally with distilled water. The seeds were made into different groups for germination experiment.

Preparation of potting mix

The potting mix for the experimental purpose was made following Deb *et al.,* (2012) by mixing soil and chopped coconut coir at 1:1 ratio. The garden soil was crushed into fine powder, sun dried and mixed with the coconut coir in the ratio of 1:1 and put in a plastic pot and transparent poly bags. The poly bag and plastic pot were made perforated for better aeration. They were kept moist before sowing the seeds for germination.

Experimental process

The protocol developed by Deb *et al.,* (2012) was followed with suitable modification in the present study. A part of the seeds were processed and sowed immediately after the harvest while others were treated differentially at 4ºC in a refrigerator for 0, 24, 48, 72, 96 hours and sowed as described below:

1. A set of stratified seeds were sowed in filter paper in a humidity chamber of 90 mm in diameter and kept in a laboratory (25°C).
2. Another set of processed seeds (stratified seeds) were sowed in the potting mix and kept in an incubator at the constant temperature of 30°C.
3. While another set of stratified seeds were sowed in seed bed (poly bag) and maintained in a poly house.
4. To test the post harvest tolerance of the seed for various periods, the processed seeds were stored at 25ºC (in the laboratory) in sealed poly bags before they were sowed in the seed bed for seed tolerance experiment.
5. The seedling morphology and seedling mortality rate was also studied.

To study the emergence, survival and growth of seedlings of *Aconitum nagarum* under each condition, 4 replicates of 13 seeds each (for filter paper test, N=52 seeds/test) and 20 seeds each (for seed bed germination, N=80 seeds/test) were used. In each poly bag, the soil mixture was packed and 20 seeds were sowed. In filter paper test 13 seeds were sowed. The seed beds were watered at regular intervals. The experimental design was completely randomized. The data were collected daily based on seed germination, seedling morphology; seedling mortality; percent response etc. For the study

Table 1. Distribution pattern of *Aconitum nagarum* at different location of Nagaland and Manipur

Site	GPS Coordination	Altitude (mASL)	Distribution	Locality
Nagaland				
Southern Dzukou valley	N25 34 30.4, E 94 02 43.3	2400	Common	Valley and hill slope
Western Dzukou valley	N 25° 36 44.8, E 094 00 03.4	2648	Common	Valley and hill slope
Mount Saramati	N 26 2 26.7, E97 6 97 13	2000-3841	Common	Valley and hill slope
Japfu Hills	N 25 35 86.3, E 094 04 047	3020	Common	Hill slope
Manipur				
Shirui Hils	N 25 06 39.6, E 094 27 13.3	2427	Less common	Hill slope and top of hills
Dzukou valley	N 25 34.40.5, E 094 04 48.9	2550	Common	Valley and hill slope

the seedlings were maintained in the respective polybags and watered at regular interval and allowed to grow un till becoming normal plantlets. Once the seedling showed normal functioning like rooted plantlets, emergence of normal leaves etc., the seedlings were transferred to the poly house. Once the seedlings were established in the poly house, the seedlings exhibited differential growth and many seedlings died.

Filter paper test at room temperature (25ºC)

The seeds were treated at 4ºC in a refrigerator for different periods (0, 24, 48, 72, 96 h). The seeds were then placed on moist filter paper in a humidity chamber of 90 mm diameter and kept for germination at the laboratory (25°C). The seeds were kept moist throughout the study period. The germination process gets completed by the emergence of radical followed by leaf with seedling formation. A total of 13 seeds were used for each treatment with 4 replicates (N=52 seeds/ treatment). The seedlings were transferred to poly house for further seedling growth studies. The experimental design followed was based on Deb *et al*., (2012).

Germination test in incubator

The stratified seeds (stratified at 4ºC for 0, 24, 48, 72 and 96 h) were placed on potting mix to test the role of stratification on germination. Each treatment consists of four replicates with 20 seeds each (N=80 seeds/treatment). The differently stratified seeds were sowed in the potting mix and incubated at 30°C in the incubator. The seeds were monitored at regular intervals for seed germination, seedling morphology and seedling mortality etc.

Germination test in seed beds (poly bags)

The differentially stratified seeds were placed on potting mix in a perforated polybag of 150 mm in diameter. Each treatment consists of four replicates with 20 seeds (N=80 seeds/treatment). The plants are monitored regularly for germination. Germination percentages were calculated after eight weeks of seed culture.

Post harvest storage tolerance test for *Aconitum nagarum*

The seeds are stored at a temperature of 25ºC. The seeds were tested for the post harvest storage tolerance. Every month sets of processed seed were sowed in the seed bed. Their germination rate for each experiment was carried out and the results obtained were recorded monthly. The result obtained was compared and checked to see how far *Aconitum nagarum* seeds can tolerate storage. All the above experiments were based on the works reported by Deb *et al*., (2012) with *Cinnamomum tamala*.

Table 2. Floral display of *Aconitum nagarum*

Parameters	Observation
Inflorescence	Alternate raceme
Number of inflorescence/plant	8-28 nos.
Flower type	Hermaphrodite
Anthesis	6-6.30A.M
Mode of anther dehiscence	Longitudinal
No. of anther/flower	49
No. of pollen grains/anther	1000-2000
Stigma type	Pentacarpellary
Ovary type	Pentalocular
Seed	Obpyramidal, brown
Seed/plant	270-540 nos.
Root	Hearth shape, dark brown

Data are compiled from successive two years of study/observations.

Seedling mortality and seedling morphology

The seedling mortality was observed by transplanting the seedling grown from the differently treated seeds in the poly house and percent seedling survival was recorded till the plant mature or till its survival period which ever is earlier.

The transplanted seedlings in the poly house were also checked for changes/modifications in the seedling, which was observed after the transplantation of the germinated seeds (like modification in the leaf, roots etc).

RESULTS AND DISCUSSION

Associated species, floral biology and morphology

Present study was conducted in Nagaland and Manipur at the altitude between 2000 m to 3841 m above mean sea level (Table 1) in six different geographical areas. In all the study areas the populations were found in the hill slopes of the valley. In the present study, an interesting feature was observed as common associated species growing in all the study areas; they are, *Sinarundinella* species, *Gaultheria* species and *Fragaria* species**.** In all the areas *A. nagarum* was growing healthy where these associated species were available. This information on associated species could be used for the identification of new niches of this species for rehabilitation of the species.

In the present study, it was observed that the plants of *Aconitum nagarum* growing at Dzukou valley started budding from September second week onwards (Figure 1c) with peak flowering in October first week (Figure 1d). The flowers are blue in colour, in slender raceme, petals and filaments glabrous, carpel 5, and bisexual. The flowers bloom acropetally i.e. flower starts blooming from the base of the inflorescence to the tip of the inflorescence. Thus the fruits also mature acropetally. The anthesis was observed between 6.00 - 6.30 AM. Anther dehisced longitudinally from 7.00 AM till 9.30 AM. The number of anther was 49 per flower. During the study on floral phenology, it was found that there is a strong correlation between anther development and the stigma development (Table 2). The flower colour changes as the plant fully dehisced. The flowering duration per flower varies from 4-6 days followed by fruit formation. Fruits mature within 10-15 days. Fruit formation starts from the second week of October (Figure 1e) and by the third week of October, fruits mature and the plant dries up (Figure 1f**)**. While, in Shirui hills of Manipur at an elevation of 2427 m ASL 25º 06' 39. 6" N and 094º 27' 13.3" E peak flowering of *Aconitum nagarum* was observed by the first week of November and by the second week flowering decreases with the formation of fruits. By the third week, fruits are mature and the plants were all dried up. In local dialect of the Shirui village, it is known as the summer blue. The

Table 3. Difference in the floral phenology of *Aconitum nagarum* at Dzukou valley, Nagaland and Shirui hills, Manipur

Parameter	Dzukou valley	Shirui hills
Budding	September	October
Flowering time	October 1st week	November 1st week
Anthesis	6-6.30A.M	6-6.30 A.M
Seed setting	Oct 2nd week	Nov 2nd week
Seed maturity	Oct 3rd week	Nov 3rd week
Sprouting of plants	March/April	April/May
Duration of flowering	4-5 days	5-6 days
Altitude and GPS Coordinates of study area	2684m ASL, N 25º36'44.8 Latitude E094º00'03.4 Longitude	2427 m ASL, N 25º 06' 39. 6" latitude and E 094º 27' 13.3" longitude

Temperature during flowering at Dzuku valley-16°C
Temperature during flowering at Sirui Hills-17°C

number of flowers per plant varies from 8-28. The most common pollinator was found to be honey bee. Seeds per carpel varies from 9-13 i.e., mean seeds per flower was 45-65 and per plant was 270-540 (Table 3). Pollen per anther varies from 1000-2000 which means an average of 98000 pollen grains per flower. In the present study it was found that all the flowers to fruits ratio was not 1:1, it was 3:2 i.e., one third of the flowers did not support fruit formation. An average of 21.35 flowers developed per inflorescence while of the 21.35 flowers 13.65 flowers ended with fruit formation and remaining flowers did not form any fruits.

Seed biology

In the present study it was found that emergence of radicals from the germinated seeds, percent germination, morphology of seedling and seedling establishment are influenced by various factors. The light requirement of seeds for germination and seedling morphology appears to be species specific. Seedling survival on the seed beds/forest floor is governed by the availability of light, water and nutrients (Kitajima, 2007). The requirement of plant species differ greatly with reference to their habit preference, temperature requirement, and post harvest storage, specific pre-treatment for seed germination, seedling emergence and survival. Many plant species exhibit differential correlation with reference to vegetation cover and light requirements, temperature etc. (Kwit and Platt, 2003, Pages *et al.,* 2003). Storage containers have also great influence on the germination of seeds (Verma *et al.*, 2009). Seed treatment with chemical and low temperature enhances seed germination (Pandey *et al.*, 2000).

In the present study different techniques were adapted for seed germination. In the filter paper test, maximum germination was achieved from the seed

Table 4. Effect of stratification on the seed germination of *Aconitum nagarum* on filter paper (in a humidity chamber of 90 mm diameter)

Stratification period (h) at 4°C	% response (±SE)*	Types of plant response
0	67.00 (0.20)	Healthy roots
24	15.00 (0.25)	Healthy roots
48	38.00 (0.30)	Root healthy, hairy at the zone of maturation
72	15.40 (0.20)	Root healthy, hairy at the zone of maturation
96	15.00 (0.25)	Elongated healthy roots

* ±SE: Standard error from mean; Data represents the mean of three replicates.
Initiation of the roots was considered as breaking of dormancy.

Table 5. Effect of stratification on the seed germination of *Aconitum nagarum* in seed bed (Polybag)

Treatment type	Avg. time taken to germinate (days)	% response (±SE)*	Types of response
Without stratification	29	06.7 (0.2)	Healthy rooted seedlings
Stratified for 24h at 4°C before sowing	29	10.0 (0.2)	Healthy rooted seedlings with cotyledonary leaf
Stratified for 48h at 4°C before sowing	28	03.3 (0.3)	Healthy rooted seedlings
Stratified for 72h at 4°C before sowing	25	03.3 (0.1)	Healthy rooted seedlings
Stratified for 96h at 4°C before sowing	23	20.0 (0.2)	Healthy rooted seedlings with cotyledonary leaf

* ±SE: Standard error from mean; Data represents the mean of three replicates.
Emergence of the root was considered as breaking of dormancy.

Table 6. Effect of post harvest storage (at 25°C) on seed germination and viability of *Aconitum nagarum* on seed bed

Storage duration at 25°C (months)	Time for first sign of germination (days)	% germination (±SE)*	Types of response
0	10-30	6.7 (0.2)	Healthy rooted seedlings
1	30-60	6.5 (0.7)	Healthy rooted seedlings
2	60-90	6.0 (0.6)	Healthy seedling with stunted growth
3	90-110	5.5 (0.5)	Delayed germination
4	110-130	4.5 (0.6)	Delayed germination
5	130-160	2.2 (0.3)	Delayed germination with stunted growth
6	0	0	No germination

* Standard error from mean.
Data represent the mean of three replicate without any stratification.

stratified for 48 h at 4ºC followed by 96 h when maintained in the laboratory at 25ºC with minimum days taken to germinate. Under this condition 38% and 15.38% seed germination recorded after 13 days and 10 days of sowing respectively (Table 4 and Figure 1g). Seeds without stratification exhibited very poor germination (6.7%), on comparison to filter paper test then stratified seeds sowed on seed beds (prepared in poly bags supported better germination).

Seeds sowed in the poly bags exhibited 20% germination within 23 days from the seeds stratified for 96 h at 4°C (Table 5). But there was no seed germination recorded from the seeds maintained in the incubator at 30ºC across the stratification period. Seed germination was achieved within 29-30 days with emergence of roots with cotyledonary leaves while true leaves are formed within 58-60 days (Figure 1h). In the present study it was found that seed germination rate and germination time were greatly influenced by pre-treatment of seeds. There was significant difference in the germination period and germination rate with the stratified and non-stratified seeds. During the present study, investigation was carried out on the relationships among rate of cold stratification to determine whether the seeds require pre treatment of low temperature for germination. The finding in the present studies thus support that seeds of *Aconitum nagarum* prefer low temperature for seed germination. The lowest temperature tolerance by the recalcitrant seeds appear to be species specific (Fu *et al*., 1990, Oliveira and Valio, 1992, Barbedo and Cicero, 2000, Tommasi *et al*., 2006, Sharma and Gaur, 2012). Tommasi *et al.,* described that *Ginkgo biloba* seeds could be stored at 4°C for one year but when stored at 25°C, seeds died after six months (Tommasi *et al*., 2006). During the present study a similar response was recorded where seeds stratified at 4°C germinated better over seeds stored at 25°C.

Post harvest storage tolerance of seeds of *Aconitum nagarum*

Though, the seed preservation practices are as old as agricultural civilization but organized and systemic storage facilities have been developed only in the 20th century. There are over 1500 seed or gene banks present world over and accessions are increasing regularly. Longevity of seeds is not universal and is of species specific. After harvest the viability of seeds decline with time, seeds may exhibit differential germination and seedling morphology and field establishment (Walters, 2004). So, for some plant species, using relatively fresh seeds give superior germination over stored seeds.

During the present study, efforts were put to examine the post harvest storage tolerance of the seeds. The seeds were stored at 25°C for 0-6 months and seeds were sowed in the seed beds at one month interval (Table 6). Data collected in the present study exhibited gradual

decline in the germination response after one month of storage and from the third month, germination rate declined significantly. The germination rate declined from 6.7% to 4.5% in the fourth month and in the sixth month, there was no germination i.e., seeds lost viability completely The findings of the present study clearly showed that the seeds of *A. nagarum* are recalcitrant in nature. The seed in the natural habitat does not get the appropriate environment immediately after maturation for germination which in turn affects the seed propagation of the species in the natural habitat.

Seedling morphology and seedling mortality

The first sign of seed germination was the emergence of radical from the seed coat followed by a pseudo cotyledonary leaf formation. The cotyledonary leaf was replaced by a true leaf. The true leaf starts emerging from the base of the pseudo leaf. As the true leaf progress in size, the pseudo leaf turn yellowish in appearance and slowly wither giving way for the true leaf to succeed after 58-60 days of seed germination.

The seedling mortality was observed by transplanting the seedling in the poly house. Monthly basis survey was conducted to access the seedling mortality in the poly house. Seedling survival was very high till the month of April/May and starts decreasing by the last week of June/July. The growth of the plant became stunted. The seedling establishment was very low though seed germination was high in the forest as well as in the poly house.

CONCLUSION

The plant reproductive study is crucial for conservation strategies of this endemic plant. Present study suggests that the seeds of *A. nagarum* are of recalcitrant in nature and demand cold stratification prior to germination. For seed propagation of *A. nagarum,* one should take good care of the seed after collection to avoid desiccation and storage of seeds beyond 5-6 weeks.

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