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RESEARCH ARTICLE

Studies in the seed ecology and seedling phenology of *Andrographis paniculata* (Brum. f.) Wall. ex Nees in West Bengal, India

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Abstract

Andrographis paniculata (Brum. f.) Wall. ex Nees is an important medicinal plant of tropical Asia and has been used widely for a long time for different clinical ailments. Despite profuse flowering and a high percentage of fruit set, the number of seedlings in the natural population is the bare minimum. The knowledge regarding the seed ecology and seedling phenology of *A. paniculata*, is needed to formulate conservation strategies for the species. Therefore, a detailed study on seed ecology and seedling phenology has been conducted. During seed ecological studies, seed production, seed-set percentage, seed-ovule ratio, mechanism of seed dispersal, seed structure along with germination status, viability, dormancy and its breaking, and percentage of moisture content loss have been studied. Seeds exhibit some kind of dormancy [germination percentage (GP) = 28.51 ± 0.98, mean germination time (MGT) = 33.07±0.89 days, and germination index (GI) = 0.73 ± 0.08] which can be effectively overcome through acid scarification using H_2SO_4 and hot water treatment. The species shows epigealphanerocotylar germination. The hypocotyls exhibit rapid growth, and cotyledons emerged at 7.52 ± 0.27 TARA (Time After Radicle Appearance in days). Seedling is characterised by a thickened, hairy hypocotyl, a distinct collet, a pair of paracotyledons, and three pairs of eophylls in opposite decussate phyllotaxy. The studies on seed ecology and seedling phenology play a significant role in understanding how the shortening of the inception period enhances the germination status, and distinct seedling morphology provides the opportunity to formulate conservation strategies in their wild habitat.

Keywords

eophyll; paracotyledon; scarification; seedling phenology; seed dormancy; seed viability

Introduction

Plants are vital sources of different drugs (1). Therefore, *in-situ* conservation of plant species is an essential criterion for getting a continuous supply of drugs (2). One of the most fruitful ways to maintain their existence in nature is to study the details of their seed ecology and seedling phenology. Long-term existence is only possible when these plants reproduce sexually, produce seeds, have successful germination (3), and are kept safe during their initial stages of development (4). In flowering plants, the seed-ovule ratio is an important index to conclude their reproductive fate (5). Seed dispersal behaviour, dormancy period, germination ability, and the viability status of

a plant species are the important parameters that provide the opportunity to understand the species' seed ecology with maximum efficacy (6). Seed anatomy, especially the seed coat structure in many cases, is responsible for dormancy, viability, and moisture regulation (7). Therefore, all aspects of seed ecology are very significant in drawing a fruitful line from an existing one to the next (8).

Besides seeds, seedlings also suffer maximally, with the highest mortality rate within their entire life cycle of a sexually reproduced flowering plant (9). Seedlings of many taxa, mainly herbs, are so tiny that they lose our attention in wild habitats (8). Phenological studies of the seedlings are very helpful in identifying them in their miniature state. These characteristics are quite different from their adult ones in many cases and provide keys for systematic and phylogenetic studies (10). A typical seedling of dicotyledons comprises a primary root along with secondary, tertiary, and subsequent branches, a hypocotyl, a distinct collet, an epicotyl, a pair of cotyledonary leaves, and a plumular bud with following nodes and internodes, and eophylls if present. These characters are used in categorisation and identification. In the context of conservation and sustenance, seedling establishment and their care in their habitat represent the most crucial phase in fruitful regeneration.

Andrographis paniculata (Brum. f.) Wall. ex Nees is an important medicinal plant in tropical Asia, including India (11). Since ancient times, the plant has been widely used as a traditional medicine for several clinical purposes (12). The mature leaves are used to treat diarrhoea, fever, throat infections and many other infectious and chronic diseases (11). It is ethnobotanically used for snake bites, bug bites, dysentery, malaria, diabetes, skin infection, herpes, helminthiasis, and enteritis(13, 14).The different pharmacological effects and its uses have been reported by many researchers, such as anti-hepatitis (15), antihyperglycemic (16), antimalarial (17), anti-inflammatory (18), cardiovascular (19), antioxidant (20), hepatoprotective (21), anti-HIV (22), anti-cancer (23), immunostimulatory (24) and sexual dysfunctions (25). Despite its immense medicinal use, no attention was given to the seed ecology and seedling phenology of this medicinally important plant species. With such an idea, the present study was performed to portray aprecise scenario of seed anatomy, seed storage, seed viability, seed germination, and seedling morphology.

Materials and Methods

Fruit and seed collection

Mature fruits of *A. paniculata* were collected (on the 15th day of each month, October to December of 2016 to 2018 and January to March of 2017 to 2019) from the wild habitat of the Midnapore Sadar area (22.4309° N-87.3215° E) of West Midnapore district and the rural regions of Chandannagarsub-division (22.5153° N - 88.2147° E) of Hooghly district, West Bengal, India. Seeds were carefully isolated and then sun-dried (35 ± 2 ˚C) for two consecutive days, 5 hours each day. These sun-dried seeds with healthy conditions (visually recognised) were divided into several seed lots (each with 1000 seeds) and stored in Borosil glass vials in a airtight container. They were kept in laboratory conditions (27 ± 2°C).

Seed-ovule ratio and seed set

Seed production was studied based on fruits collected randomly in each month (October to March) for three consecutive years. The seed-ovule ratio was determined by investigating the number of ovules per flower by collecting the flowers (20 flowers in each month) randomly and an average number of seeds per fruit (flower). The seed set percentage was calculated by multiplying the seed-ovule ratio by 100.The weight of the seeds was measured based on seed lots (each with 1000 seeds).

Seed dispersal

The distance of dispersed seed at the time of fruit dehiscence was measured from the mother plant using a measuring tape in feet. For accurate measurement, the mother plants (N=20, in each year) were cultivated in artificial pots and kept in clean surface areas to recognise the minute seeds during dispersal. The data was recorded after continuous 1 hour observation with intervals of 2 hours on a particular day (10.00 a.m. to 5.00 p.m.) of each week (October to March) during the work. The distance of each dispersed seed for each fruit was measured from the mother plant. Finally, the data that were recorded for all the studied fruits were considered, and the mean value of seed dispersal distance was calculated.

Morphology and anatomy of seeds

The morphology of the seed was studied under a ZEISS Stemi 305 steriobinoculor microscope with an Axiocam 105 colour digital camera attachment. The detailed anatomical structure was worked out by sectioning, followed by the study under a Leica DMLB compound bright field microscope. Photomicrographs were taken with a Leica DFC 295 digital camera attachment.

Seed germination, dormancy and scarification

Seed germination experiments were performed as per the guidelines of the International Seed Testing Association (2015)(26). Before seed germination, seeds were surface sterilised with 0.1% (w/v) $HgCl_2$ solution for 90 seconds and then washed thoroughly with double distilled water to remove traces of $HgCl_2$, if any. These seeds were kept in double distilled water overnight, and then such imbibed seeds were sown on moistened filter paper with double distilled water in sterilised Petri dishes. The Petri dishes were kept at room temperature with diffused natural light. Germination status was recorded after each 24-hour interval in six replicates with 20 seeds in each set. Well-known scarification techniques such as hot water treatment, alternating hot-cold temperature treatment, and acid scarification treatment were employed (27). Seeds were subjected to hot water treatment with different temperatures (50˚C, 55˚C, 60˚C, 65˚C and 70˚C) for different periods (3 min, 5 min, 7 min and 10 min). Alternative hot water and cold water treatments: seeds treated with hot water in each said category were immediately followed by expo-

sure to cold water (30˚C) for another 5 min and 10 min. In acid scarification techniques, sulphuric acid (H_2SO_4) with different concentrations [12 (N), 16(N), 20(N), 24(N) and 30 (N)] [N = Normality] was used for variable time durations (2, 5, 7, 10, 12 and 15 min). Germination percentage and T_{50} value (time required for 50% germination) were calculated in different categories as per performed experiments. The Mean Germination Time (MGT) and Germination Rate (GR) were calculated using the formulae as proposed by Ellis and Roberts (1981), (28) as follows:

$$MGT = \frac{\sum(n \times d)}{N}$$

[n = no of seeds germinated on each day.

d = no of days from the beginning of the test.

$$GR = \frac{1}{MGT}$$

N = total no of seeds germinated at the termination of the experiment.]

The Germination Index (GI) was calculated as described by the Association of Official Seed Analysis (AOSA, 1983), (29). The formula is mentioned below:

$$GI = \frac{\text{No of germinated seeds}}{\text{Days of first count}} + \cdots + \cdots + \frac{\text{No of germinated seeds}}{\text{Days of final count}}$$

Loss of moisture content under storage

The loss of moisture content of seeds was measured from the difference in weight between the freshly harvested seeds and dry seeds. The weight of the seeds was measured based on seed lots, each with 1000 seeds. Seeds were kept in a LABARD Hot Air Oven for two days consecutively at 80 °C to remove the maximum moisture. The percentage of loss of moisture content with respect to fresh weight was determined as follows (30):

$$\frac{(\text{Weight of freshly harvested seeds} - \text{Weight of dry seeds})}{\text{Weight of freshly harvested seeds (before drying)}} \times 100$$

The loss of moisture contents of stored seeds was measured at an interval of 90 days up to 360 days since storage.

Viability of seeds under storage

A TTC (2,3,5-Ttriphenyl tetrazolium chloride) test was performed to analyse the seed viability status at 90-day intervals up to 360 days under storage conditions, and the same was also compared by performing a germination test after optimum scarification treatment. Freshly harvested seeds were scarified with 12 (N) H_2SO_4 for 10 min and then stored in Borosil glass vials in a tight air state. Such seeds were used to perform germination tests for up to 360 days at an interval of 90 days to analyse their viability under storage.

Seedling phenology

The phenological changes associated with seedling development of *A. paniculata* were primarily studied in the wild habitat of the Midnapore Sadar area (22.4309° N - 87.3215° E) in West Midnapore District and rural regions of Chandannagar sub-division (22.5153° N - 88.2147° E) in Hooghly District, West Bengal. Day-to-day (at 12.00 noon) detailed developmental changes were studied from the time after radicle appearance in days (TARA) from the seedlings grown naturally in the research garden of the Botany and Forestry Department, Vidysagar University campus, Midnapore. The individual seedlings were tagged and appropriately numbered to record their day-wise morphological changes. Observations were based on 300 seedlings for three consecutive years (2017-2019).To describe the architectural details of seedlings, we have used the terminologies mentioned by Hickey (1973) (31), Vogel (1980) (32) and LAWG (1999) (33). Photographs were taken using a Nikon D 5000 Digital Camera (Japan).

Statistical analysis

Raw data were statistically analysed by using the software Microsoft Excel (ver. 10) and SPSS (var. 16.0) to explore the Analysis of Variance (ANOVA) with Duncan's multiple range test [DMRT] ($P<0.05$).

Results

Fruit morphology and seed set

The fruit of *A. paniculata* develops from a two-celled ovary. Each locule contains six ovules attached in axile placentation. Fruits are simple, dry, and dehiscent (capsule) in nature, slightly flattened with two lobes marked by a central longitudinal depression. Mature fruits are dark brown, pointed at the apex, and narrowed at the base (Fig. 1a). Generally, the fruit contains 11 to 12 seeds, though it varies from 6 to 12 seeds per fruit. The fruits with six seeds are very rare, and seven seeded fruits were not found in this study. Though exceptionally few fruits (3.66%, N = 300) were found without any seeds. The overall seed production scenario per fruit was 10.10 ± 0.24 (Mean ± SE, N = 300). The weight of the seed lots (1000 seeds) of freshly harvested seeds varies from 1.69 g to 1.85 g with a mean value of 1.765 ± 0.01g (Mean ± SE, N = 18). Thus, the mean weight of individual seeds was 1.765 mg. The size of the fruits ranges from 16 to 29 mmin length with a mean value of 20.05 ± 0.37mm (Mean ± SE, N=120) and 2.5 to 3.0 mm in breadth with a mean value of 2.75±0.03mm (Mean± SE, N = 120) (Fig.1a).

Seed-ovule ratio and seed set

The number of ovules per ovary (flower) in *A. paniculata* is twelve, though the species produces 10.10 seeds per fruit. So, the seed-ovule ratio of the species per flower is 0.84:1, and the seed set percentage is 84.16.

Seed dispersal

The seeds are attached through the funiculus. The slender extension of the funiculus, i.e., jaculator (± 2 mm in length), remains adpressed with the seed laterally like a hook (Fig. 1b-c). This jaculator is responsible for the ballistic dispersal of seeds after the complete maturity of the fruit. Here, the jaculation mechanism is of the xerostatic type. The jaculation occurs after achieving the highest dry condition of the fruit, that facilitates the degeneration of the longitudinal cementing tissues of the fruit wall. At that

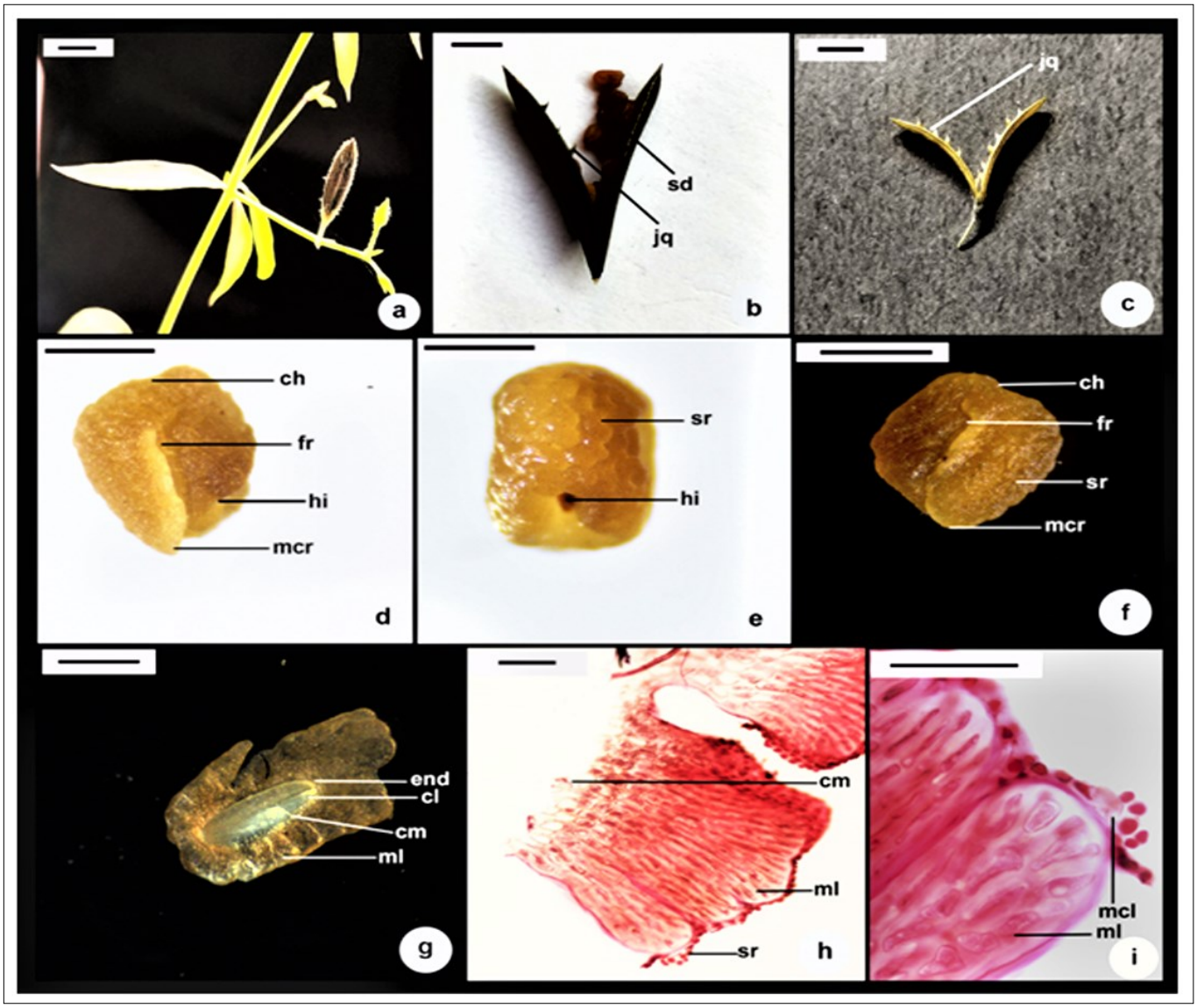

Fig.1. Fruit and seed structure of *A. paniculata*: (**a**) Mature fruit,(**b**) Dehisced fruit**, (c)** An empty fruit case showing the arrangements of jaculators, (**d**) Morphology of seed with deep furrow and somewhat pointed micropylar end, (**e**) Seed with deeply grooved hilum and rugose surface, (**f**) Seed with flattened chalazal end and notched micropylar end, (**g**) T.S. of seed showing thick seed coat with mechanical layer followed by crushed mesophyll, remnants of endosperm and centrally situated embryo, (**h**) Seed coat with thick mechanical layer and crushed meshophyll tissue and (**i**) Mechanical layer of seed coat with palisade like cells and surface with mucilaginous patches. [**sd**– seed, **jq**–jaculator, **ch**–chalaza, **fr**–furrow, **hi**–hilum, **mcr**– micropyle, **sr**–surface, **end**– endosperm, **cl**– cotyledons, **cm** - crushed mesophyll, **ml** - mechanical layer, **mcl** -mucilage. Scale bars: 5 mm (a-c), 2 mm (d-g), and 100 µm (h-i)].

time, the pressure generated by the jaculators within the fruit causes optimum stress that leads to fruit dehiscence in two valves with a cracking sound. These two valves are separated from the apex to the base through their peripheral wall, at the right angle to the longitudinal depression (Fig. 1b-c). The seeds are tiny, nearly compressed, light weight and disperse aerodynamically. The seeds were dispersed 2 ft to 7 ft from the mother plant by force generated by the jaculator. It is categorized into three types: low distance (2.14 ± 0.24 ft dispersed seeds), medium distance (4.27 ± 0.19 ft dispersed seeds), and long-distance (6.25 ± 0.21 ft dispersed seeds) (mean ± SE, N = 300).

Morphology and anatomy of seeds

This species develops the seeds from anatropous, unitegmic, and tenuinucellate ovules. At maturity, seeds are yellowish-brown in colour, 1.35 ± 0.02 mm (length) × 1.05±0.02 mm (breadth), almost rectangular, somewhat flattened, rugose, and deeply furrowed by two curved, unequally flattened portions. The narrower portion comprises the micropylar region, and the broader part includes the hilum region (Fig. 1d-f). The seeds have a profoundly grooved hilum and laterally placed notched micropyle, whereas the chalazal end is more or less flattened (Fig. 1e). The seed surface is rough and warty, devoid of waxy deposition but with discrete patches of mucilage (Fig. 1e-i).The sectional view of the seed showed a highly thick seed coat with a centrally situated embryo (Fig. 1g). Seeds are albuminous and exarillate. Anatomically, the seed coat is highly lignified and derived from the outer epidermis of the testa. This lignified tissue consists of thick-walled palisade like cells arranged in many layers (Fig. 1h-i).The endosperm is cellular. The embryo is straight with two distinct cotyledons (Fig. 1g).

Seed germination

The freshly harvested seeds of *A. paniculata* showed a meagre percentage of germination. The seed coat of the species is rigid and thick; after being sun-dried, it becomes more hardened. The rate of germination of such immediately harvested and sundried seeds in distilled water is meagre (26.66-30%) and also takes a prolonged inception

period for commencement (22.15 ± 0.81 days; mean±SE) and completion of germination (57.34 ± 1.42 days; mean ± SE). The average maximum germination percentage in three consecutive years is only 28.51 (Table 1).

Dormancy and scarification

We did not measure the germination percentage (GP) only; rather, we also analysed several other orthodox parameters of seed germination to evaluate the scarification efficiency, viz., the time required for 50 percent germination (T_{50} value), mean germination time (MGT), germination rate (GR) and germination index (GI). All these parameters were analysed statistically. The most significant effect ($F_{5,12}$ = 1.7688, P < 0.001, ANOVA in respect of GP) was shown by the 12(N) H_2SO_4. It not only increased the germination percentage but also significantly reduced the mean germination time (MGT=10.89, $F_{5,12}$ = 281.32, P < 0.001) and the T_{50} value (11.00, $F_{5,12}$ = 213.30, P < 0.001) indicates a huge shortening of the inception period. The most reliable parameter in respect of germination percentage, germination speed, and overall impact was analysed by the germination index (GI). The highest GI was usually shown by said one (GI = 8.78, $F_{5,12}$ = 74.34, P < 0.001) (Table 2). As per all these parameters, such ameliorating effect followed by 16 (N) H_2SO_4 for 5 min, 24 (N) H_2SO_4 for 2 min, hot water (60˚C) for 5 min and then hot water (50˚C) for 7 min, respectively (Fig. 2). These manifest the overall effectiveness of each treatment and their significant variation.

Table 1. Cumulative percentage of germination of freshly harvested unscarified seeds of *A. paniculata.*

Seed lot	Germination (%) in days						
	1-10	11-20	21-30	31-40	41-50	51-60	61-70
Set-I (2016-2017) [N= 90]	0	0	12.22	23.33	26.66	28.88	28.88
Set-II (2017-2018) [N= 90]	0	0	8.88	25.55	26.66	26.66	26.66
Set-III (2018-2019) [N= 90]	0	0	15.55	24.00	28.88	27.00	27.00
Mean ± SE [N= 270]	0 ± 0	0 ± 0	12.21 ± 1.92	25.18 ± 0.97	27.40 ± 0.74	28.51 ± 0.98	28.51 ± 0.98

SE - standard error

The freshly harvested seeds in normal conditions showed a maximum 28.51 ± 0.98% (mean±SE) of germination. Though the TTC (2,3,5-triphenyl tetrazolium chloride) test performed immediately after harvest of such seeds exhibited 95.92 ± 1.61% (mean ± SE) positive results that indicate the presence of viable embryos within these non-germinated seeds. This finding reveals that there was some sort of primary dormancy. Different scarification experiments were performed to overcome such dormancy. Scarification treatments with hot water showed some promising results. The two most effective hot water treatments were seeds pre-treated at 60 ˚C for 5 min, which exhibited 78.14 ± 1.96% (mean ± SE) germination and 50˚C for 7 min, exhibited 71.47±1.61% (mean ±SE) seed germination. Though hot water-cold water alternate treatments did not show satisfactory results. The most successful results came from the scarification treatments performed with Conc. H_2SO_4. Among the different treatments with conc. H_2SO_4, the most effective three treatments were 12 (N) for 10 min, 16 (N) for 5 min, and 20 (N) for 2 min. The seeds pre-treated with these three treatments showed 94.07 ± 2.25% (mean ± SE), 85.55 ± 1.92% (mean ± SE) and 79.25±1.33% (mean ± SE) of germination, respectively. The detailed results of all five most effective (germination percentage > 70%) treatments (two with hot water and three with conc. H_2SO_4) are graphically presented in Fig. 2. These scarification treatments increased the germination percentage most effectively and reduced the inception period significantly (Fig. 2).

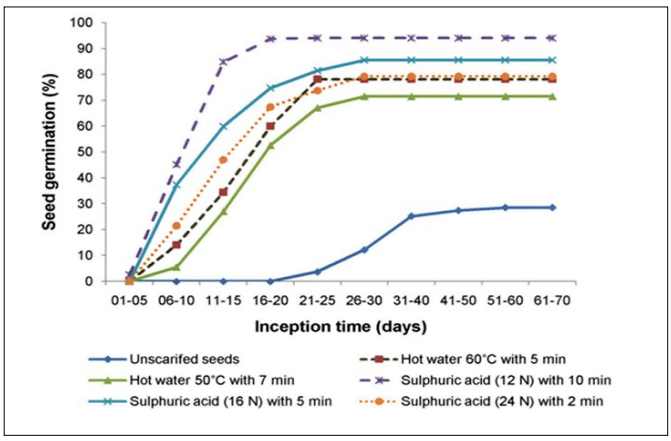

Fig. 2. Percentage of seed germination of *A. paniculata* after different scarification treatments in day wise performance.

Loss of moisture content of seeds under storage

Freshly harvested sun-dried seeds showed 8.20 ± 0.15% (mean ± SE), moisture, which decreased gradually during storage up to 180 days. After that, the moisture level of those seeds remained the same, and there was no further moisture loss. The maximum loss of moisture level, i.e. 3.07 ± 0.15% (mean ± SE), decreased in the first three months during storage. Then the rate of moisture loss was diminished. Only 0.99±0.08% (mean ± SE), of moisture was lost in the next three months. After that, the moisture level of such stored seeds remained unchanged (Fig. 3).

Viability of seeds under storage

The seeds immediately after harvest, treated with the best scarification treatment [12 (N) H_2SO_4 for 10 min], showed 94.07 ± 2.25% (mean ± SE), of germination. After 90 days of storage, such seeds showed 64.80 ± 3.03% (mean±SE), of germination. At the end of the 180 days of storage, the germination became 44.07 ± 3.23% (mean ± SE), and after 270 days, it became 31.10 ± 1.69% (mean ± SE). Finally, after 360 days of storage, the germination percentage of such seeds was 18.51 ± 0.98% (mean ± SE), only. The results clearly showed that the seeds exhibited a gradual loss of germinability with time under storage (Fig. 3). The result also justified the result obtained from the TTC test by counting the stained seeds.

Table 2. Mean value (Mean ± SE, N= 270) of different parameters of seed germination status in respect to different treatments. The different lowercase letter indicating the means (column wise) were significantly different by DMRT($p \leq 0.05$), among the treatments for the same parameter [column wise data analysis].

Treatment	GP	T_{50}	MGT	GR	GI
Control (Unscarified)	28.51^{e}±0.98	--	33.07^{a}±0.89	0.0302^{e}±0.00085	0.7317^{e}±0.08
Hot water (60˚C) for 5 min	78.14^{c}±1.96	18.66^{a}±0.33	16.26^{b}±0.17	0.0615^{d}±0.0063	4.8947^{cd}±0.13
Hot water (50˚C) for 7 min	71.47^{d}±1.61	19.66^{a}±1.61	17.47^{b}±0.32	0.0572^{d}±0.00104	4.2240^{d}±0.28
H_2SO_4 [12(N)] for 10 min	94.07^{a}±2.25	11.00^{d}±0.0	10.80^{e}±0.29	0.926^{a}±0.00255	8.7833^{a}±0.54
H_2SO_4 [16(N)] for 5 min	85.55^{b}±1.92	13.33^{c}±0.57	13.05^{d}±0.37	0.0767^{b}±0.00222	6.9080^{b}±0.32
H_2SO_4 [24(N)] for 2 min	79.25^{c}±1.33	15.66^{a}±0.88	14.68^{c}±0.42	0.0681^{c}±0.00198	5.5667^{c}±0.28

GP - germination percentage; **T_{50}** - time required for 50% germination (in days); **MGT** - mean germination time (in days); **GR** - germination rate; **GI** - germination index

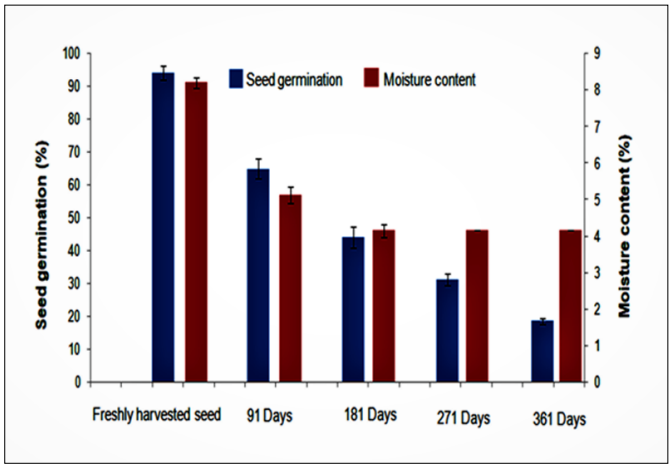

Fig. 3. Seed:Percentage of germination and percentage of moisture content of *A. paniculata* under storage conditions after 90 days of intervals.

Seedling phenology

After scarification treatment, the seeds took 4-5 (4.78±0.21) days for the inception of germination. The commencement of germination was marked by the emergence of a whitish, quite thickened radicle from the seed coat (Fig. 4a). The hypocotyl exhibited rapid growth that led to lifting the seed coat above ground level (nearly 3-5 mm; 4.12 ± 0.29). The germination pattern was strictly epigeal (Fig. 4b-c) and the entire cotyledons remained within the seed coat. The hypocotyl was relatively thickened, greenish in colour, had abundant whitish hairs, and often formed a bending appearance (Fig. 4b).

The greenish cotyledons started to come out by rupturing the seed coat through the deep furrow at 3-4 (3.45±0.19) TARA (time after radicle appearance). The fleshy green hypocotyl reached a height of about 5-7 (6.14±0.12) mm, and the embryonic root grew up to a length of 4-6(5.09±0.17) mm (Fig.4c). The cotyledons (2 mm × 1.5 mm), almost equal in appearance, come entirely out with the shedding of seed coats at 7-8 (7.52±0.27) TARA (Fig. 4d). This kind of germination pattern is mentioned as a phanerocotylar-epigeal type. The cotyledons were greenish with profuse hairs, fleshy, thick, and photosynthetic, a primary root had grown to 5-7 (6.26±0.22) mm, and hypocotyl reached up to 5 mm (Fig.4e). As the cotyledons were photosynthetic, they were considered paracotyledons. Such cotyledons are mentioned as epigealphanerocotylar with a foliaceous (EPF) nature. At 15-17 (16.25±0.41) TARA, the plumule was recognised as a minute projection (1 mm) at the flap of the cotyledons (Fig. 4f). Finally, the emergence of plumules revealed the completion of germination and, subsequently, the commencement of the seedling phase.

At 19-21 (20.31 ± 0.15) TARA, epicotyl differentiation occurred with the formation of the second node by the emergence of the first pair of foliage leaves (2 mm × 2 mm). The leaves are light green in colour, unequal in size, and arranged in opposite decussate phyllotaxy. Hypocotyl attained a 6-8(7.27 ± 0.31) mm with a greenish, hairy appearance and a thick, distinct collet (Fig. 4g). The cotyledons (5 mm × 4 mm) were still growing and exhibited somewhat unequal size, and the tap root grew up to 6-8 (7.31 ± 0.22) mm in length with five to nine secondary branches. At 25-28 (26.84 ± 0.41) TARA, foliage leaves (1st pair) reached 4 mm × 3 mm with the hypocotyl 7-9 (8.31 ± 0.34) mm in height. These unequal paracotyledons reached their maximum size, 10 mm × 8 mm (the larger one) and 9 mm × 7 mm (the smaller one) during 32-35 (33.21 ± 0.51) TARA and hypocotyl also reached their maximum height (12 mm). The paracotyledons were ellipsoidal, with entire margins, rounded-obtuse apex, an obtuse base, and a hairy surface with weak brochidodromous venation. At 37-40 (38.54 ± 0.37) TARA, the first pair of juvenile foliage leaves appeared slightly unequal and attained their sizes of 12 mm × 8mm and 9 mm × 6 mm with an epicotyl height of 7-9 (8.12 ± 0.29) mm. These juvenile foliage leaves were differentiated into a short petiole (2 mm) with ovate lamina, sinuate margins, obtuse to sub-acute apices, rounded base, glabrous surface, and brochidodromous venation. The second pair of juvenile foliage leaves appeared as a minute projection from the axils of the first pair at 41-44 (42.35 ± 0.61) TARA, with a height of 9 (8.26 ± 0.23) mm of the epicotyl (Fig. 4h). The overall shoot height reached up to 30 (28.75±0.71) mm, and the primary root increased in length to 45 (43.19 ± 0.47) mm (Fig. 4i). The paracotyledons remain attached and became yellowish, indicating their time of senescence**.**

At 45-50 (48.11 ± 0.71) TARA, both the paracotyledons dropped off in an interval of 2-3 days, and the epicotyl reached its maximum length (10 mm). The second pair of juvenile foliage leaves was also unequal in size, reaching 11 mm × 6 mm and 9 mm × 5 mm and arranged similarly in the opposite decussate pattern. The architecture of these leaves was precisely alike, as mentioned for the first pair.

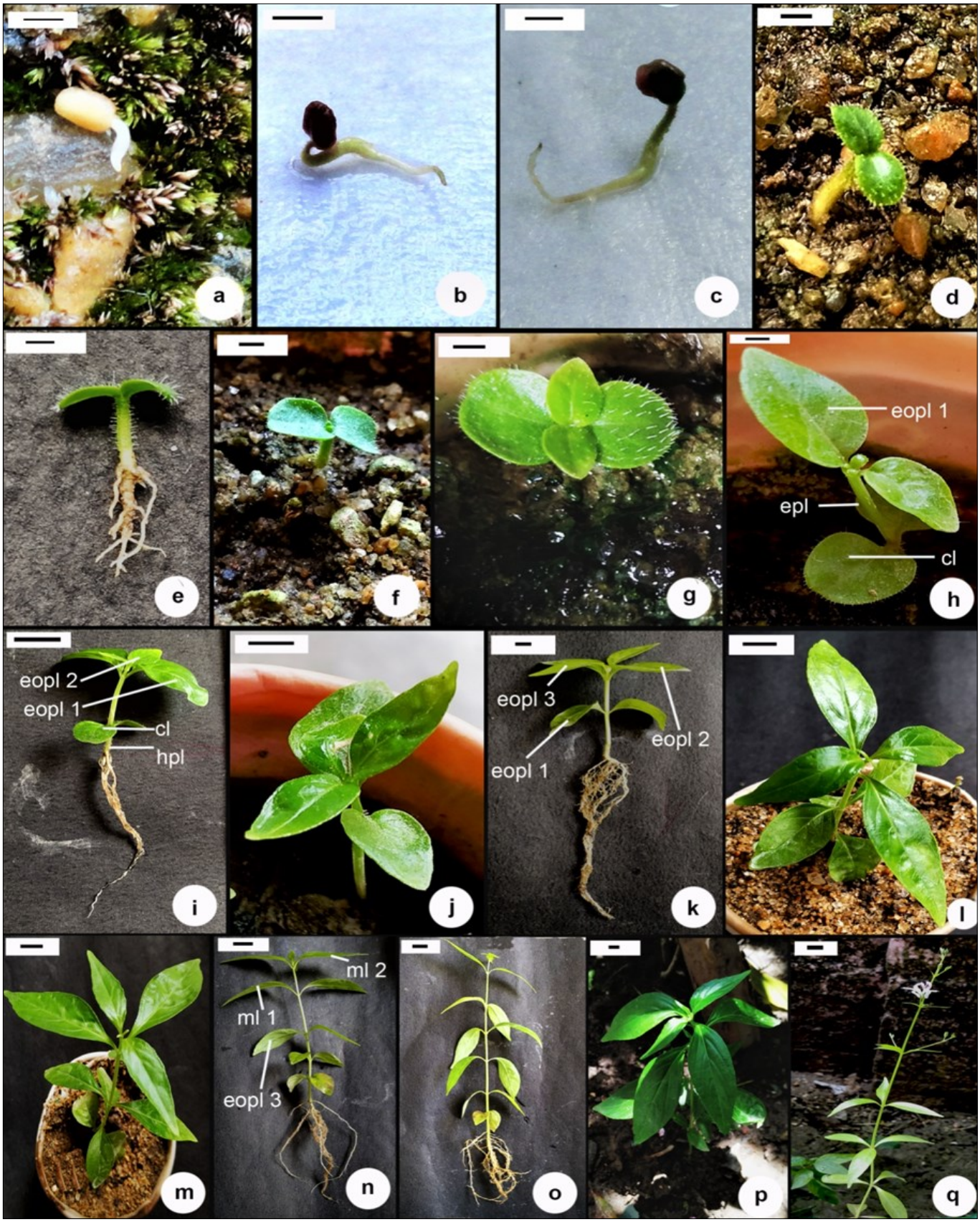

Fig. 4. Seed germination and seedling phenology: Seed germination and seedling phenology of *A. paniculata* through the 'Time After Radical Appearance [TARA]. (**a**) Seed showing emergence of radical,(**b**) Rapid growth of hypocotyl lifted the seed coat; (**c**) Seed coat with emerging cotyledons,(**d-e**)Seedling at 7-8 TARA exhibiting complete emergence of paracotyledons,(**f**) Seedling at 15-17 TARA with a recognizable plumule,(**g**) seedling at 19-21 TARA with first pair of eophylls, (**h-i**) Seedling at 41- 44 TARA showing emergence of second pair of eophylls in opposite decussate disposition and overall seedling growth,(**j**) A seedling at 48-51 TARA with the emergence of third pair of eophylls,(**k**) Seedling at 54- 57 TARA showing emergence of fourth pair of foliage leaves with overall root-shoot growth, (**l**) Seedling at 65- 68 TARA with emergence of fifth pair foliage leaves,(**m**) A seedling at 77- 80 TARA showing emergence of sixth pair of foliage leaves and maximum size achieved by fourth pair of foliage leaves,(**n**) Seedling at 86- 90 TARA with emergence of seventh pair of foliage leaves and overall seedling growth in respect of root-shoot length,(**o**) Seedling at 97- 102 TARA showing emergence of eighth pair of foliage leaves with the first pair of eophylls in almost senesced condition,(**p**) A seedling at 141- 157 TARA with the emergence of tenth pair of foliage leaves and abscission of all three pairs eophylls indicates onset of adulthood and (**q**) Flowering twig developed through seed germination with details record of seedling phenology showing heteromorphisim. [**cl** – cotyledon, hpl – hypocotyls, **epl** – epicotyls, **eopl 1-3**: - first to third pair of eophyll, **ml1-2**: - first and second pair of mature leaf. **Scale bars**: 1 mm (a-h), and 5 mm (i-q)].

The third pair of foliage leaves just emerged as a tiny leaf bud (2 mm) at the 48-51 (49.31 ± 0.51) TARA, and at the same time, the first pair reached its maximum size, i.e., 20 mm × 16 mm and 17 mm × 14 mm (Fig. 4j). The heights of the first (between 1st and 2nd pair of juvenile foliage leaves) and second (between 2nd and 3rd pair of juvenile foliage leaves) internodes were 9-11 (10.12 ± 0.14) mm and 1-2 (1.08 ± 0.08) mm, respectively. At 54-57 (55.45 ± 0.61) TARA, the fourth pair of foliage leaves was come out (1 mm) and the third pair attained 12 mm ×5mmin size, where both leaves appeared almost in equal size, noticeably quite different from the first two pairs. The architecture of the third pair of leaves was also somewhat different from the first two pairs, as leaves were ovate-oblong in shape, sub-acute apices, margins slightly undulating, surface glabrous, and weak brochidodromous venation. The third pair of leaves was considered transitional between the juvenile foliage leaves (1st and 2nd pair) and the adult foliage leaves (4th pair and so on). At this stage, the total shoot length reached 45-51 (48.15 ± 0.72) mm though the primary root grew more rapidly and achieved a length of 82-91mm with five to eleven secondary branches (Fig. 4k). The second pair of juvenile foliage leaves reached their maximum size of 26 mm × 18 mm and 22 mm × 16 mm, and the first internode also attained its maximum height (19 mm).

At 65-68 (66.41 ± 0.41) TARA, the fifth pair of foliage leaves just emerged (2 mm), and at the same time, the third pair of foliage leaves attained their maximum size (36 mm × 14 mm) (Fig. 4l). The second internode also reached its maximum height, 25 mm. The fourth pair of foliage leaves has grown to the size of 38 mm × 14 mm. Morphologically, these leaves (4th pair) were distinct from the juvenile ones in terms of shape (lanceolate), apex (acute), margins (entire), base (cuneate), surface (glabrous), and venation (reticulodromous). At 77-80 (78.54 ± 0.55) TARA, the sixth pair of leaves just emerged from the axils of the fifth pair. The 4th pair of leaves reached their maximum size (41 mm × 15mm), and the third internode (between the 3rd and 4th pairs) also attained its maximum height (35 mm) and between the 4th and 5th pair internode increased in length up to 15 mm. The 1st and 2nd pairs of juvenile foliage started to turn yellowish, indicating their onset of senescence (Fig. 4m). The total shoot height reached 120-125 (123.14 ± 0.84) mm, and the primary root grew to 95-100 (97.16 ± 0.75) mm. In this stage, we observed the more rapid growth of the shoot than the root, just opposite to initial seedling growth.

At TARA 86-90 (88.42 ± 0.56), the emergence of the seventh pair of foliage leaves took place, with the attainment of shoot height 142-150 (146.52 ± 1.14) mm and the primary root increasing up to 102-110 (107.16 ± 1.08) mm with profuse secondary and tertiary branching (Fig. 4n). The fifth pair of leaves achieved a maximum size of 50 mm × 15 mm, and the 4th internode also attained its final length of 40 (38.42 ± 0.57) mm. The architecture and arrangements of the successive fifth and sixth pairs of leaves were precisely identical to those of the earlier fourth pair. The eighth pair of leaves just emerged on 97-102 (100.29 ± 1.24) TARA, with the overall increase in shoot length 161-167 (164.82 ± 1.31) mm and root length 106-112 (109.57 ± 1.14) mm (Fig. 4o). The sixth leaf pair achieved its final size (52 mm × 15 mm), and the fifth internode also attained its maximum height to 44 (42.39 ± 0.43) mm, and the sixth and seventh internodes were 14 (12.72 ± 0.37) mm and 2 mm, respectively. The first pair of foliage leaves is still attached, though almost in senesced condition. At TARA110-115 (113.14 ± 1.21), the ninth pair of foliage leaves just emerged, and the shoot length reached up to 175-184 (180.35 ± 1.42) mm, and the emergence of the tenth pair of leaves took place at 124-128 (126.35 ± 1.31) TARA associated with the abscission of the first pair of juvenile leaves (eophylls). Within this period, the sixth and seventh internodes reached their maximum length of 45 (43.41 ± 0.53) mm and 47 (45.39 ± 0.41) mm, respectively, and in a few seedlings, branches started to come out from the axils of the fifth node onwards. At 141-157 (150.43 ± 1.72) TARA, along with subsequent growth of the tenth pair of foliage leaves, abscission of the second and third pairs of eophylls was observed, and finally, the plantlet achieved adulthood (Fig. 4p). It denoted the completion of the seedling phase and the onset of the adult phase. The interesting observation in the present work is that few seedlings (3.36 ± 1.20%, mean ± SE, N= 300) developed three leaves from each node beyond the cotyledonary node, and those leaves were arranged in whorled phyllotaxy (Fig. 4q). This is a significant observation marked as seedling heteromorphism.

Discussion

A. paniculata has been traditionally used as an important medicinal plant in India, including West Bengal. It is now widely used in drug isolation for several clinical treatments. However, plant species are currently very limited in a managed landscape. The species' reproductive success is very satisfactory regarding the floral display, fruit set, and seed set. Thus, we undertook a detailed study on seed ecology and seedling phenology to map the ambiguity that creates a severe threat to existence.

Though *A. paniculata* showed massive flower production for several months with a steady-state flowering pattern, it ultimately led to moderate fruit set and seed set. The seed set outcome (84.16 %) was quite satisfactory, but seedling development in the natural population is almost negligible. The wild population's seed-ovule ratio (0.84:1) also does not indicate substantial reproductive failure. The mature aerodynamically suitable seeds were dispersed mainly through jaculation, like many other members of the family Acanthaceae (30). The species precisely follow the xerostatic ballistic dispersal mechanism (34). This dispersal mode may create gathering in limited areas (35) with a high mortality rate (36). Though seed dispersal is entirely independent of any vectors, there is massive competition with the parents and/or siblings for seedling establishment (37). Seeds are typically acanthaceous in nature, with thick, lignified seed coats made up of several layer palisade like cells that are exotestal in origin (38). The surface of seed coats is warty, with discrete mucilaginous patches derived from the pulp. The additive structure

of those seeds referred to them as 'diaspores' rather than seeds (39). Due to a thick mechanical layer over the seed coat, the seed germination percentage was low (28.51 ± 0.98) with a prolonged inception period (40). To overcome the germination difficulties, different scarification treatments were performed. The most effective treatment (GP > 75.00) was by scarification using sulphuric acid (Conc.) as recorded in other seed species by different workers, such as in *Hyphaene thebaica* Mart. (41) and pre-treatment with hot water. The most significant effect on seed germination status was shown by seeds treated with conc. H_2SO_4 (12 N) for 10 min followed by 16 (N) for 5 min and 24 (N) for 2 min, and hot water treatment also showed a significant effect. Hot water pre-treatment positively ameliorated seeds with tough, impermeable seed coats by increasing water and oxygen permeability (42). Similar observations were also recorded in different plant taxa viz. *Acacia nilotica* (L.) and *Leucaena leucocephala* Lam. (de Wit) by Duguma *et al*. (43) and also in *Atropa belladonna* L. percentage of seed germination enhanced from only 26% to 82.5% through different temperature treatments reported by Genova *et al*. (44).

Seeds stored under controlled conditions also lose their moisture content gradually up to the sixth month (4.05%), then remain unchanged. The discrete mucilaginous patches on the seed coat play a significant role in moisture regulation under storage conditions (31). Similarly, the germination percentage of stored seeds decreased gradually for up to one year. However, the maximum reduction in germination was found during the first six months (50%), which was later reduced only by about 25.56% for the next six months. The result indicates a gradual loss of seed viability under storage conditions. Therefore, we may say that the germination percentage, which indicates the viability status of seeds, and their moisture content under storage conditions, are two parallel phenomena, and there is a clear positive correlation between them (Fig.5) (45).

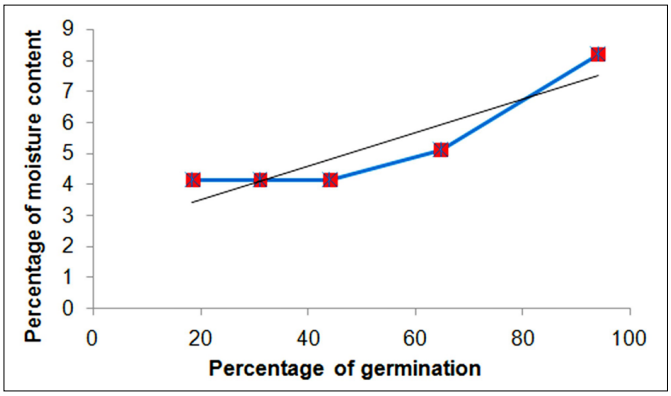

Fig.5. A correlation between germination percentage and moisture content of seeds under storage condition of *A. paniculata.*

The loss of moisture content associated with the loss of viability under storage was recorded by Ellis *et al*. (1991) (46). It is usually mentioned as the third category of seeds, an intermediary between conventional orthodox seeds and recalcitrant seeds. The initial loss of moisture content immediately after seed harvest did not affect their germination or viability, but the loss of moisture content below the threshold level exhibited immense effect.

A. paniculata exhibited a phanerocotylar-epigeal type of germination with very significant phenological developments during its seedling phase. Such a type of germination pattern is considered an advanced characteristic of the plant species (47). Phanerocotyly is an important characteristic of herbaceous, small-seeded plants with scanty endosperm. The species with phanerocotylar development exhibits more seedling growth than any other type when exposed to incident light. The seedling was very distinct from its adult one by a pair of green, fleshy, thick, hairy, sessile paracotyledons (EPF). The EPF seedlings revealed a wide range of morpho-physiological characteristics that favour their survivability as an understory. Most interestingly, the paracotyledons were unequal, i.e., isocotylar in nature (32). These cotyledons were truly photosynthetic and retained for 45 to 50 (48.11 ± 0.71) days from the onset of the seedling phase, and within this period, the 2nd pair of foliage leaves also developed. It indicates the role of the paracotyledons in the initial establishment of the seedlings. The species marked by heteroblastic development has three pairs of eophylls. Eophylls were morphologically very distinct from the adult foliage leaves by their ovate lamina, sinuate margins, obtuse to sub-acute apices, rounded base, glabrous surface, and brochidodromous venation. These observations distinctly signify the heteroblastic development of the seedling species. The species with EPF and heteroblastic development are conventionally marked as gap-dependent species, and similar observations were recorded in several plant species belonging to different families, viz., Lamiaceae, Moraceae, Rubiaceae, and Melastomaceae (48). Seedling morphology is a stable and reliable characteristic feature for identifying plant species at the juvenile stage (38), though in rare instances, heteromorphism was observed. In this species, we got seedling heteromorphism in a significantly lower percentage (3.66).

In the end, we get several intimations of seed ecology, viz., a limited range of seed dispersal (2 to 7 ft), a meagre percentage of seed germination (28.51), and a prolonged inception period (22.51±0.81 days). The low percentage of seed germination leads to a low percentage of seedling development. And such a long inception period suffers from the unavailability of water as the time of seed set is mainly in the winter season. In this part of the country, rainfall is significantly less. If those seeds are kept intact for the next rainy season, they lose their viability by almost 50%. Restricted dispersal also causes massive loss of seed germination and a high percentage of seedling mortality (36). In wild conditions, we observed that the seedlings are very tiny in size and enclosed by many types of grass or other herbaceous species that lead to the loss of the population either by herbivores or anthropogenic activities. So, we can conclude that suitable scarification [12(N) sulphuric acid for 10 min] treatments of freshly harvested seeds significantly increased the seed germination percentage (94.07) and also maximally reduced the inception period by 4-5 (4.35 ± 0.44) days. Introducing such treated seeds into the different landscapes may give us a fruitful result in the restoration and reintroduction of wild populations. Recently, the controlled placement of effec-

tive seeds and seedling establishment into a natural habitat or managed habitat have been used as the most successful tool for plant species conservation (49). Seedling morphology with distinct, unique characters allows one to obtain at least some sort of attention in their natural habitat. The knowledge of seedling phenology is vital for their sustenance in wild habitats, especially in tropical rain forests with highly mixed and dynamic species compositions. So, the present study formulates a way of sustaining *A. paniculata* through regeneration, reintroduction, and successful establishment of the seedlings.

Conclusion

A. paniculata exhibits satisfactory reproductive success, though it has limited occurrence in wild habitat. This is mainly due to very low seed germination with prolonged inception periods. Thick, hard, and lignified seed coats mostly imposed such dormancy. This is overcome through acid scarification (Conc. H_2SO_4) and hot water treatment. It also shortens the inception period. Ultimately, these treatments may be used to enhance seed germination status, leading to the development of many seedlings in wild habitats to mitigate our demands. Another approach is to identify the plant species in its seedling phase by its juvenile characters. It gives the plant more attention in their wild habitat to protect them from herbivores and adverse anthropogenic activities. So these are the fruitful findings that may be used to enhance the *A. paniculata* population in wild habitats.

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Authors' contributions

AK did the material collection, field data collection (Chandannagar locality, West Bengal, India) and processing, laboratory experiments, taking photographs, figure drawing and writing the manuscript. SB collected the field data (Midnapore locality, West Bengal, India) and performed laboratory experiments. UL did the microscopic analysis, statistical analysis and manuscript editing. PK conceived the idea and designed the experimental work, manuscript writing, final editing and endorsement. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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