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REPRODUCTIVE BIOLOGY OF *EMBELIA RIBES*: AN IMPORTANT MEDICINAL PLANT OF THE WESTERN GHATS

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ABSTRACT
Embelia ribes is a climbing shrub belongs to the family Primulaceae and is distributed in warmer regions of northern and southern hemisphere. *Embelia ribes* commonly known as 'VayuVidang' is an important medicinal plant used in different traditional systems of medicine in India. The fruits of *Embelia ribes* contain Embelin and other high valued secondary metabolites which have a wide range of clinical applications. The plant was located in forest areas of Ponmudi of Thiruvananthapuram district which comes under Agasthyamala Biosphere reserve and carried out reproductive biological studies. A comprehensive study on reproductive biology of this plant has not so far been made. *E. ribes* is polygamodioecious with low male and female ratio is 1:5. Honey bees, wasps, stingless bees and butterflies are the major pollinators. Seeds are dispersed by birds and remain dormant for 15-30 weeks. Natural regeneration is very poor due to over exploitation and low percentage of seed germination.

KEY WORDS: Embelia *ribes*, honey bees, Ponmudi, Polygamodioecious, pollinators.

INTRODUCTION

India has been recognized as one of the 17 mega diversity centers of the world and nurtures enormous plant diversity (Von *et al.*, 2017). The immense applications of the medicinal plants are widely accepted all over the world from time immemorial. The demand of potential medicinal plants for health care has been amazingly increased by the last decades throughout the world. The unscientific and over exploitation damaged the potential habitat of many red listed medicinal plants growing in the Western Ghats. In addition to this, some medicinal plants become rare or endangered due to their own reproductive anomalies or unadaptive nature to the changing environment. These combined effects on medicinal plants over a long period accelerated the process of rarity/endangerment and finally led to the reduction of resource base for sustainable utilization. In this context, a detailed study on reproductive biology of a high value medicinal climber, *Embelia ribes* was carried out. *Embelia ribes* commonly known as 'Vizhal' in Malayalam is an important medicinal plant used in different traditional systems of medicine in India. Over exploitation due to its medicinal importance in wild condition had led to the total depletion of its genetic resources in the Western Ghats. It is used in 75 ayurvedic formulations under the name of "Vidanga". The fruits of *Embelia ribes* contain Embelin and other high valued secondary metabolites which have a wide range of clinical applications (Kuichi *et al.*, 1998). Fruits are used in Ayurvedic preparations like Abhayrishtam, Ayaskrithi, Pippallyasavam, Anuthailamand Kachuradithailam (Iyer, 1983.) Ayurvedic products like Vidangadi Churna, Vidanga Taila and Vidangarishta are also available. The fruits are acrid, astringent, digestive, diuretic, carminative and contraceptive (Shah and Khanna, 1961; Warrier *et al.*, 1994). The paste is locally applied against skin infections. The plant is used as anti-inflammatory drug to relieve rheumatism and fever (Khan *et al.*, 2010). The fruit cures tumors, ascites, bronchitis, Jaundice and mental disorders. Knowledge on reproductive biology is a prerequisite for both evolutionary and conservation studies (Anderson, 1995). In flowering plants, at natural population level possess a wide array of morphological and physiological mechanisms that influences breeding systems (Radhamani *et al.*, 1998). Conservation programmes stressing the need for the protection of endemic species of tropics but rarely focus on breeding system, reproductive success and regeneration in natural populations. Species recovery and afforestation programme are not effective without the knowledge on reproductive biology of plants. Studies on reproductive biology of *Embelia ribes* has not been conducted so far. Hence, an investigation was carried out over a period of 4 years in Ponmudi forest areas of the Western Ghats to quantify phenology, pollination, seed germination and natural regeneration of this plant.

MATERIALS AND METHODS

Field survey was conducted to locate the viable populations of the selected species from the Western Ghats. Morphology of the candidate species was studied by using hand lens and dissection microscope.

Phenology – The following pheno-events were recorded (Dafni *et al*., 2005).

- Emergence of vegetative buds and leaves.
- Flower initiation and duration of development.
- Peak flowering period, the time of flower opening (anthesis), flower longevity
- Fruit initiations and development.

- Fruit maturation and dehiscence.

Pollen biology- Pollen morphology was studied by the Acetolysis method proposed by Erdtman (1952).The SEM photos were taken from the Acetolysed samples. Pollen-ovule ratio was calculated as per the method suggested by Cruden (1977). Pollen fertility was assessed by Acetocarmine glycerine staining technique (Shivanna & Rangaswamy, 1992).

Assessment of pollen viability by using

- Fluorochromatic reaction test (FCR) (Heslop-Harrison,1970)
- 3,3'-Diaminobenzidine test DAB, (Dafni *et al*., 2005)
- Pollen germination –both *in vitro* and *in vivo* of *Embelia ribes* was carried out during the peak period of receptivity (Shivanna & Rangaswamy, 1992). Stigma receptivity was studied by Hydrogen peroxide test and surface stigma esterases using α napthyl acetate

As part of pollination biological studies, (Dafni *et al*., 2005), the number of floral visitors and their behavior, foraging time and the frequency of visit were recorded. All the visitors were collected and identified with the help of experts. As part of fruit and seed biological studies, flower fruit ratio and ovule seed ratio was calculated. Seed moisture content and percentage of seed germination were calculated both in lab and natural conditions (Bewley and Black, 1982). To elevate the seed physiological dormancy, the seeds were treated with GA_3 of different concentrations.

RESULTS AND DISCUSSION

Embelia ribes, an important medicinal plant commonly known as 'Vayuvidang'. Now it is considered as vulnerable due to over exploitation; being used in about 75 Ayurvedic preparations. The fruits, leaves and roots are used to cure various diseases such as lung diseases, indigestion, mouth ulcer, pneumonia and heart diseases. A detailed study on the biology of this species has not so far been made. Therefore, a study was conducted for a period of 4 years (2013-17) in Ponmudi forest areas of Thiruvananthapuram district which comes under Agasthyamala Biosphere reserve at an altitude between 600- 850 masl. The population consists of more than 80 individual plants. The plant grows in association with *Xanthophyllum arnottianum*, *Glycosmis pentaphylla*, *Syzygium cuminii*, *Syzygium gardenerii*, *Morinda reticulata*, *Neolitsea scrobiculata*, *Litsea floribunda*, *Bischofia javanica*, *Grewia latilifolia*, *Canarium strictum* etc. Periodical observations were made by frequent field visit in the population during the study period. The details of geographic and climatic data of the study area are given below.

Phenology

Knowledge on phenology and floral biology is essential for conducting studies on pollination biology. *Embelia ribes* is a polygamodioecious plant shows prolific flowering which establishes in open well lit areas. The plant starts blooming in the month of mid January and continues up to March (Plate 1a). Peak flowering was noticed in the last week of February (Fig. 1) Male plants produced flowers ahead of females. In both cases flowers are small, greenish white with mild fragrance. In case of late flowering plants in the study site, flower initiation was noticed during mid April and extends up to May. Usually fruit initiation was noticed in March and attains maturity during last week of June (Plate 1 e-f). Fruit are black-blue in color with single seed. There is no difference in anthesis pattern across elevation. Majority of the female flowers open in the night between (1900-0300h) but male flowers open in the morning around 0600h and continues up to 0900h (Table 1). Anther dehiscence was observed one hour after anthesis. The longevity of female flowers is more compared to male flowers. *Embelia ribes* is polygamodioecious with low male and female plant ratio is 1:5. Male flowers were short lived compared to females. Male flowers consists of well developed anthers and reduced pistillode whereas in female flowers are with pistils and anther contain no pollen grains (Plate 1 b-d).

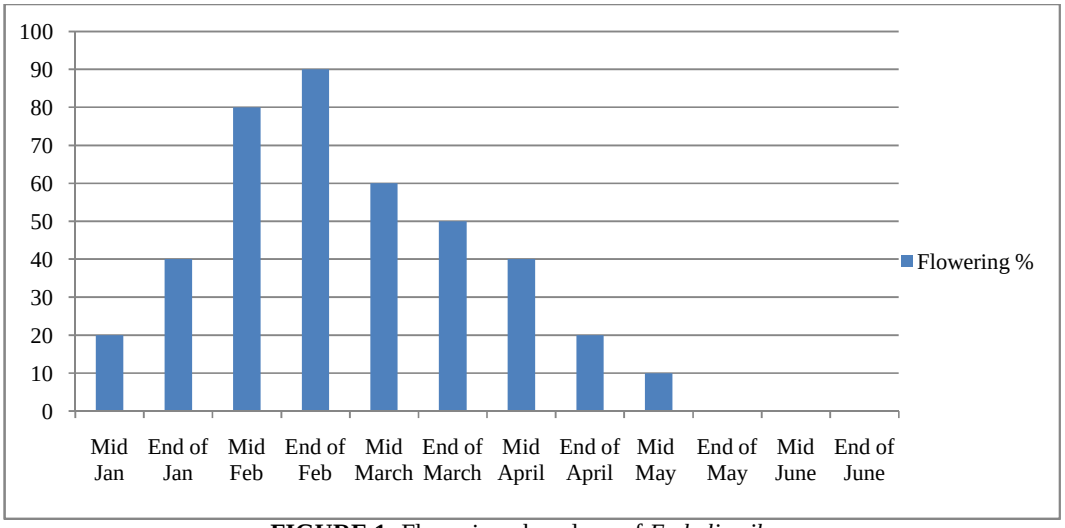

FIGURE 1: Flowering phenology of *Embelia ribes*

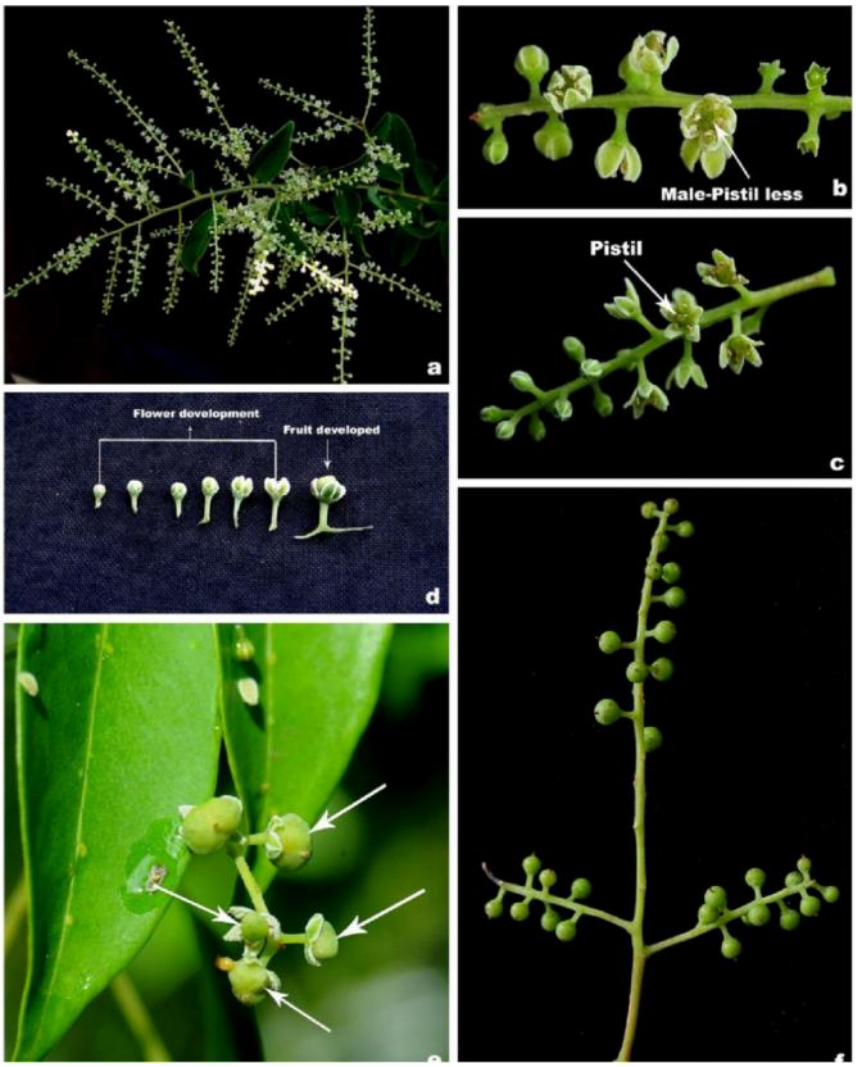

PLATE 1: Floral biology of Embelia ribes- a flowering twig, b. Male flower, c. Female Flowers, d. Flower and fruit development, e. Fruit developmenton female twig, f. Developed fruiting twig

TABLE 1. Floral characters of *Embelia ribes*

Sl No.	Floral Characters	Observations
1	Flowering period	January-March
2	Flower type	Polygamodioecious
3	Flower colour	Greenish white
4	Odour	Mild fragrance
5	Presence of nectar	Present
6	Anthesis time	Night, 1900-0300h
7	Anther dehiscence time	2000-2200h
8	No.of anthers /flower	5
9	Total No. of pollen grains in male flower	±4750
10	No.of ovule/flower	1
11	Pollen size	10.6 – 13.2 µm
12	Stigma type	Wet and papillate
13	Pollen fertility	80%
14	Fruit type	Drupe

Pollen biology

Pollen grains are tricolporate with 10.6-13.2µm in diameter (Plate 2d). The male flowers have a reduced pistilode and well developed anthers. An anther consists of around 950 pollen grains. Therefore 4,7500 pollen grains are there in a flower. But female flowers have anthers, they do not produce pollen. Pollen fertility test by Acetocarmine–glycerine staining technique indicated that maximum percentage of pollen fertility (80%) was noticed on the day of anthesis. Similarly different test by pollen

viability (FCR, and DAB) revealed that highest percentage was observed on the day of anthesis (Table 2 and Plate 2, b&c). Pollen viability gradually reduced with time. *In vitro* pollen germination studies agree the results of pollen viability. The best pollen germination along with 682 µm tube elongation was noticed in Brewbakers medium containing 10%sucrose after 24 hours of incubation (Plate 2a). The sucrose is the best carbohydrate source for pollen germination and tube elongation of many plants. But the concentration of sucrose varies from species to species. Higher concentration of sucrose resulted in low percentage of pollen germination and tube growth (Sidhu and Malik, 1986). This observation agrees with the findings in *Embelia ribes.*

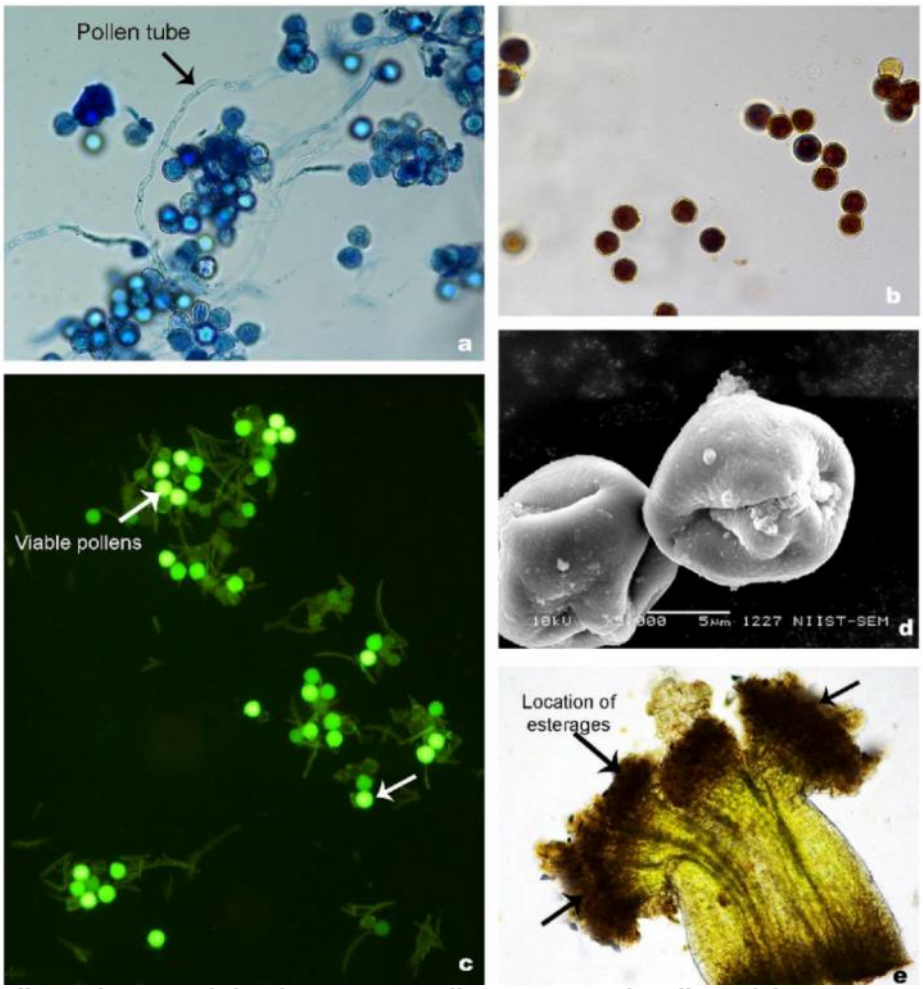

PLATE 2. Pollen Biology in Embelia ribes- a in vitro pollen germination, b. Pollen viability test in DAB, c. Pollen Viability by FCR, d. SEM of Pollen grains, e. Non specific stigma Esterases on Stigmatic head

TABLE 2. Pollen fertility and viability of *Embelia ribes* under different tests

Different tests	One day before anthesis (%)	On the day of anthesis (%)	Second day of anthesis (%)
ACT	20.04±1.09	80.69±1.76	15.02±2.15
FCR	5.72±1.98	76.53±1.21	10.68±1.72
DAB	7.62±0.56	75.04±1.76	12.24±1.86

ACT-Acetocarmine-glycerine test, FCR-Flourochromatic reaction test, DAB-Diaminobenzoidine test Values mean± SD

Stigma receptivity and *In vivo* pollen germination

The stigma receptivity refers to the ability of the stigma to accept and support pollen germination and tube elongation of compatible pollen grains. The receptivity of the stigma is an important factor for the completion of post pollination events in many flowering plants (Heslop-Harrison, 1981).Female flowers usually open in the night and stigma receptivity was noticed soon after anthesis continues up to the next day of anthesis. Stigma receptivity was checked by cytochemical localization of esterases and Hydrogen peroxide (H_2O_2) test. Non specific stigma esterase was located on the stigmatic head as well as on the lateral surface of the stigma as indicated reddish brown colour (Plate 2e). The presence of esterase over the stigmatic surface coincides with its receptivity. Hydrogen peroxide test also indicated that maximum number of bubbles produced at the time of anthesis. *In vivo* pollen germination of 46.25% along with 462µm tube elongation was noticed at the peak period of stigma receptivity. Pollen grains adhered on the receptive surface of the

stigma and germinated pollen grains produced pollen tubes and it grows towards the stigmatic tissues. Finally it reaches the ovary and fertilize the ovules. Fertilized ovules developed into seeds. If the pollen is compatible, all the events such as pollen adhesion, germination, penetration of pollen tube and pollen tube entry in to the ovule proceed normally, resulting fertilization and seed development (Shivanna and Johri, 1985). The present investigation shows a positive correlation with the above findings.

Pollination and reproductive success

In *E. ribes*, male and female flowers are of the same size and shape. The nectar production is comparatively high in male flowers than in females. Nectar is golden yellow in color and thick in consistency. The nectar secretion is high during 0600-0900h and after that fades down. The flow of Nectar in female flowers is relatively lower but is secreted throughout the day. Once the nectar flow is lowered in male flowers, the floral visitor's starts moving towards the female flowers. The enhanced nectar production in male flowers allows the visitors to first converge on male flowers and then move to the females; this facilitates the pollination.

TABLE 3. Pollinator behaviour of *Embelia ribes*

Visitors	Family	Visiting time	Visiting status	Foraging nectar	Foraging hours
Apis cerana	Apidae	Day	Regular	Pollen+nectar	0700-1200
A. millifera	Apidae	Day	Occasional	Pollen +nectar	0700-1100
Trigona irridipennis	Apidae	Day	Regular	Pollen+nectar	0600-1200
Wasp(Vespa spp)	Vespidae	Day	Regular	Nectar	0800-1100
Eurema blanda	Pieridae	Day	Regular	Nectar	0920-1130
Eupolia core	Nymphalidae	Day	Occasional	Nectar	0830-1030
Danaus genutia	Nymphalidae	Day	Occasional	Nectar	0730-0930
Black ants (*Componotus compressus*)	Formicidae	Throughout	Regular	Nectar	Throughout
Small bees (*Mellipona* sps)	Apidae	Day	Occasional	Nectar	0830-1100

TABLE 4. Reproductive output and success

Sl. No.	Flowers	Fruits	Seeds	Fruit / Flower	Seeds /Ovule	Success
1	2432	486	480	0.199	0.98	0.197
2	1378	375	272	0.272	0.72	0.192
3	8602	1978	1665	0.229	0.84	0.190
4	6836	1467	1357	0.214	0.92	0.194
5	1024	289	228	0.282	0.78	0.222
6	3321	656	647	0.197	0.98	0.194
7	9453	1967	1853	0.208	0.94	0.198
8	918	287	280	0.312	0.97	0.305

Honey bees, wasps, Stingless bees, Butterflies, Black ants and small bees are the pollinators of *E. ribes*. The pollinators were attracted by mass blooming of flowers with fragrance. The flowers provide both pollen and nectar as rewards to the visitors. Among the pollinators, honey bees and black ants (*Componotus compressus*) foraging in the day time but ants are active throughout the day (Table 3). About 80 individuals were tagged in the study site. Being a dioecious plant, it is an obligatory out-crossed pollination system. None of the emasculated and bagged flowers sets the fruits, which indicates that *E. ribes* is not an apomitic. The un-emasculated bagged flowers failed to set fruits at any time of flowering indicating that the plant is self-incompatible. But manual cross pollination (pollen collected from male flowers and transferred to the receptive stigma of female flowers) produced fruits (40%). The present study revealed that, there is a low fruit to flower ratio. But the seed - ovule ratio is very high (Table 4). Flower abortion ranges from 40-60%.Scarcity of pollinators may be one of the reasons for flower abortion. *In-vivo* pollen germination studies indicated that only small quantity of pollen grains are loaded on the stigmatic surface of the flower. Percentage of fruit set was 40-45%, during the developmental stage they abort up to 12%. Fruits are eaten by birds and insects

Seed biology

Fructification takes place after the drooping of the flower. The fruits of *Embelia ribes* are globose, ovoid drupe having 3.5-4mm in diameter Scarcity of natural pollinators, low male-female ratio of flowers will adversely affect the fruit production in the population. Certain minor infestation caused by some unidentified insects larvae affect the fruits which influence the percentage of fruit set. Young drupes are green and it changes to dull red or black when mature. Fruits weight was 0.031 gm. Seeds are black, globoid, smooth covered with a thin membrane, 1.5-2 mm in diameter. The seed is horny, depressed at the base and has a ruminate endosperm. Seed weight is 0.016 gm. The fruit takes 5-6 months to attain maturity.

Fruits ripen during June and are retained during mid August. Seeds are dispersed by birds Seeds remain dormant for 15-30 weeks. Fresh seeds have 28.47 ±1.1% moisture content, when tested, they registered 20 ±2.2 % germination. Natural regeneration of *E. ribes* is poor due to overharvesting and exploitation, development of abortive embryos, low seed viability and germination percentage. Seed dispersal occurs through birds which consume the fruit. Due to the bird dispersal of large number of fruits, regeneration is always noted as solitary

seedlings and never in patches owing to seed dispersal. Regeneration of seedlings was observed randomly in well-lit areas. Vegetative propagation also has not been found effective. To alleviate the physiological dormancy, the seeds were treated with GA3 of different concentrations. The highest seed germination 75% was recorded in GA3(1000ppm) treated seeds. The similar results were noticed in *Asparagus sprengerii* (Dhoran and Gudadha, 2012).

The seedlings were transferred to the field gene bank of JNTBGRI. It is an attempt to produce quality plant stock and thus to enhance the cultivation protocol of this plant.

CONCLUSION

Embelia ribes is polygamodioecious species with male and female plants that promotes out-crossing by limiting self-pollination. The study revealed that the male plants flowered ahead of females this is also a natural adaptation of the plant to utilize maternal resources. The present investigation indicated that there is a low fruit to flower ratio but the seed-ovule ratio is very high. Scarcity of native pollinators due to less rewards and low male female ratio adversely affected the fruit production in the population. Natural regeneration of *E. ribes* is poor due to overharvesting and exploitations, habitat destruction, slow germination of seeds with low viability and germination percentage. Pre-germination treatment of seeds (GA_3 1000ppm) was done to alleviate physiological dormancy. This is an attempt to produce Quality Planting material (QPM) and enhanced the cultivation protocol of this important plant.

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