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Pollen morphology, viability and germination of *Elaeocarpus munroii* (Wight) Mast., An Endemic tree species of the Western Ghats

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Abstract
Elaeocarpus munroii belongs to the family Elaeocarpus is endemic to the Western Ghats. This plant is included in the red list under near threatened category of IUCN. A study has been carried on pollen morphology, viability, germination using light and scanning electron microscopy. The pollen viability was found to be 87-90% on the day of anthesis and it gradually reduced successive days after anthesis. *In vitro* pollen germination studies indicated that, maximum percentage pollen germination was 72% along with 2034.87µm tube length was noticed in Brew baker's medium containing 20% sucrose. Pollen grains are isopolar, spherical to oblate spheroidal, tricolporate and 8.6±0.49 µm×8.8±0.40 µm in diameter.

Keywords: *Elaeocarpus munroii*, pollen viability, anthesis, endemic, Western Ghats

Introduction
The genus *Elaeocarpus* has more than 360 known species worldwide which is the largest genus of the family Elaeocarpaceae, distributed in the regions of South Asia, South China, Japan, Malaysia, Australia, New Guinea, Fiji and Hawaii (Coode, 2007; Joshi *et al* 2014) [8, 19]. Twenty-six species have been reported from India mostly confined to Northeast and South India (Khan *et al* 2003) [20]. In Peninsular India, 13 species were reported, of which 6 species are endemic to the Western Ghats (Irudhayaraj and Ramasubbu, 2017) which includes *E. munroii*. The selected species *Elaeocarpus munroii* is included in IUCN red list as near threatened (WCMC, 1998). Most of the *Elaeocarpus* species are medicinally important. In *Elaeocarpus sphaericus* the leaves and seeds are used to cure stress, anxiety, depression, asthma, hypertension, arthritis and liver diseases. Rudraksha beads were obtained from the seeds of *Elaeocarpus ganitrus* are used for religious purpose (Murthi, 1993) [22]. In most of the *Elaeocarpus* species, the leaf extract or the whole plant part consists of indolizidine alkaloid which reduce enzymatic activity of glucosidase. Hence used in the treatment of diabetes and cancer (Wiart, 2006) [39]. In case of *E. munroii* the leaf, stem and fruit extracts are rich in phytochemicals and have potent anti-oxidant activity (Anusuya Devi *et al* 2018) [4, 5] also the ethanoic extract of leaf have anti-inflammatory and anti-arthritic activity (Anusuya Devi *et al* 2018) [4, 5]. The leaf extract of *E. munroii* have anti-fungal and anti-bacteria activity (Sharvani *et al* 2015) [29]. In view of potentiality of the selected species, a study has been conducted on pollen biology of *E. munroii.* In Elaeocarpaceae, pollen grains are 3-aperturate (Coode, 2004; Fabian *et al* 2016; Rathnasiri Premathilake and Siwert Nilsson, 2001) [7, 14, 28]. Counting the pollen grain number is helpful to determine the pollen ovule ratio also the pollination efficiency and dynamics of pollen dispersal (Shivanna, 1992) [32]. The pollen grains have to be viable for successful fertilization of fruit and seed set (Shivannah, 2003; Cruzan, 1989) [9]. The sperm cells produced by the Pollen grains is necessary for the sexual reproduction of angiosperm species (Simpson, 2006) [34]. So it is important to measure the viability of pollen grain. It is possible by direct method like *in vitro* germination (Alcaraz *et al* 2011) [2] and by indirect methods by conducting staining tests (Abdegadir, 2012). The medium for i*n vitro* germination, some pollen requires aqueous solution of sucrose to germinate but some require special substrate for germination (Dafni, 1992) [12]. By observing the pollen viability and pollen vigor the quality of pollen can be measured (Ottaviano and Mulcahy, 1989) [24]. If the quality of pollen reduces, which brings down the success of sexual reproduction of natural population (Amat *et al* 2011) [3] The present study gives an account on pollen morphology and production of pollen grains of the threatened species *E. munroii*. Also evaluate the pollen viability, assess *in vitro* pollen germination and pollen tube growth.

Materials and method
Study area
E. munroii was collected from Ponmudi hills of the Western Ghats Thiruvananthapuram district. The species located at 8°45'27.36" N - 77°06'47.26" E, at altitude between 780m - 1000m above sea level. The study was carried out during the period of 2018-2020. The pollen grains were collected in the peak flowering period (September- October). Bisexual flowers are produced in auxiliary raceme inflorescence. The flowers can remain in the inflorescence for 5-6 days. From the reproductive mature individual we randomly select 6 tree species. From each plant, 20 inflorescences were selected.

Pollen morphology and production of pollen grain per anther
The pollen grains collected were acetolysed according to the method proposed by Erdtman 1960 [13]. The acetolysed

pollen grains mounted in glycerin jelly were observed under light microscope (NIKON HL600) at a magnification of 100X. The size, shape, exine thickness were taken based on observing 10 pollen grains. Also the pollen grains subjected to Scanning Electron Microscopy (SEM) studies. The exine ornamentation and aperture characters were examined under the SEM and microphotographs were taken. The number of pollen grains per anther was calculated by the method proposed by (Dafni *et al*. 2005) [11]. Pollen suspension were taken on a clean micro slide and counted the total number of pollen grains under microscope. The procedure was repeated for 10 sample suspension. Calculated the total number of pollen grains per anther using the formula

$$\text{Mean no. of pollen grain} = \frac{\text{No. of pollen per suspension} \times \text{no. of pollen suspension}}{\text{Total no. of suspension used for counting}}$$

Pollen viability

Pollen viability was assessed two staining methods; FCR (fluorochromatic reaction) and DAB (3-3' Diaminobenzidine) test. The fluorochromatic reaction (FCR) test (Heslop-Harrison and Heslop-Harrison, 1970) [15] was used to examine esterase activity and membrane integrity. The viable pollen grains show fluorescence by the enzymatic reaction. DAB test used for knowing the peroxidase activity of viable pollen grains (Dafni *et al* 2005) [11]. The viable pollen grains showed brown colour due to the precipitation of the reaction. The pollen grain collected from the day before anthesis and after anthesis subjected to viability test. Pollen grains from a total of twenty flowers were used. The pollen viability was scored according to staining level and observe under the microscope (NIKON HL600) at 40X magnification. Twenty fields were selected to observe the viable and non-viable pollen grains. The viability calculated in terms of percentage.

***In vitro* pollen germination and tube growth**

The pollen grains collected from freshly dehisced flowers were subjected to *in vitro* pollen germination using Brewbaker's and Kwack's medium (Brewbaker and Kwack,1963). The effect of sucrose concentration on pollen germination and tube growth was evaluated. The pollen grain collected from the day before anthesis and after anthesis subjected to viability test. The sucrose concentration of 5%, 15%, 10%, 20%, 25% and 30% medium were used. The pollen grains were incubated for 24 hours in moist chamber. The pollen tube growth was observed after 1h, 8h, 16h and 24h and measurements were taken. Pollen tube was considered to be germinated when the pollen tube length is equal to or exceed the pollen diameter. The slides observed under light microscope (NIKON HL600) at 40X.magnification. Hundred pollen grains were considered in a single field to find out mean pollen germination.

Data analysis

Mean and SD of all measurements were taken. Regression analysis performed between pollen viability and pollen germination. Statistical analysis was performed using Gretl.

Results

Pollen morphology and production of pollen grains

The pollen grains of *E. munroii*,(Figure1a-c) are isopolar, spherical to oblate spheroidal in shape. An average of 8.6±0.49 µm×8.8±0.40 µm in size. Polar outline is circular or slightly angular. Equatorial outline is more or less circular. Tricolporate. Colpi elliptic 6.5±0.45 µm long 1.35±0.52 µm wide at equator, sides tapering towards the pole, tip acute. Ora more or less circular 2±0.27 µm across. Apocolpia 1.5±0.22 µm across. Exine 0.55±0.10 µm thick. Sexine is psilate in light microscope. Sexine is microreticulate with granular texture in SEM observation. *E. munroii* produces 7490±138 number of pollen grains per anther.

Pollen Viability

Pollen viability was determined by using FCR and DAB test. In case of FDA test the viable pollen grains produce green florescence under UV filter counted as viable (Figure1 d). The highest pollen viability recorded was 90% in the day of anthesis (Table 1). There is a gradual decline in viability percentage day after day from time of anthesis. The same can be observed in case of DAB test. The pollen grains stained brown colour counted as viable (Figure1 e). According to the viability rate in DAB the highest percentage is obtained in the day of anthesis (87%). I n both cases no pollen grains are viable before anthesis. In both the cases on the second, third day an average of 70% and 60% pollen grains was viable respectively.

Table 1 Pollen viability test

Days	FDA test	DAB test
One day before anthesis	0	0
1st day	90.18±6.20	87.38±2.56
2nd day	77.51±9.97	72.42±3.34
3rd day	61.67±6.16	56.16±4.56
4th day	11.64±8.35	61.73±3.14
5th day	0	0

***In vitro* pollen germination**

The pollen grain subjected to *in vitro* pollen germination using Brewbaker's and Kwack's medium. The effect of different sucrose concentration on pollen germination were examined. Observation were done after 24 hours of incubation. On the first day of anthesis 72% pollen grains get germinated (Figure 2) which is the maximum percentage obtained in this experiment, from the medium consist of 20% sucrose (Figure1 f). Above 50% pollen grains germinated only in 10% and 15% sucrose containing medium. Compared with the sucrose free medium (control) the germination percentage increased with increase in sucrose concentration up to 20%, then show a decrease in 25 and 30%. The germination percentage declined each day after anthesis. After the 3rd day of anthesis up to10% pollen get germinated. Below 40% of pollen grains get germinated in 3rd day of anthesis. There is no pollen germination was noticed day before anthesis.

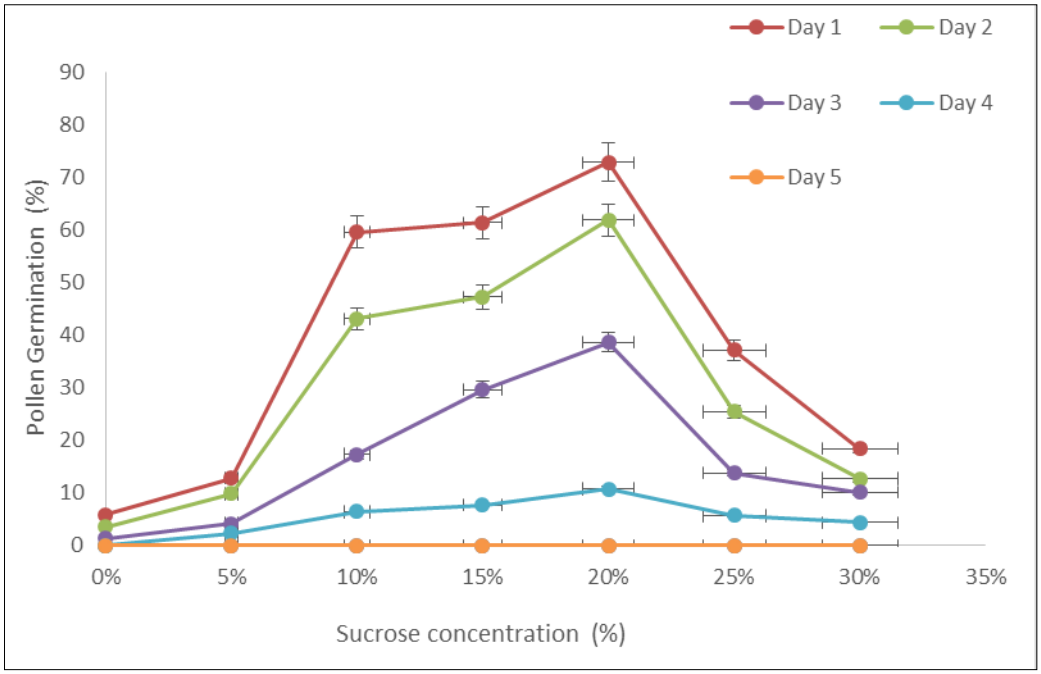

Fig 1: Effect of sucrose concentration on pollen germination of different time interval after anthesis

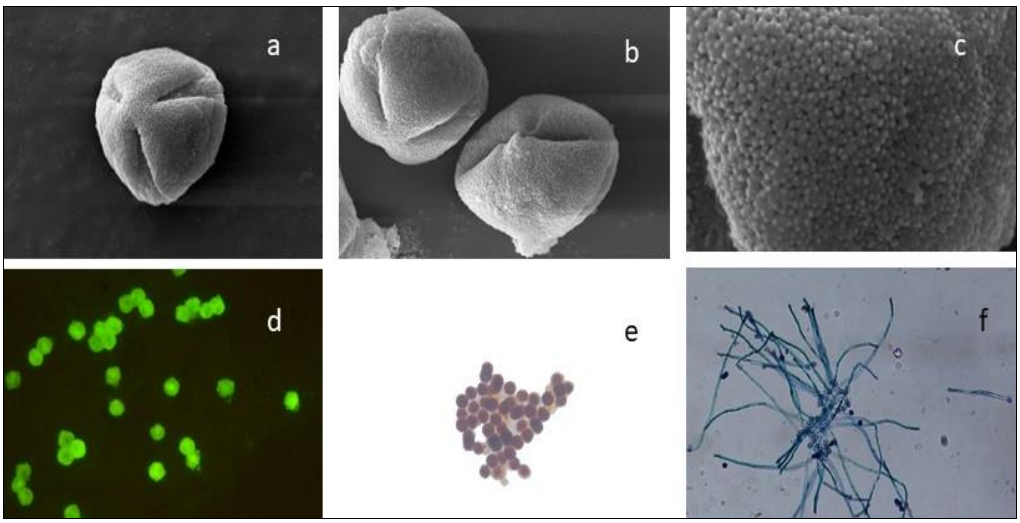

Fig 2: a-c scanning electron micrography of mature pollen grain. d- FDA stained pollen showing green flurescence. e-DAB stained pollen showing brown colour. f- *In vitro* germination.

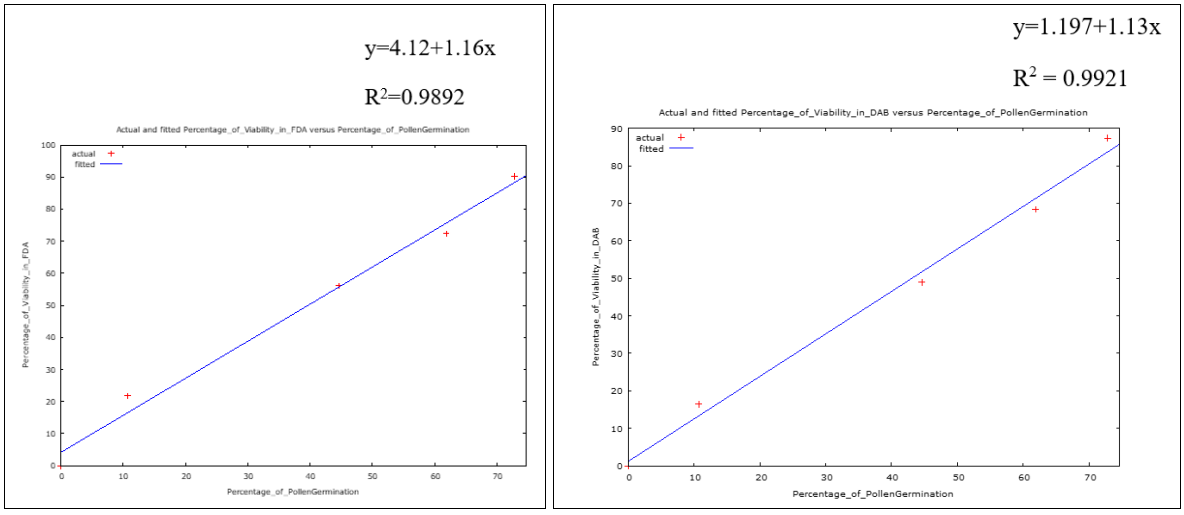

Fig 3: Regression analysis between pollen staining and *in vitro* germination.

A regression analysis between the FDA test, DAB test and the *in vitro* germination was performed (Figure 3). The viability of pollen was strongly correlated with pollen germination (r^2 = 0.9892, r^2 =0.9921 for pollen germination with FDA and DAB respectively) and highly significant (P < 0.001).

Pollen tube growth

The effect of sucrose concentration on pollen tube growth were monitored. Pollen grains started to germinate after 6h of incubation. Pollen tube length were measure after 8h, 16h and 24h.The pollen tube length varied according to the concentration of sucrose (Figure 4). The highest pollen tube growth obtained from the medium contains 20% sucrose. Compared to control tube growth increases from medium contain 5% sucrose to 20%. After that there is no further increase in the pollen tube length in 25 and 30% of sucrose. An average of 2034.87µm in length is observed. The sucrose concentration influences the pollen tube length up to 20%. Fairly good pollen tube length can be observed in 10% and 15% sucrose containing medium.

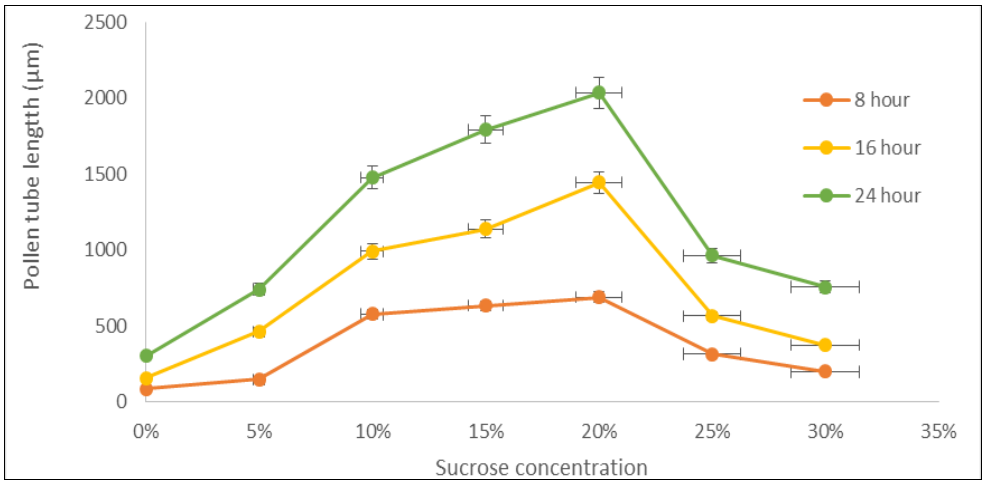

Fig 4: Pollen tube growth on different concentration of sucrose after 8h, 16h and 24h.

Discussion

The species *E. munroii* and most of the *Elaeocarpus* species produce tricolporate pollen grains, isopolar, circular to elliptical in outline (Vasanthy, 1976) [38]. This observation is similar to *E. serratus* and *E. tuberculatus* (Tissot, 1994) [37]. In *E. firdausii*, 3 colporatre and 2 colporate pollen grains are present (Fabian *et al* 2016) [14]. A small difference in pollen size and outline is present as compared to other related species. It is reported that pollen in *Elaeocarpus* species under Light microscope sexine is smooth (Tissot, 1994) [37]. SEM observation showed reticulate sexine in *E. sylvestris* var. *elipticus* and *E. photiniaefoius* (Ikuse, 1956) [17]. But in *E. montanus* and *E. obovatus* scabrate to perforate sexine is present (Huang, 1972) [16]. In *E. blascoi* pollen grains are of 10-12 µm in size (Ramasubbu and Felix, 2016) [22]

In angiosperms pollen morphological characters are positively correlated with pollination system (Osborn *et al* 1991) [23]. The pollen walls with thick exine, high ornamentation and high amount of sporopollinine are characteristic feature of zoophilous plants (Paccini and Hesse, 2005) [25]. The reticulate pollen grains often pollinated by means of insects (Thanaka *et al* 2004). The pollen morphological features of *E. munroii* support entamophilous pollination. The FDA and DAB test are not much differing in determining the viability of pollen grain in *E. munroii*. Both the staining methods are enzymatic and reliable to test the viability. FCR test (Heslop-Harrison and Heslop-Harrison, 1970) [15] can monitor esterase activity and membrane integrity. DAB test indicate the receptive stigma as well as viability of pollen grains (Dafni, 2007) [10]. Comparing the viability and germination tests carried out, the staining method is time efficient, find out the viable pollen in short time (Sulusoglu, 2014) [35]. The result obtained from the *in vitro* pollen germination of *E. munroii* indicated that Brewbaker's medium containing 20% sucrose is good for the high pollen germination and pollen tube formation. Sucrose concentration up to 20% can influence the pollen germination and tube growth positively. It is reported that in *E. blascoi* 20% sucrose concentration give the best percentage of germination and 77% pollen grains are viable at the time of anthesis, the pollen grains recorded per anther is 50,700 ± 456 (Ramasubbu and Felix, 2016) [27]. Higher concentration of sucrose does not provide further increment in germination or tube growth in *E. munroii*. *In vitro* analysis sucrose in the germination medium can provide energy for pollen metabolism and maintain the osmotic pressure. Different sugar types and concentration is the important factor on the germination medium, it acts as the carbon source for pollen germination and tube growth. From these sugar types sucrose is the best carbon source (Shivanna and Johri, 1989; Miranda and Clement, 1990) [21]. But increase in concentration of sucrose in the medium tends to contribute more carbon to the medium which may imbalance the osmotic pressure and affect the pollen tube growth (Premachandra *et al.* 1992) [26].

In this study a strong correlation between pollen viability and pollen germination is observed. Correlation between FDA and *in vitro* pollen germination in many angiosperm species are reported (Shivanna and Heslop-Harrison, 1981) [31]. In the case of *E. munroii* the pollen viability experiment gives a strong correlation between DAB test and *in vitro* germination.

Conclusion

The structure of pollen grains of *E. munroii* can identified by studying the pollen morphology. Both the staining methods FDA and DAB test can have used to determine the viability of the pollen grains. The highest viability is

observed on the day of anthesis. But a good number of pollen grains are viable on the second day of anthesis. Twenty percentage sucrose was the best concentration medium obtained higher germination percentage and pollen tube growth. Both the staining technique and in vitro germination can used to check the quality of pollen grains. For successful fertilization and seed set, pollen grains have to be not only fertile but also viable at the time of pollination. Hence, pollen viability is an essential attribute for successful fertilization and fruit set.

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