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*Full Length Research Paper*

# Studies on the pollen biology of *Terminalia paniculata* Roth. (Combretaceae)

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***Terminalia paniculata*** **is a deciduous tree widely distributed in tropical semi-evergreen and moist deciduous forest. Flowers of** ***T. paniculata*** **have ten stamens, which remain inside the bud and anthesis is carried out at different times of the day. Pollen grains are medium spherical, tricolpate 17 µm in diameter, smooth exine and yellow in colour. The pollen: ovule ratio approximated 16,000: 1. Pollination efficiency was increased daily, from first day of flower opening (0.000076), reaching the maximum on third day (0.00083) and there was no pollen deposition on the fourth day. To effectively assess** ***in-vitro*** **pollen viability in** ***T. paniculata*****, an optimized germination medium (30% sucrose with Brewbaker and Kwak's medium) was developed. Pollen grains stored under cryopreservation (-20°C) had prolonged viability. Using the hand pollination for testing the** ***in-vivo*** **viability of pollen grains the maximum number of fruit set was obtained in the fresh pollen grains (0 h – pollen collected at the time of anthesis) on the stigma. Afterwards, increase in the age of the pollen decreased the fruit set. After 15 h of anthesis, the pollen grains lost their viability and there was no fruit set. The current findings will be useful in studying, pollen – pistil interactions, gene flow and heterozygosity of the** ***T. paniculata*** **populations.**

**Key words:** Cryopreservation, *in vitro* germination, pollen storage.

## INTRODUCTION

Establishment of forest plantations is currently of great interest in India because of rapid depletion of the natural forest. In order to enhance the forest cover and to meet increasing human needs for timber, firewood, fodder, herbs and other forest products, four restoration strategies were developed that is, Conservation forestry, Agro forestry, Industrial forestry and Environment / Revegetation forestry (Pandey, 2007). These afforestation programs have been vigorously implemented in various parts of India by Governmental organizations, Non Governmental organizations and private entrepreneurs. Most reforestation and afforestation programs use seedlings as planting materials, with improved quality planting materials obtained from rigorous genotype selection. Hybridization is another method that holds potential for improving planting materials in foresting. In either case understanding the pollen biology of particular species is important for developing a tree improvement strategy (Bosch, 1992).

Reproductive success of hermaphroditic plants is determined by both the quantity and quality of the gametes and offsprings produced. Pollen grains are reduced male gametophytes which, upon pollination, produce pollen tubes that grow through the pistil to effect fertilization and seed set. Most estimates of the reproductive success of hermaphroditic plants are based solely on the haploid contribution through the female function, ovule and seed production (Wheller and Guris, 1992) because of the difficulties inherent in estimating haploid contribution through male function. The production and the dispersal of pollen have both biological and genetic implications for the quantity and genetic value of the seed produced. Hence pollen biology is of immense significance in tree improvement programmesas it determines gene flow and heterozygosity of the population, and these in turn determine genetic variability.

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*T. paniculata* has been extensively utilized for wood and non wood products for construction and is often used to substitute teak. Other common uses of *T. paniculata* wood are timber for agricultural implements, boat building, ply-wood, block boards and packing cases (Rao and Purkayastha, 1972). Other than the wood products, *T .paniculata* yields indigenous drug preparations, tannins, gums, oils, fodder and certain organic compounds from its leaves, trunk, bark and fruits. This study was conducted to gain a better understanding of pollen biology by studying: the pollen morphology, viability and germination in *T. paniculata*

## MATERIALS AND METHODS

The study was conducted between September 2003 and November 2005. Intensive exploration trips were made Courtallam, western ghats (9° 15 ' N, 77° 30' E) during the period. The pollen biology of *T. paniculata* flowers was studied by following methods:

### Pollen structure and pollen shedding

A flower opened for three days but receptive for less than one day. All the days flowers opened, flowers in several inflorescences were tagged and anthers were periodically collected to examine morphological changes under microscope in order to determine the pattern of anthesis, time of anthesis and pollen shedding. Size of pollen grains was measured under light microscope using ocular and stage micrometer.

### Pollen production

To determine the number of pollen grains per flower, one mature anther per flower was removed from ten matured buds selected from five inflorescences in two randomly selected `trees. Anthers were gently squashed, on a slide, stained with acetocarmine, covered with circular cover slip and pollen grains in each anther were counted. From this value, pollen production per flower and the number of pollen grains per inflorescence were calculated (Bernardello et al., 1994).

### Pollen – ovule ratio (P/O ratio)

The pollen – ovule (P/O) ratio was determined by the number of ovules per flower divided by total number of pollen grains per flower. Buds and flowers were fixed in 70% ethanol. Ovule quantity was calculated using Anderson and Symon's method (Anderson and Symon,1989).

### Pollen efficiency, pollination success and pollen deposition

Pollination efficiency was determined by dividing the number of pollen grains deposited per stigma by the number of pollen grains produced per flower. To determine the number of pollen grains on the stigma, 100 receptive stigmas were randomly chosen and removed from the tagged flowers that have undergone pollination just then and stained with Alexander's stain to detect the pollen grains. The pollination success of flower was calculated by percentage of pollinated flowers out of the total number of flowers present in the inflorescence. To determine pollen deposition on the stigma, 100 stigma from tagged flowers were randomly selected, and stained with Alexander's stain (Alexander, 1969, 1987) to detect the pollen grains.

### *In vitro* pollen germination

*In vitro* pollen germination was conducted in Brewbaker and Kwack's medium (Brewbaker and Kwack, 1963) with various sucrose concentrations (5, 10, 15, 20, 25, 30, 35, 40, 45 and 50%) to detect the optimum level required for the species established empirically. The optimum medium was determined by germination trial in different germination media. Pollen germination was determined by hanging drop method using different pollen germination media with addition of a drop of surfactant, Triton x 100 which prevents clumping of pollen grains. The hanging drop culture was sealed with vaseline to prevent evaporation of the culture medium. From that the percentage of pollen germination and length of pollen tubes were assessed.

### Pollen storage

Fresh pollen from the day of anthesis was collected for pollen storage. The anther was excised before dehiscence (n = 25), gently brushed into petriplates and collected into vials. For each treatment five vials were stored at 4 – 20°C, and in benzene and at room temperature. All the vials were stored for 10, 20, 40, 80 and 120 days and at the end of each period the pollen viability was studied.

### Pollen viability

Variation in pollen viability capacity with grain age was investigated by staining experiments. The viability of stored pollen grains was tested by Alexander's staining method (Alexander, 1969; 1987) and i*n vivo* testing the pollen viability.

### *In- vivo* testing the pollen viability

Testing the viability of the pollen grains was made on the basis of hand pollination experiments. Pollen was collected at the time of anthesis and at different times afterwards that is, at the time of anthesis (0 h), three hours after anthesis, six hours after anthesis, nine hours after anthesis, 12 h after anthesis and 15 h after anthesis. The emasculation was carried out on first day of flower opening and after emasculation, stigma of the third day after flower opening, were pollinated with pollen grains with different ages that is, 0, 3, 6, 9, 12 and 15 h of after anthesis and the pollinated flowers were bagged. . Daily observations were made up to fruit set and the bags were removed from the flowers ten days after pollination. The percentage of fruit set in pollinated flowers was calculated by counting the number of fruits present in the pollinated flowers. From this the pollen viability under *in vivo* conditions was observed.

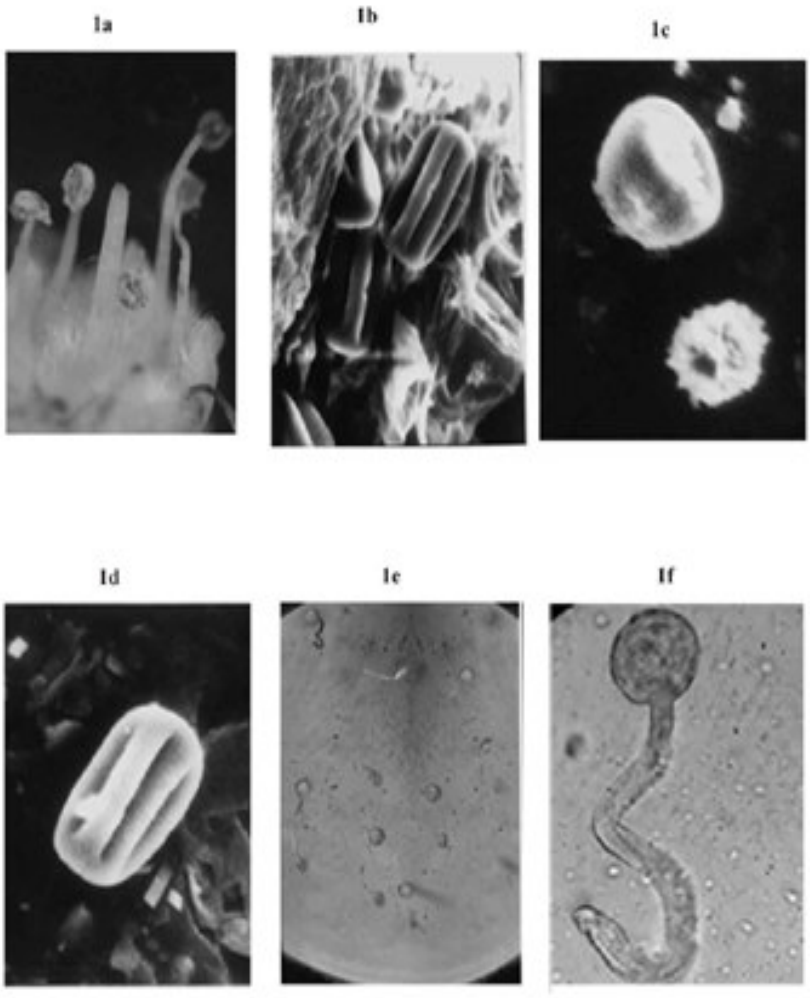
### Scanning electron microscopic studies

The pattern of anthesis, architecture of pollen grains was studied using scanning electron microscope (SEM). For SEM, anthers, stigmas and flowers of *T. paniculata* were fixed in 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.0, before being dehydrated in a graded ethanol series. Dried specimens were mounted on stubs and coated with gold. Coated materials were examined under SEM and photographed.

## RESULTS

### Anthesis and time of anther dehiscence

Flowers of *T. paniculata* had ten stamens, yellow in co-




**1a. Dechised anther**
**1b. Pollen grains released during dechiscence (1200x)**
**1c. Pollen grain polar view (1200x)**
**1d. Pollen grain lateral view (2000x)**
**1e & 1f. *In-vitro* pollen germination (100x and 400x)**

**Plate 1.** Anther dechiscence pattern, pollen architecture and *in vitro* pollen germination in *T. paniculata*

lour and were arched inside the bud until the flower opened. The calyx of mature bud started splitting at 02 00 h. The next hour (03 00 h), two stamens were erect, and by 06 00 h six more were exerted and by 08 00 h the other two stamens stood erect. The anther dehiscence started from 10 00 h, at the time the first two anthers dehisced (Plate 1a), at 17 00h three more anthers and by 08 00 h of the day after flower opening, the remaining five anthers were dehisced (Figure 1).

**Pollen shedding**

Approximately eight hours after the flowers opened, the first two stamens were found to dehisce (Figure 1). The cells along the sides of the anthers broke down forming a longitudinal slit (Plate 1b). As the anthers were subjected to wind action or brushed by insects, mature pollen was released. Next three stamens were found to dehisce at 15 h after flower opening and anthesis of remaining sta-


**Figure 1.** Schematic overview of events that occur in the flowers of *T. paniculata*.

stamens was found to occur at 28 h after flower opening. Some pollen remained in the anther for nearly three hours after anther opening. Pollen dispersal was increased by the insects. Anthers turned into brown with a bent filament six hours after anthesis and collapsed two hours later

## Pollen production

A mature anther contained approximately 1,614 ± 63.29 (ranging from 1,300 – 1,800) pollen grains. The mean number of pollen grains per flower was 16,540 ± 685.9 (ranging from 12,500 – 18,800). The mean number of pollen grains per inflorescence was 5, 32,620 ± 73,363 (ranging from 2, 90,520 – 7, 74,720).

## Pollen architecture and pollen-ovule ratio

Pollen grains were medium spherical, tricolpate, 17 µm (15 - 19 µm) in diameter, exine smooth and yellow in colour. Pollen coat substances (pollen kitt) were occasionally found on the maturing *T. paniculata* pollen surface, in particular on pollen collected during the early receptive period (Plate 1c and 1d). There was an ovule for every 16,000 pollen grains. The pollen – ovule ratio was around 16,000: 1.

## Pollination efficiency

Pollination efficiency was correlated positively between the numbers of pollen grains that reach stigma with number of pollen grains produced by flower. The pollination efficiency varied day by day after flower opening and is shown in Table 1. Pollination efficiency was increased day-by-day, beginning from first day of flower opening (0.000076), reaching the maximum on third day (0.00083). Pollination efficiency on the fourth day of flower opening was found to be zero, as there was negligible amount of pollen deposition.

## Pollination success and pollen deposition

The number of pollen grains deposited on the stigma gradually increased from the day on which flower opened to the second day after flower opening. On the third day after flower opening, the pollination success was found to be almost nil due to the deposition of negligible amount of pollen grains (0.98 ± 0.26) on stigma (Table 3). There were differences in the number of pollen grains deposited on the stigma at different times during different days. About eight percent of flowers were pollinated on the first day and the number of pollen deposited was 1.24 + 0.2182 (range from 0 - 3) pollen grains per pollinated flower. There was a significant increase in the number of pollinated flowers from day of flower opening, to two days after flower opening.

Around 54% of flowers were pollinated on day two while on day three, about 72% of flowers were pollinated. The number of pollen grains deposited on stigma also varied by the day. On day of flower opening 1.24 ± 0.2182 pollen grains were deposited on the stigma reaching a maximum on second day after flower opening (13.48 ± 1.2920) afterwards, pollen deposition declined (Table 1).

## *In - vitro* pollen germination

Table 2 shows that the optimum pollen germination that is, 90.10% was obtained in 30% sucrose with Brewbaker and Kwack's medium (BKM) and the pollen germination were low in other media irrespective of sucrose concentration. No germination was observed in distilled water. A close examination of culture in BKM with 30% sucrose indicated that the germination of pollen grains commenced two hours after dusting but the maximum pollen germination was obtained about 12 h of incubation. Varying concentrations of the media had significant effect on the pollen germination and length of the germination tubes. The mean length of pollen tube was found to be maximum (62.40 µm) at BKM with 30% sucrose (Plate 1e and 1f) while it was minimum (18.60 µm) in BKM with 50% sucrose (Table 2). The pollen tube length decreased either with increase or with decrease in the concentration from 30% sucrose along with BKM. Occasionally due to hyper concentration of sucrose in the medium bursting of the pollen tubes resulted in formation of bisiphanous tubes.

## Pollen storage

Figure 2 shows the effect of different storage conditions on the viability of pollen grains of *T.paniculata*. The pollen grains, stored in Benzene lost viability rapidly and it came down to 15% after 10 h of storage. After 18 h all the pol-

**Table 1.** Deposition of pollen grains on the stigma of *T. paniculata*

| S. No | The day on which / after flower opening | Range of pollen deposition | No. of pollen grains deposited on stigma | Pollination efficiency | Pollination success (in %) |
|---|---|---|---|---|---|
| 1 | The day on which flower opened | 0 – 3 | 1.24 ± 0.2182 | 0.000076 | 8 |
| 2 | First day after flower opening | 3 - 10 | 6.40 ± 0.5363 | 0.00039 | 54 |
| 3 | Second day after flower opening | 6 - 26 | 13.48 ± 1.2920 | 0.00083 | 72 |
| 4 | Third day after flower opening | 0 -1 | 0.98 ± 0.26 | 0 | 0.2 |

Ø Range of pollen deposition, pollination efficiency and pollination success were found to increase from the first day to third day of flower opening; on the fourth day all the three become zero. Mean value + Standard error of 100 samples.

**Table 2.** Effect of different sucrose concentration along with BKM on the pollen germination and length of pollen tube in *T. paniculata.*

| S. No | Sucrose with BKM (in %) | Percentage of pollen germination | Pollen tube length (mm) |
|---|---|---|---|
| 1 | Distilled Water | 0 | 0 |
| 2 | 05 | 15.00 | 23.50 ± 1.26 |
| 3 | 10 | 20.00 | 31.20 ± 0.81 |
| 4 | 15 | 34.00 | 32.20 ± 1.34 |
| 5 | 20 | 46.70 | 42.10 ± 1.34 |
| 6 | 25 | 65.30 | 44.10 ± 1.86 |
| 7 | 30 | 90.10 | 62.40 ± 1.57 |
| 8 | 35 | 76.50 | 52.50 ± 2.25 |
| 9 | 40 | 40.80 | 34.80 ± 1.90 |
| 10 | 45 | 21.80 | 29.40 ± 2.17 |
| 11 | 50 | 10.50 | 18.60 ± 1.31 |

len grains lost their viability. Pollen stored in normal temperature as well as 4°C exhibited nearly the same level of viability during first six hours. and afterwards the decline percentage viability was more pronounced in pollen stored at room temperature than those stored at 4°C. The complete loss of viability under room temperature was at 26 h after storage, but pollen-stored at 4°C had an extended viability up to 38 hrs after storage. Pollen grains stored at –20°C showed loss of viability slowly corresponding to the age of pollen grains. A significant drop in the percentage of viability from 90 to 45% was apparent after 14 h of storage. Afterwards the pollen viability decreased to 20% at 26 h of storage. The complete loss of viability prolonged to 44 hrs of storage under cryopreservation

### *In-vivo* testing of pollen viability

Table 3 shows the results of *in-vivo* testing of viability of pollen grains based on the fruit set. The percentage fruit set was found to decrease towards the collection time of the pollen grains after the anthesis that dusted on the stigma. The pollen grains collected after 15 h of anthesis lost their viability and hence fruit did not set.

## DISCUSSION

Studies of pollen viability, fertility and storage are important for breeding programmes. Pollen germination studies are essential for the estimation of the quantity of the pollen required for controlled pollination. Artificial germination of the pollen grains is a sure test of pollen fertility, which is important for undertaking any breeding program. The medium components required for pollination of different plant species varies (Vasil, 1960). From the present investigation shows, 30% sucrose with BKM was the optimum medium for the germination of pollen grains of *T. paniculata*.

The present investigation shows that pollen germination and tube elongation are two distinct processes differing in their sensitivity to different concentrations of the medium. In many instances due to hyper – or hypo nutrition the percentage of pollen germination and length of the tube and abnormal pollen tube were considerably reduced. Bursting of pollen also increased and occasionally the pollen tubes were observed to eject their contents. *T. paniculata* though characterized by the presence of monosiphonous conditions, at low frequencies bisiphonous conditions were recorded in the present study. In addition to this was various pollen tube

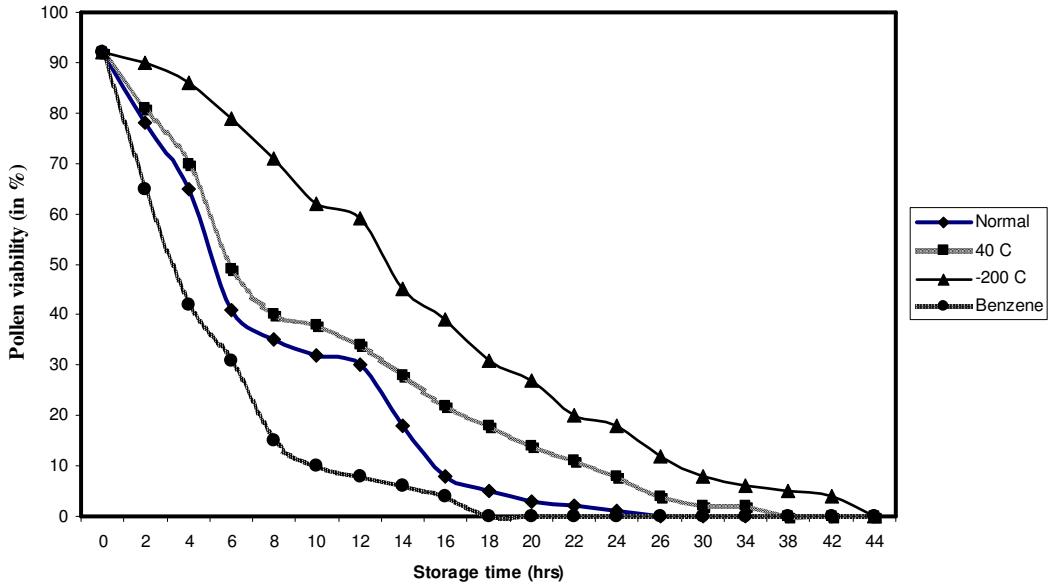



**Figure 2.** Pollen viability in relation to different conditions of pollen storage.

**Table 3**. *In-vivo* testing the viability of pollen grains of *T. paniculata*

| S. No | Pollen collection time (hrs)* | No. of fruits formed | Fruit set (in %) |
|---|---|---|---|
| 1 | Fresh (0) | 164 | 82 |
| 2 | 3 | 148 | 74 |
| 3 | 6 | 122 | 61 |
| 4 | 9 | 94 | 47 |
| 5 | 12 | 66 | 33 |
| 6 | 15 | 0 | 0 |

* Pollen collection time indicates that the pollen was collected at the time of anthesis (0 h) and after the anthesis (3, 6, 9, 12 and 15 h after the anthesis) (n = 100)

deformilities viz. bloating or 'bulla' formation resulting in the swelling of the tip of the pollen tube. Pollen tube grown in a coiled manner was also observed frequently which was due to unstraight tube wall.

Stored pollen grains very much useful to breeding programme to develop the new genotype and conservation. Storage of pollen that retains its viability is a useful method therefore for providing gametes synchronously. Temperature and other factors like humidity, organic solvents appear to be most important factors for the maintenance of pollen viability (Visser, 1955). In the current study, the pollen grains of *T. paniculata* were stored under different conditions. Among them, the cryopreserved pollen grains retained their viability for prolonged time than those stored under other conditions. The factors affecting pollen viability, like the duration for which anthers continue shedding pollen and the range of environmental factors to which they are exposed, are critical for cross-pollinated species of *T. paniculata*, where it has been observed that the stigma becomes receptive 2 – 3 days after flower opening and anthers continue shedding pollen from anther dehiscence. Whether fresh pollen grains were being shed day 1 and day 2 or those shed at the start of anther dehiscence remain adherent to the anther surface could not be ascertained. However, there was a gradual reduction of pollen germination from 92% at the beginning of anther dehiscence to reduce up to 30% after 12h of anther dehiscence. After 24 h of anthesis, all the pollen grains lost their viability. So the present investigations about the pollen biology of *T. paniculata* will assist to study the breeding system, pollen – pistil interactions, gene flow and heterozygosity of the *T.paniculata* populations in future.

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