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Impacts of Canopy Position, Plants' Age and Pollinators on Pre-Pollination Pollen Viability of *Tectona grandis* Linn.

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Abstract: *Tropical trees may change in pre-pollination pollen viability according to the types of pollinator, flower exposure, canopy position, plants' age and pollen predacious ants and thrips. A canopy study system was used and in vitro pollen viability measures, to evaluate relative impact of such factors in one forest tree species, monsoon forest teak, Tectona grandis Linn. of the family Verbenaceae. Relative pre-pollination pollen viability was measured with Alexander's stain, TTC, MTT stain and in vitro pollen germination. The impact of ants and thrips was gauged from bagged and open flowers, and direct counts on flowers in Tectona grandis; canopy position impact (affecting light, exposure, temperature) was gauged using collections made with pole clippers, and by reciprocal transfer of inflorescences on branches. A few native insect species were scored for viable pollen as they first left their nests in early morning. Viability decreased according to ants and thrips presence in Tectona grandis. Pre-pollination pollen viability was higher on the higher flowers. Pollen viability in transplanted inflorescences changed. Relative pollen viability was in agreement using different in vitro pollen viability tests. The frequency of pollen viability increased steadily from low to high canopy height. The percentage of viable pollen was different for different assays showing similar trend to increase. The middle and high canopy height did not show much variation in pollen viability compared to low canopies. So the tree canopy height may attain a vital role in determining pollen viability. The proportion of viable pollen, varied greatly, showing a steady increase with plant age, and the number of pollen grains per flower also increased with age. These results support the qualitative prediction that pre-pollination pollen viability gradually decreases with ageing, reflecting an increase in genetic load with age.*

Key words: *canopy biology, teak plant, pollen, insects*

I. Introduction

Before a pollen grain is released from an anther the environment may alter its germination capacity, and after release it may also undergo modification. Impacts of the floral environment and interacting organisms in this 'pre-pollination' phase of pollen biology have scarcely been investigated in the field (Brewbaker, 1967; Heslop-Harrison, 1986; Oni, 1990; Thomson and Thomson, 1992; Pacini et al., 1997). *In situ* exposure of pollen to thrips that feed on grain protoplasm causes damage before pollen is removed by pollinators (Kirk, 1985), while pollinators may further modify pollen germination capacity (Kearns and Inouye, 1993; Hatjina et al., 1999). I investigated the potential impact of biotic and abiotic environmental factors— exposure and insulation, bee visitors or potential pollinators, and thrips within inflorescences— to test their influence on pre-pollination pollen viability. Although some primarily botanical rules at anther and pistil level, which may vary among individuals (Kearns and Inouye, 1993 & Bhattacharya and Mandal, 2003), might control the physiological functioning of viable pollens. Pollen viability could be assessed by different methods like staining with non-vital dyes, *in vitro* germination test (Heslop-Harrison et al 1984; Shivanna and Johri, 1985) or by *in vivo* test as analyzing the final seed set (Shivanna and Johri, 1985; Razora and Zsuffa, 1986). Non-vital stains are useful to determine pollen viability quickly (Kearns and Inouye, 1993). Analyzing the final seed set is the most reliable method (Shivanna and Johri, 1985 & Bhattacharya and Mandal, 2004) but it is not useful because it takes so much time to get proper information. According to Barrow (1983) *in vitro* germination method is reliable under assumption that pollens capable of germination would be fertile, although there are some difficulties due to arising problems in pollen tube development at *in vitro* conditions (Mulcahy and Mulcahy, 1988). Viability is considered as a phase in which pollen remains able to germinate on an appropriate (receptive and compatible) stigma (Dafni, 1992; Dafni and Firmage, 2000), which varies from species to species, ranging from minutes after shedding to months (Shivanna and Johri, 1985). The importance of studies dealing with pollen viability has been greatly recognized in pollen biology as a priority for helping to understand species reproductive performance and for successful breeding programme implementation (Dafni and Firmage, 2000). The present work has been done in order to know (a) The potential impact of biotic and abiotic environmental factors— exposure of heat, sunlight and insulation on pollen grains of selected plant at pre-pollination stage, insect visitors or potential pollinators, and thrips within inflorescences are investigated to test their influence on pre-pollination pollen viability of selected taxon. (b) The effect of plants' age and canopy height on pre-pollination

pollen viability of selected taxon is also investigated to know the biological and reproductive efficiency of pollen grains with reference to variable canopy height and age of selected taxon. (c) This investigation might be required for further studies on the genetic improvement in the context of ecological model development of this important timber tree species.

II. Materials And Methods

Use of canopy study using pole clipper system allowed sampling of several pollen samples of *Tectona grandis*. Sampling was conducted from the ground, using pole clippers of 5 m in length, at different study sites of West Bengal. Individual open flowers were collected by hand between 8,00 and 13,00 hours, and kept in plastic vials until transported to the laboratory for pollen assays. Up to five treatments were incorporated: 1) flowers collected in the exposed upper canopy (25-35 m) or in full sunlight, 2) flowers in shaded conditions, not at canopy height, 3) flowers that had been bagged to exclude thrips in the bud stage, 4) inflorescences on branches transferred from sun to shaded positions, and 5) inflorescences transported from shade to exposed, sunny conditions. To minimize possible experimental bias introduced by weather or other variables, the displacement experiments were reciprocal, made in the morning between 0730 and 0900 hours, and the flowers were then collected between 1200 and 1300 hours. A branch with all inflorescences intact was removed and tied to a different position, including 20-30 cm of stem. When thrips were present on flowers, their abundance was evaluated by counting the insects in the plastic collection vials which held sample flowers. Ten flowers were collected for each sample of a particular inflorescence. For pollen viability studies on live bees, grains were collected on paper soda straws that were fixed in the bee nest entrance, thereby forcing bees to pass through them. The insect species employed were *Apis indica*, *A. dorsata*, *Xylocopa*, *Ceratina*, *Vespa*, *Haplothrips*, all of which possess small, approximately bee-sized nest entrances. Straw tubes 2-3 cm in length were positioned at bee nest entrances before the first bees emerged, in early morning. The inside portion of the paper tube was then washed with Alexander's stain, TTC and MTT stain on a microscopic slide, then mounted and examined under a light microscope. Ten flowers from each sample were taken to the laboratory where the pollen was removed and then subjected to Alexander's stain, TTC and MTT, using the same mix of pollen from flowers for both assays. Alexander's stain (Alexander, 1969) makes grains appear green when cytoplasm is absent. Although no correlation with germination (Marcellan and Camadro, 1996) was obtained using this stain but it can distinguish viable from non-viable pollen (Alexander, 1969). An *in vitro* test for pollen tube growth termed 'pollen-germination' (Johri and Vasil, 1961) was made using Brewbaker's medium of different strength on pollen samples. As the *in vitro* pollen germination test produces 'pollen tubes' so it may be considered for rapid assessment of pollen viability (Shivanna and Johri, 1989). The Brewbaker's (Brewbaker and Kwack, 1963) solution produced the best initial results and was applied as a small drop (ca. 40-50 μL) with a fine needle to a glass microscope slide, then covered with a cover slip. After two hours had elapsed, 100 grains were scored for pollen tube growth. Pollen grains were counted under a microscope at 400x, examining a total of 100 grains.

Statistical analyses

The experiment was conducted following completely randomized design using at least three replications for each treatment. As random effects were followed, the data were analyzed for two – way analysis of variance (ANOVA, Model II) at test of significance ($P<0.05$) between each treatment. In order to determine the impact of thrips on pollen viability of *Tectona grandis*, the independence of attributes in contingency table was tested using χ^2 statistics. Mean values from all replicates were pooled and standard error of these values was calculated and pair-wise comparisons of all combinations with student's t-tests was made using SPSS software (SPSS Inc., USA) and Zar (1984). Statistical analyses were done using SPSS 14.1 version of statistical software. Mean value of 37 days in each year, collecting pollen samples 3 times at 4-hour intervals in each day were considered. Number of trees were considered to be 17; in 3 canopy heights: L (low), ca.10-15m; M (middle), ca. 15-30m; H (high), ca.>30m. The mean values of pollen viability were calculated by finding the sum of all the individual observations and then dividing the total by the number of observations in each viability test. Standard deviations were obtained from the variance of each test by extracting the square root and were expressed in the units in which the measurements were taken. The standard errors of the mean were calculated from the standard deviation of samples, by dividing it by $\sqrt{n}$ (n is sample size). The correlation coefficient value (R) was calculated by dividing the sum of products of deviations from their respective means by the square root of the products of the sums of squares of deviations from the respective means of the two variables (canopy height vs. plants' age).

III. Results

Pre-pollination pollen viability using Alexander's stain, TTC, MTT and *in vitro* pollen germination tests differed significantly among and between the trees (ANOVA, $F= 5.127$; d.f. = 89, 137; $P< 0.05$) and according to flower height and exposure (high/exposed versus low/shaded flowers, ANCOVA, $F=4.276$; d.f. =

23, 237; P< 0.05). The pollen germination assay using Brewbaker's solution confirmed the same trend. Significant differences occurred in pollen viability between different trees belonging to same species at different eco-zones (F= 5.178, d.f. = 79, 247; P<0.05) and exposure/ heights (F= 6.381, d.f. = 73, 187; P<0.05). Pollen registered lower viability in higher flowers but was significantly higher in the higher flowers in *Tectona grandis* (Table 1). However, the flowers did not display equivalent results according to the four viability tests (Table 1); no differences were revealed at *in vitro* pollen germination. The presence of many thrips in flowers of *Tectona grandis* markedly affected pollen viability and was not independent of flower position in the canopy (contingency table tests of flower height versus viability, χ^2 = 8.925, df = 39, P < .0001). There was no relationship between flower height and pollen viability (contingency table tests, χ^2 = 7.139, df = 84, P > 0.1). Therefore, the thrips had a greater impact than exposure or flower position, but there was no general trend in the position of flowers with more thrips damage. Pollen viability was 49% to 71% less according to Alexander's stain and *in vitro* germination, respectively, in the presence of many thrips (t = 5.91, df = 148, P < 0.001 in Alexander's stain test and TTC and t = 9.36, df = 172, P < 0.001 in MTT stain and *in vitro* pollen germination test). The inconsistencies between the four methods showing a gap of 28 % might be due to conservative appraisal of relative viability, in experimental tests and it is presumed that the four techniques provide a conservative evaluation. An optimal technique might employ sucrose solutions with boric acid (Brewbaker, 1967) that allow natural pollen tube growth to occur. Further pollen viability measurements by fruit/seed set through hand pollination, *in vivo* pollen germination, TTC and/ or FCR stain tests, *in vitro* pollen germination using semi solid agar medium when feasible are of considerable importance to continue to assess viability determinants. Pollen viability was 89.1 ± 4.04 % through Alexander's stain and 71.5 ± 1.9 % through the *in vitro* germination test, compared to 85.4 ± 1.29 % and 56.2 ± 2.52 %, respectively, when thrips were excluded. In *Tectona grandis* pollen damage from thrips in unbagged flowers indicated a 24 % decrease by the Alexander's stain and TTC, but no comparisons were made using the *in vitro* pollen germination and MTT stain tests. 37.5 ± 1.89 % viable pollen was obtained from flowers exposed to some thrips (1-2 per flower) and 37.2 ± 1.97 % viable pollen from flowers exposed to several thrips. The percentage of viable pollens varied between species when flowers on branches were displaced to more insolated or shaded (high versus low) positions. There was no significant change in viability between branches that were transferred reciprocally (from low to high and from high to low positions). The viability of pollen in flowers of *Tectona grandis* increased when inflorescences were transferred from shaded to sunlight positions. The pollens are less frequent viable in low canopy height (ca.10-15m) compared to middle (ca.15-30m) and high (ca.>30m) canopy heights. The highest pollen viability (86.6%) was recorded in middle canopy height in Alexnader's stain solution, TTC, MTT and *in vitro* test where as it was found to be minimum (29.5%) at *in vitro* germination. The percentage of pollen viability in each canopy of different trees differs, but the general trends of pollen viability in different canopy heights remained same, showing the sequence of low<middle<high canopy height. The mean percentages of viability vary in 4 assays. The high frequency of viable pollens was obtained using Alexander's stain, while it was less in TTC and *in vitro* culture medium. Pollen abortion differed significantly (df = 15, 64; F = 26.8; p ≤ 0.001) among the tree age groups, with highest abortion rates in the oldest age groups.

Table 1: Pollen viability measured in *Tectona grandis*. Mean percentage, standard error and student's t-tests of differences between replicates at P < 0.05.

Pollen viability (%)

Plants' name	Canopy position	Alexander'sstain	TTC	Significance
Tectona grandis	High canopy	87.9 ± 8.63, n= 149	72.3± 9.09, n=146	NS, NS df=174, 162
	Low canopy	83.5 ± 7.15, n=127	65.98 ± 8.26, n=118	
	Shifted- high to low	78.5 ± 3.79, n=125	52.7 ± 8.49, n=122	S, S df=136, 130
	Shifted- low to high	85.3 ± 5.2, n=113	45.8 ± 2.83, n=110	

Plants' name	Canopy position	MTT stain	*In vitro* germination	Significance
Tectona grandis	High canopy	77.9 ± 8.57, n=120	73.8 ± 5.6, n=120	S, NS df=142, 84
	Low canopy	66 ± 5.42, n=124	62.3 ± 7.12, n=124	
	Shifted-high to low	69.5 ± 8.27, n=110	65.4 ± 7.06, n=110	NS, S df=118, 58
	Shifted- low to high	83.2 ± 5.09, n=110	44.9 ± 2.91, n=110	

Plants' name	Canopy position	Alexander's stain	TTC	Significance
Tectona grandis	High canopy	88.4±9.32, n=132	79.9±1.87, n=132	S, S df=162, 82
	Medium canopy	79.9±3.49, n=132	56.8±3.03, n=132	
	Low Canopy	79.5±3.02, n=118	44.9±3.28, n=118	S, S df=133, 63

Plants' name	Canopy position	MTT stain	In vitro germination	Significance
Tectona grandis	High canopy	88.2±2.32, n=142	79.9±5.87, n=132	S, S df=162, 82
	Medium canopy	82.3±3.49, n=132	36.5±2.03, n=132	
	Low Canopy	89.4±4.02, n=118	64.3±2.28, n=118	S, S df=133, 63

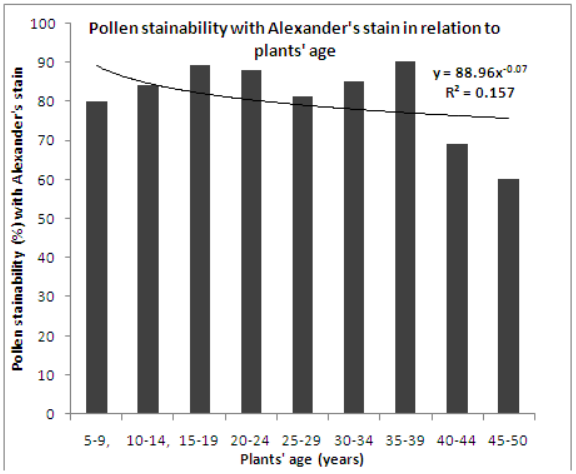

Fig. 1: Pollen stainability with Alexander's stain in relation to plants' age

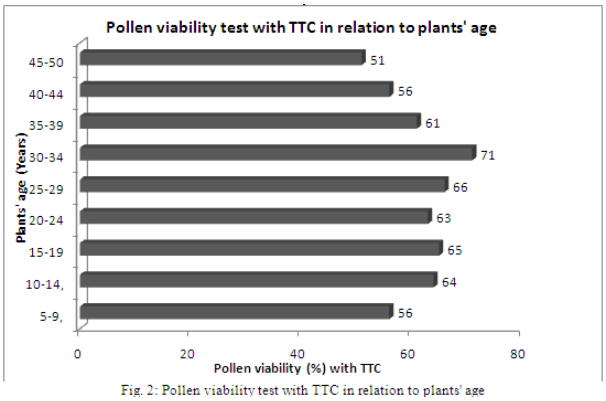

Fig. 2: Pollen viability test with TTC in relation to plants' age

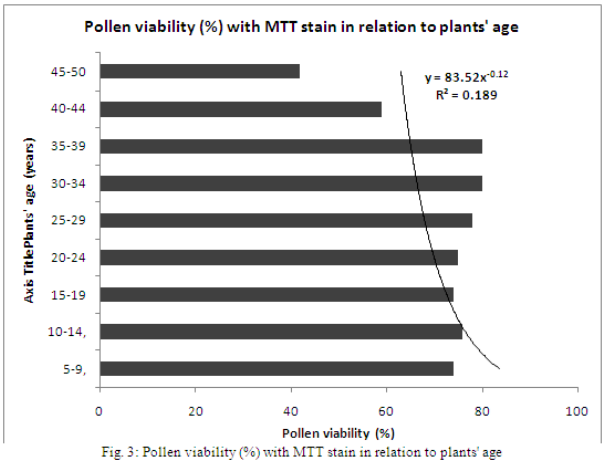

Fig. 3: Pollen viability (%) with MTT stain in relation to plants' age

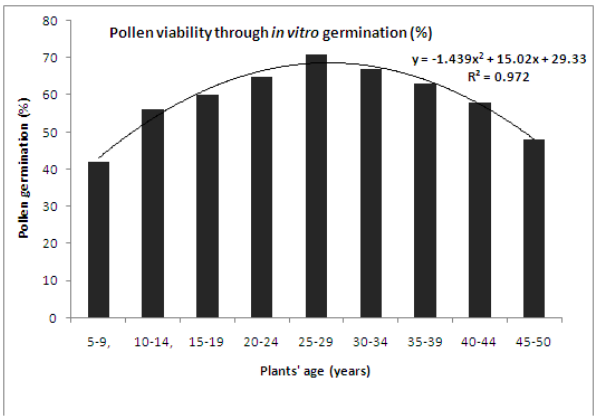

Fig. 4: Pollen viability through *in vitro* germination (%)

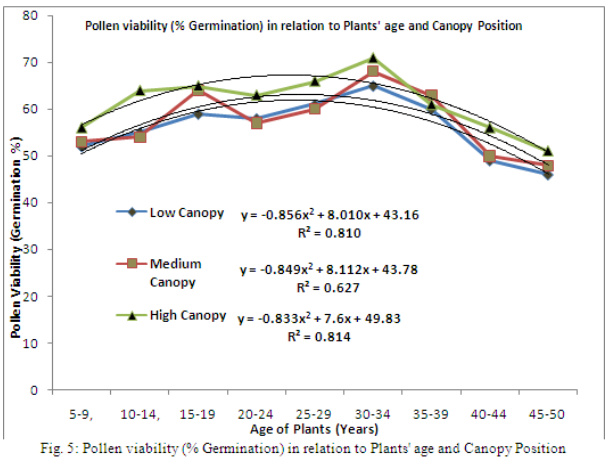

Fig. 5: Pollen viability (% Germination) in relation to Plants' age and Canopy Position

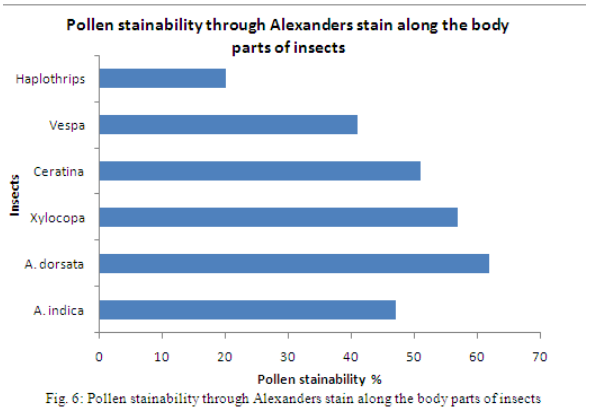

Fig. 6: Pollen stainability through Alexanders stain along the body parts of insects

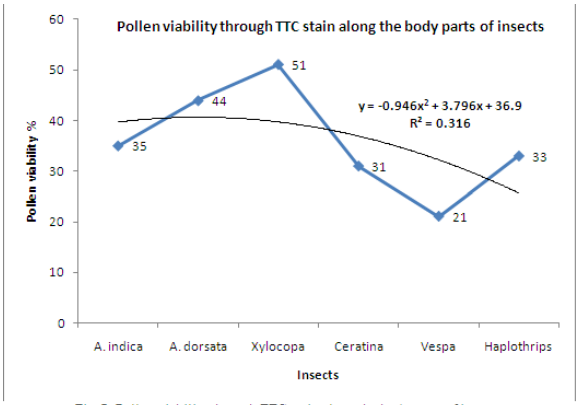

Fig. 7: Pollen viability through TTC stain along the body parts of insects

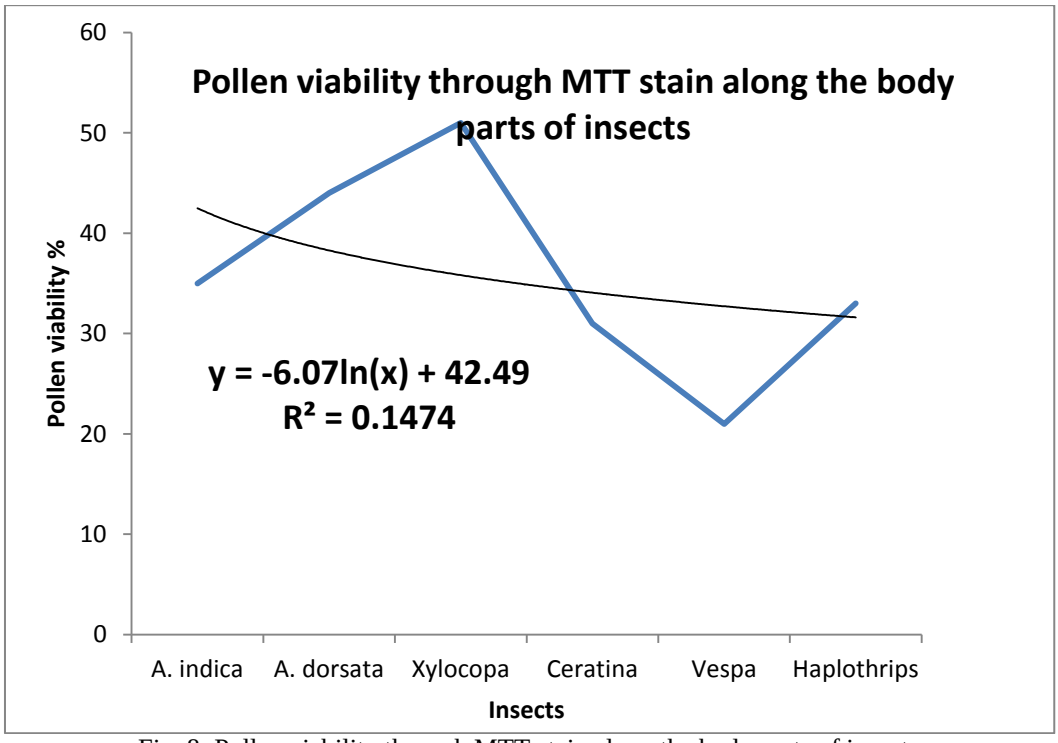

Fig. 8: Pollen viability through MTT stain along the body parts of insects

IV. Discussion

After release from the anthers pollen grains are exposed to potentially hostile environments, such as dry or humid conditions, and yet they perform well and germinate to pollinate the stigma and fertilize the ovule in varied conditions (Heslop-Harrison, 1986). Thomson and Thomson (1992) have shown that pollen viability schedule deserve equal attention to pollen exposure schedules as an object of quantitative study for evolutionary ecologists. The pollen viability might be influenced by different factors like time of anthesis, temperature, humidity, seasonal effect, flower position, anther protection, age of pollen, pollinator type, pollen packaging, number of nucleus, carbohydrate content and desiccation risk, pollen metabolism and genetic variability (Dafni and Firmage, 2000). There are known limitations of tri-nucleate pollen viability that depend upon the elapsed time since anthesis (Brewbaker, 1967). As indicated by this author, the species studied are bi-nucleate. Assessment of pollen viability is critical for the study of pollination and gene flow in natural conditions (Shivanna et al., 1991; Dafni, 1992; Mulugeta et al., 1994; Dafni and Firmage, 2000). The term viability has been used to describe pollen grains capable of germinating on the stigma (Niesenbaum, 1992), germinating *in vitro* (Lindgren et al., 1995), taking up stains (Nyman, 1992), and causing fruit set (Smith-Huerta and Vasek, 1984). However, flower height in the canopy, heat and sunlight, and viability on pollinator bodies (not where pollen is stored after collection as food) have seldom been considered for tropical trees. The present work shows the variations of pre-pollination pollen viability in selected taxon investigated and is also dependent upon sunlight exposure, canopy position, and presence of thrips and insects' activity. The impact of such biotic and abiotic factors upon pollen viability of selected tropical trees in natural conditions might be reflected with regard to ecological implications upon pre-pollination pollen viability. The variations may be due to the desiccation factor, type of pollen exposure, pollinator behaviour and environmental factors. According to Pacini and Viegi (1995) and Speranza et. al. (1997) the pollen viability is influenced by carbohydrate type and water content. Dafni and Firmage (2000) mentioned that the type of pollen exposure and pollinator behaviour impose as ecological factors and pose impacts upon pollen viability. Available literature suggests that pre-pollination pollen viability is often greater than 48 % in laboratory conditions, depending on pollen age and taxon (Eisikowitch and Woodell, 1974; Sahar and Spiegel-Roy, 1984; Vasil, 1987; Steer and Steer, 1989; Demeke and Hughes, 1991; Mulugeta et al., 1994; Aizen and Rovere, 1995; Marcellan and Camadro, 1996), but still it may vary widely between individual plants (Free and Williams, 1976; Oni, 1990) and often within same individual. The present work also suggests abiotic and biotic factors do determine the pollination potential of pollen in field conditions, that biotic factors may play a greater role than previously realized, and that there are consistencies of results from Alexander's stain, TTC, MTT and *in vitro* germination tests. As emphasized by some authors (Owens et al., 1991; Dafni 1992) Alexander's stain does not demonstrate germination capacity but only that the pollen is intact. This was a convenient method for assay of damage caused by thrips and other insects which remove pollen contents. The results of all tests seemed conservative, particularly the low values found for *in*

vitro germination assay; failure of this technique with bees means that other assays should be attempted. An optimal technique often employs sucrose solutions with boric acid (Brewbaker's medium, adjusted for tri or bi-nucleate pollen (Brewbaker, 1967) that allows natural pollen tube growth to occur. The influence of thrips on pollen viability was demonstrated to have a pervasive influence, more than the physical variables included in shaded and exposed positions, in the species studied. It is unclear if there was active selection of higher or lower flowers where thrips concentrated, but pollen viability in the field cannot be studied without considering the location and abundance of thrips, which may vary within a tree, between individual trees, and between species of one tree genus. There is poor explanation for the wide variation in pollen viability caused by inflorescence transfers to different conditions, but it is interesting that the large tree, *Tectona grandis* which has small flowers with exposed anthers, responded in contrasting ways to heat, exposure and sunlight. High canopy flowers of *Tectona grandis* produced pollen of higher viability, but the pollen in flowers transferred from low to high positions was reduced. In selected taxon, the position within the canopy in normal conditions did not influence pollen viability, but thrips damage was concentrated on higher flowers, thus pollen viability may tend, as in *Tectona grandis*, to be higher in canopy flowers. Also, transfer of flowers to more exposed positions in the high canopy resulted in higher pollen viability. According to Thomson and Thomson (1992) and Dafni and Firmage (2000) the pollen viability becomes variable as influenced by flower/canopy position, light exposure and presence or absence of pollinators. Exposure is likely to become accentuated in converted or degraded tropical forests, or in forest gaps (Dixon et al., 1994; David, 1998), and the response of *T. grandis* would seem better adapted to such conditions. Viability of pollen has been defined as having the capacity to live, grow, germinate or develop. It has been reported that pollen viability is so liable that it may differ when pollen is collected at different times of the day (Baez et al., 2002 and Davarynejad et al., 2008). Pollen collected from flowers in anthesis for one-hour show decreased germination (Shivanna and Tangaswamy, 1992). Pollen viability has a genetic component; results may be different depending on the genetic variability of individuals used as donors (Meo, 1999). The use of Alexander's procedure in present investigation may have led to overestimation of pollen viability since staining capacity depends not on the viability but on protoplasm content of the pollen grains. So, this measure of pollen stain ability may depart considerably from real value of pollen viability (Dafni, 1992). The proportion of viable pollen grains steadily increases with canopy height. Canopy height >30 m exhibited an average proportion of viable pollens 1-1.5 times greater than the one found in trees <30 m. Pollen may express genetically based traits during its development, maturation and free dispersal phases. Reproductive effort, physiological stress, resource availability may be the factors for variation in pollen viability. Populations of out crossing plants are far from being genetically uniform (Heywood, 1991 and Bhattacharya et al., 2005), and may constitute important sources of variability. Accumulation of somatic mutations with increasing heights might be a concept to our understanding of the pollen viability. Regarding germination *in vitro* the culture is dependent on the quality of pollens (Heslop-Harrison et al., 1984). Temperature has appeared as a critical factor for *in vitro* germination. The percentage of stigma receptivity increased steadily from low to high canopies showing minor differences between them. Receptivity of the stigma is a critical factor for the successful completion of post-pollination events. Receptivity is generally maximal soon after anthesis. The period of receptivity varies from species to species, and is influenced by temperature and humidity. Alternation in temperature and humidity drastically reduce the period of stigma receptivity. The duration of stigma receptivity varies from a few minutes to two or three weeks (Dafni, 1992 and Fohouo et al., 2008). The age of the flower, time of the day, and the presence or absence of stigmatic exudates may attain importance in determining stigma receptivity (Dumas and Gaude, 1983). The results of this study show a steady increase in pollen viability with tree age. Even though the average pollen size was similar between the two pollen types, viable pollen had a smaller frequency maximal size than non-viable pollen. This suggests that environmental and physiological stress does not play a role in pollen viability in this species. Instead, increasing viability levels are probably due to mutation during cell division in meristematic cells, or attributed to the accumulation of genetic load with increase in age. According to Klekowski and Godfrey (1989) the number of mutant cells should increase linearly with time and age with an increase in genetic load. Pollen grains might be aborted due to developmental breakdown at pollen development stage within anthers when several genetically mutated traits express. Mascarenhas (1992) reported that pollen expresses genetically based traits during its development including lethality. Higher P:O ratio with increase in age should not be ignored as a general cause of pollen viability. The older trees produced a greater number of pollen grains per flower than the younger ones through random meiotic division from somatic cells, which probably maximize genetic pressure leading to viability. The effect of genome-plastome interactions on meiosis during pollen development may lead to pollen viability (Chapman and Mulcahy, 1997). Moreover, this study on the pollen viability of *T. grandis* with increase in plant age provided primary information for further analyses of pre-pollination pollen viability and its dependence on tree age at the community level.

V. Conclusions

Various factors influence pre-pollination pollen viability of *Tectona grandis* in tropical field conditions. Thrips may effectively remove a large portion of pollen and exert more influence than physical exposure. Some flowers increase in pollen viability when exposed to gap conditions, but others show the opposite trend, with implications for degraded habitats. Bees and other insects may secondarily disperse viable pollen from their nests, in which it retains viability from one day to the next. Relative pollen viability was in agreement using different stains and *in vitro* tests. The tree canopy heights attain a vital role in determining pre-pollination pollen viability, the major factor for new offspring production in natural/cultivated forest settings. Appropriate management of pre-pollination pollen viability in this tree species is valuable for success of pollination programmes within and between genotypes of forestry interest. Comparisons of the expression of the accumulated genetic load in the gametophytes within single individual or across individuals of same species differing in heights may represents the basic points of making forest tree population genetics models. This study on the pre-pollination pollen viability of *Tectona grandis* with increase in plant age provided primary information for further analyses of pre-pollination pollen viability and its dependence on tree age at the community level.

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