Table 1. Production of chimaeric mice from R1.9 ES cells

Technique	No. of manipulated embryos	No. of recipient females	Total pups born	No. of chimaeras	Sex ratio of chimaeras male : female
Morula aggregation (wild-type R1.9 cells)	65	5	14	6	5 : 1
Blastocyst injection (targeted R1.9 cells)	136	10	20	7	6 : 1

(from ES cells) in the background of albino coat component of CD1 host embryos (Figure 2). The chimaeras exhibited varying degree of ES cell contribution (15 to 95%) as judged from the extent of agouti coat colour. Sex ratio was skewed in favour of males (R1.9 is a male ES cell line) among the chimaeras as was expected after introduction of male ES cells into unsexed embryos. Breeding of the chimaeras resulted in progeny with the chinchilla and agouti coat colours (Figure 3) proving the germ line potency of the R1.9 ES cell line. Subsequently, we have used R1.9 ES cells to introduce targeted deletion of κ-casein gene. R1.9 clones mutated at this locus were injected into FVB/N or C57BL/6J blastocysts (Table 1). Blastocyst injection involved microinjection of 10–15 disaggregated ES cells into the blastocoel. The manipulated blastocysts were transferred without any further culture to the recipient females[7,10]. We obtained pups carrying the mutation. Three chimaeras, out of seven tested for germ line transmission, produced pups from the ES cell component. The efficiency of germ line transmission in these experiments (42.9%) based upon the number of chimaeras bred was better than that of original R1 ES cell (25.3%)[5]. R1.9 ES cell line, isolated and established in our culture conditions, is now routinely being used for inserting defined genetic modifications into mouse germ line.

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Age-dependent pollen abortion in cashew

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The effect of plant age on pollen abortion of *Anacardium occidentale* L., family Anacardiaceae, was tested. Using Alexander's stain, which differentiates aborted and non-aborted pollen grains, the proportion of aborted pollen versus plant age, size variation in aborted and non-aborted pollen grains, and number of pollen grains per flower were estimated. Aborted pollen was smaller than non-aborted pollen, regardless of plant age. The proportion of aborted pollen, varied from 22.5 to 46.8%, 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 pollen abortion gradually increases with ageing, reflecting an increase in genetic load with age.

CASHEW, *Anacardium occidentale* L. (Anacardiaceae), is a hardy, drought-resistant, tropical or subtropical tree. It is distributed in tropical America, from Mexico and West Indies to Brazil and Peru[1]. Ranging from warm temperate moist to tropical very dry to wet forest life zones, cashew is reported[2] to tolerate annual precipitation of 7–42 dm, annual temperature of 21–28°C, and pH of 4.3–8.7. Globally, India is the leading producer; other countries producing cashew include Mozambique and Tanzania[3]. The demand for cashew is increasing, but the availability of seed is often a limiting factor for several reasons[4–6]. Consequently, an understanding of the factors affecting cashew seed production has impor-

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tant practical applications for both genetic improvement and operational seed production. In most vascular plants, gametophytes and gametes are produced by apical meristematic tissue differentiation. As long-lived perennials age, apical meristems are temporally far removed from the first mitotic divisions in the embryo, and may also be spatially far removed as the plant grows in size. Some mutant and deleterious alleles may be expected to accumulate in dividing merestimatic tissues over time as a result of DNA-copying errors with successive cell divisions[7]. Pollen grains are haploid, and commonly express the consequences of genetic load accumulation by sporophytes through abortion. Molecular analyses have also shown that a large proportion of the plant genome is actively transcribed during the life span of a male gametophyte[8–10]. Thus, studying the phenomenon of pollen abortion in tree species could aid in the development of tree-improvement programmes. The present investigation was designed to examine the effect of plant ageing on pollen abortion using the long-lived, outbreeding *Anacardium occidentale* L.

A. occidentale is an insect-pollinated, spreading, evergreen, perennial tree. Trees commonly attain a total height of 2.5–12 m (ca. 8.5 m tall) and ages of > 40 yrs. During blooming, the tree canopy is loaded with thousands of flowers in panicles. Flowers numerous, male or hermaphrodite, radially symmetrical, each flower has ten small anthers and one pistil containing one ovule. Pollen grains are oblate spheroidal, three-colporate and psilate type, being 17.1 ± 1.2–19.5 ± 1.4 μm in diameter[11].

The study was carried out during peak flowering (April–May) in an open population of *A. occidentale* (10 to > 40 yrs of age and ca. 2.5 to 12 m height). Twenty-one trees were randomly selected within 0.35 km^2 area that was relatively homogeneous with respect to sunlight exposure, soil texture and slope. Tree height was measured with the help of metallic measuring tape. Age was determined taking stem cores at breast height and counting annual rings. According to Veblen and Lorenz[12], ring counts give a reliable estimate of tree age. On each of the seven sampling dates, ten newly opened flowers, chosen at random from low, middle and high branches of each tree, were collected in the morning just after flower anthesis (before insects/thrips visit), which occurred between 7 and 11 am; three slides per collection were prepared. Immediately after flower collection, a drop of Alexander's stain[13] was put onto the slide, pollen from immediately dehisced anthers (not foraged by flower visitors) was put into the stain solution with the help of a platinum needle, gently heated, and mounted on a microscope slide. Using 400x magnification, 200 pollen grains per slide were scored as aborted or not aborted. A pollen grain was considered to be aborted if it was empty or had highly degenerated protoplasm[13]. Non-aborted pollen is characterized by the presence of thick walls (ca. 3.5–5 μm), while the walls of aborted pollen become thin (ca. 1.5–2.5 μm) and optically refringent. As pollen production per flower is an accurate measure of reproductive success, it was counted following the procedure of Dafni[14], with plant age. The mean pollen number per flower was estimated by crushing ten mature and undehisced anthers of a flower in a drop of 10% glycerin and safranin on a microscope slide. Under 400x magnification, the total number of pollen grains was counted. Ten flowers per tree were sampled to obtain mean number of pollen grains per flower, which was converted into log value. Correlation analysis was used to examine the relationship between plant age and height. Size differences between aborted and non-aborted pollen grains were compared by using Student's *t*-test. The effect of plant age on changes in pollen abortion and pollen production was analysed using analysis of variance and pairwise comparisons of all combinations with Student's *t*-test[15].

A. occidentale varied in age from 10 to > 40 yrs and height from 2.5 to 12 m (Figure 1). The positive correlation between the two attributes (age and height) is highly significant ($y = 0.2228x + 1.685$; $R^2 = 0.5113$; df = 20, $P < 0.05$). Thus, in *A. occidentale*, tree age can be estimated by measuring tree height. While average pollen grain size was similar (18.35 ± 1.21 μm), the grain size frequency of aborted and non-aborted pollen differed (df = 513; $t = 11.87$, $P \le 0.001$; Figure 2). The maximum frequency (71 ± 8.23%) of the size of aborted pollen ($n = 285$) was 13–16 μm and the frequency maximal (78 ± 7.51%) of the size of non-aborted pollen ($n = 230$) was 17–19 μm. Exponential increase of pollen

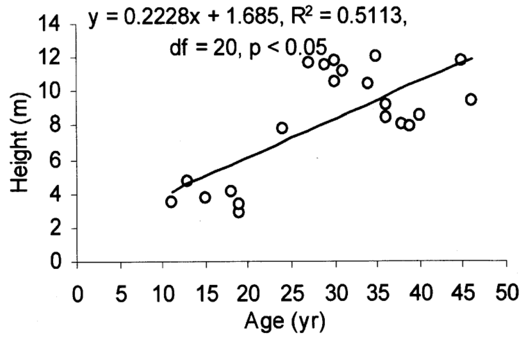

Figure 1. Relationship between height and age of *Anacardium occidentale.*

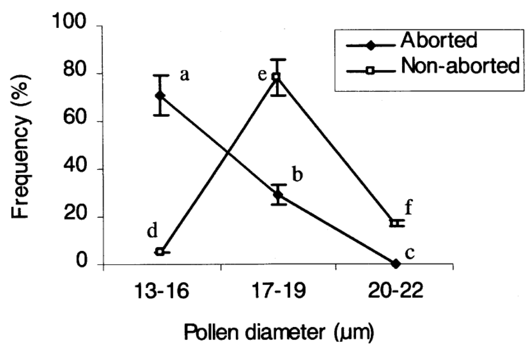

Figure 2. Frequency (%) distribution vs pollen diameter (μm) of aborted and non-aborted pollen grains of *A. occidentale.*

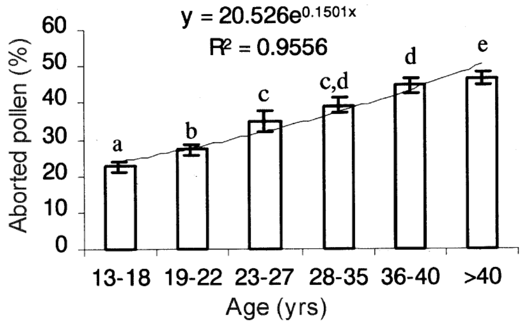

Figure 3. Mean proportion (± SE) of aborted pollen grains for each age group of *A. occidentale*. Means followed by the same letter do not differ significantly.

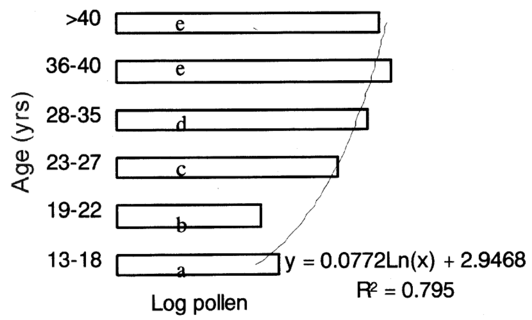

Figure 4. Pollen production (log value) per flower for each age group of *A. occidentale*. Different letters indicate significant differences.

abortion with increase in plant age ($y = 20.526e^{0.1501x}$; $R^2 = 0.9556$) was found, which differed significantly (ANOVA; $F_{5,48} = 22.8$, $P \leq 0.05$) among the different age groups (Figure 3), with highest abortion rates in the oldest age groups. Age-wise variations on the number of pollen grains per flower (log value of pollen number) were found ($y = 0.0772\ln(x) + 2.9468$; $R^2 = 0.795$), with significant differences (ANOVA; $F_{5,48} = 12.9$; $P \leq 0.05$) among the trees of different age groups showing an increase with the increase in plant age (Figure 4).

The results of this study show a steady increase in pollen abortion with tree age. Even though the average pollen size was similar between the two pollen types, aborted pollen had a smaller frequency maximal size than non-aborted pollen. This suggests that environmental and physiological stress does not play a role in pollen abortion in this species. Instead, increasing abortion 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[16], 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 the pollen development stage within the anthers, when several genetically mutated traits are expressed. Mascarenhas[10] reported that pollen expresses genetically based traits during its development, including lethality. Higher pollen production with increase in age should not be ignored as a general cause of pollen abortion. 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 maximizes genetic pressure leading to abortion. The effect of genome–plastome interactions on meiosis during pollen development may lead to pollen abortion[17]. Moreover, this study on the pollen abortion of *A. occidentale* with increase in plant age has provided basic information for further research of pollen abortion and its dependence on plant age, environmental and/or physiological stress and genetic pressure on plant population.

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