

The Reproductive and Seed Biology of *Glycosmis pentaphylla* (Rutaceae)

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ABSTRACT The floral, fruit and seed biology of *Glycosmis pentaphylla* (Rutaceae) was investigated. Anthesis occurred at 0700 - 1300 hours. Flower abortion was 27% and fruit abortion was 95%. Anther wall formation was of the basic type and pollen grains were shed at the 2-celled stage. Pollen grains are trizonocolporate, radiosymmetric and prolate spheroidal. They showed maximum germination in 4% sucrose solution and lost their viability four days after anthesis. The coefficient of uniformity of germination for extracted seeds sown was 0.04 compared to 0.01 for intact fruits sown. Seed germination was less than 10% after storage in 5°C, 15°C and 28°C for 25 days although the viability of fresh seeds tested by germination was very high (97%). Seed germination is hypogeal and both fasciation and polyembryony occurred rarely.

ABSTRAK Biologi bunga, buah dan biji *Glycosmis pentaphylla* (Rutaceae) telah dikaji. Waktu antesis adalah di antara jam 0700 dan 1300. Kadar keguguran bunga adalah 27% dan kadar keguguran buah, 95%. Pembentukan dinding anter adalah jenis asas dan debunga matang disembarkan pada peringkat dua sel. Debunga berbentuk trizonokolporat, radiosimetrik dan prolata sferoid. Percambahan maksimum debunga dicapai dalam larutan sukrosa 4% dan dayahidupnya merosot empat hari selepas antesis. Koefisien keseragaman untuk percambahan bagi biji benih yang ditanam adalah 0.04 manakala bagi buah adalah 0.01. Percambahan biji adalah secara hipogeal dan fasiasi serta poliembryoni jarang berlaku.

(Reproductive biology, *Glycosmis*, anther development)

INTRODUCTION

Glycosmis pentaphylla (Retz.) DC. belongs to the family Rutaceae, which has many species of economic importance like *Citrus grandis* Osbeck, *C. reticulata* Blanco and *C. sinensis* Osbeck. The family is outstanding for the presence of aromatic oil glands in leaves, flowers and fruits, which may be a significant source of essential oils. The genus *Glycosmis* was established by Correa de Serra in 1805, from *Limonia pentaphylla* Retz. and *Limonia arborea* Roxb. [1]. It has about 30 - 40 species in South East Asia, Malesia and South China; with 14 taxa in Malaya [2]. It is the second largest genus after *Citrus* L. in Peninsular Malaysia and many of the species are endemic [3]. *Glycosmis pentaphylla* is usually planted as an ornamental plant for its sweet-smelling

flowers and attractive fruits. The flowers are bisexual and borne in axillary panicles.

Data on the reproductive biology of *G. pentaphylla* is rather fragmentary [4, 5] and this study aims to document the pollen development, floral, fruit and seed biology of *G. pentaphylla*.

MATERIALS AND METHODS

Floral and fruit development

Floral and fruit development of *G. pentaphylla* were observed from April to July 2002 in the Rimba Ilmu Botanic Garden, University of Malaya. Fifty buds and 60 fruits from five inflorescences were randomly selected and their length and diameter measured with vernier calipers at weekly intervals. Another seven

inflorescences were tagged, left exposed to pollinators and their development was traced.

Anther development and pollen studies

Buds and flowers at various stages of development were collected and fixed in Craff III solution. Standard paraffin techniques [6] were employed in preparing serial sections of 6- μ m thickness.

Fresh pollen grains were collected, acetolysed according to standard techniques [7] and mounted in safranin-stained glycerine jelly. The length and diameter of 30 pollen grains were measured using a Leica QWin Image Processing and Analysis System. The ratio of polar length and equatorial diameter (P/E ratio) was calculated. Pollen was cultured in 0 to 10% sucrose concentrations using the sitting drop technique [8].

Seed biology

Each fruit contained a single seed. Eighty mature fruits were collected and 40 extracted seeds after removal of the pericarp while another 40 intact fruits (whole fruits) were sown in garden soil at 25 - 32°C. A seed was considered germinated when its plumule reached 1 cm above soil level. Seed germination percentage, germination rate and the coefficient of uniformity of germination (CUG) were calculated according to Bewley and Black [9].

For seed storage, no humidity control was applied. 120 seeds were washed with 2% Clorox, rinsed with sterile distilled water and oven-dried at 40°C for 24 hours. The seeds were divided into four batches, with 30 seeds each receiving treatments as follows: a) no storage (sown directly as control); b) stored at 5°C in a refrigerator; c) stored at 15°C in a refrigerator; d) stored in a laboratory drawer (temperature \pm 28°C). Seed storage was for 25 days before being sown to determine their viability which was also tested using 1% tetrazolium chloride solution. The Statistica version 5.5 programme was used for the analyses.

RESULTS

Floral development

Glycosmis pentaphylla flowers throughout the year. The young flower buds (length 1.47 ± 0.19 mm, diameter 1.27 ± 0.17 mm) were green with

brownish cilia on the margins of the sepals. After about one month, the buds started to change from green to yellow with green dotted glands all over the petal surfaces. After another 11 days i.e., on day 42 from the first observation, most of the buds (length 4.65 ± 0.54 mm, diameter 2.98 ± 0.22 mm) reached anthesis. Initially, there was hardly any noticeable growth for the first 30 days (see Figure 1), then it accelerated until anthesis.

The flowers bloomed asynchronously in the same inflorescence, anthesis occurring at random; some early and some could be delayed until three months later. One or two flowers were seen to develop faster and reached anthesis much earlier than (2 - 3 months ahead of) the others in the same inflorescence. Anthesis occurred in the early morning, even before 0800 hours and lasted until around noon. The peak hours of anthesis were from 0830 hours till 1000 hours during which many insects, such as bees, wasps, red ants and butterflies were seen visiting the flowers. The petals were not wide open but slightly loosened, and bees and wasps were seen prying open the petals to access nectar and pollen. Flowers were considered to reach anthesis when the margins of the petals were clearly visible and the petals had loosened. During anthesis, the flowers were sweet-scented; the stigmas glistened with a sticky, crystal-like substance secreted; the anthers were yellow and had started to dehisce longitudinally to release the pollen; and the pale green nectaries secreted clear, sticky nectar. The anthers with shorter filaments ("lower anthers") dehisced some time later in the day than the anthers with longer filaments ("upper anthers").

A day after anthesis, the anthers degenerated, turned brownish and gradually became black on the third day before abscission. The petals also abscised a day after anthesis, revealing the degenerated anthers, and abscised faster if insects had visited the flower or if there had been rain. They could persist up to two days if left undisturbed. Meanwhile, calyces and stigmas persisted; the stigmas were white but still looked fresh and receptive even 2 - 3 days after anthesis before turning brown. The nectaries eventually dried up and turned white.

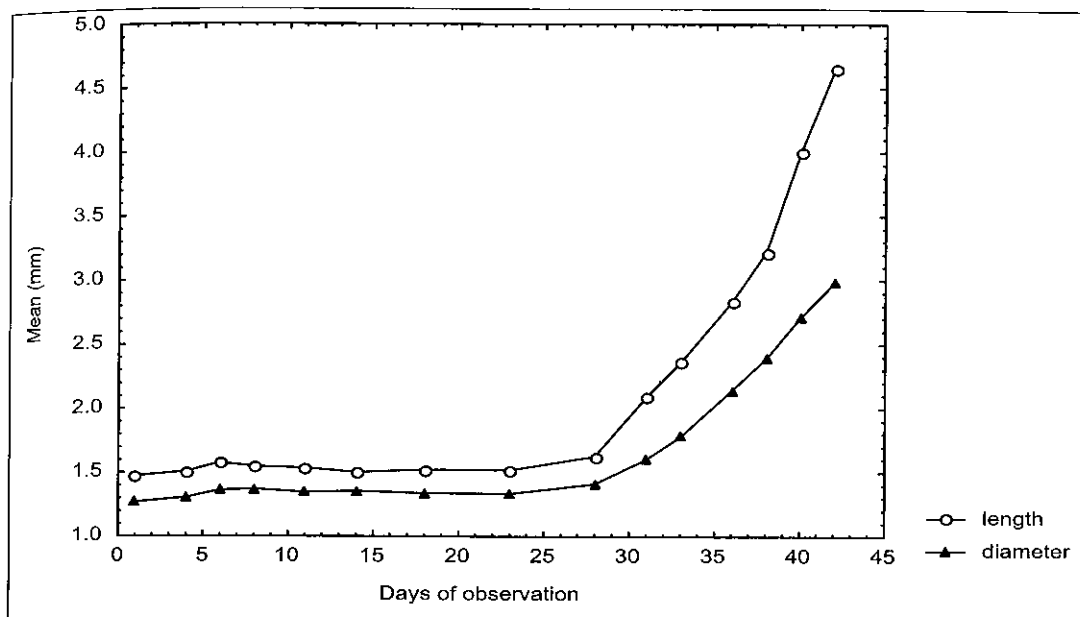


Figure 1. Mean growth in length and diameter of 50 flower buds from the first day of observation

Fruit development

Among tagged inflorescences, the first (A) to reach anthesis was on 24/5/2002, the second (B) on 28/5/2002 and the third (C) on 4/6/2002. As shown in Figure 2, the mean fruit length and diameter increment with time for the three groups all showed a sigmoid curve. The average diameter and length of the fruits one week after anthesis were 1.38 ± 0.08 mm and 1.02 ± 0.16 mm, respectively. The fruit took about 34 - 47 days from the day of anthesis to maturity before abscission. The average diameter and length of the mature fruit were 11.31 ± 0.76 mm and 9.38 ± 0.75 mm, respectively.

The young fruits were green and turned pale pink after 30 - 40 days and finally translucent pink before abscission a day or two later.

Pollen studies

From Table 1, the percentage of flower abortion varied in the inflorescences from nil to 79.1%

with the mean percentage of 27.1, which was very much lower than fruit abortion at 94.5%.

Pollen grains are trizonocolporate, radiosymmetric, of the amb type, and fossaperturate (Figure 3A, 3B). The length was 20.70 ± 1.32 μ m, diameter at polar view was 17.02 ± 1.39 μ m, and diameter at equatorial view was 16.50 ± 1.28 μ m. The P/E ratio $\times 100$ was 103.15 and under Erdtman's classification [10], the grains were prolate spheroidal and had small pores.

One-way ANOVA comparison of the percentage pollen germination and pollen tube length at the six different sucrose concentrations showed significant differences at $p < 0.05$ (Table 2). Fresh pollen grains showed maximum germination of 33.2% in 4% sucrose solution, with tube length of 0.42 ± 0.08 mm. At 4% sucrose solution, the pollen grains showed a gradual decline in viability on subsequent days after anthesis and lost their viability on the fifth day.

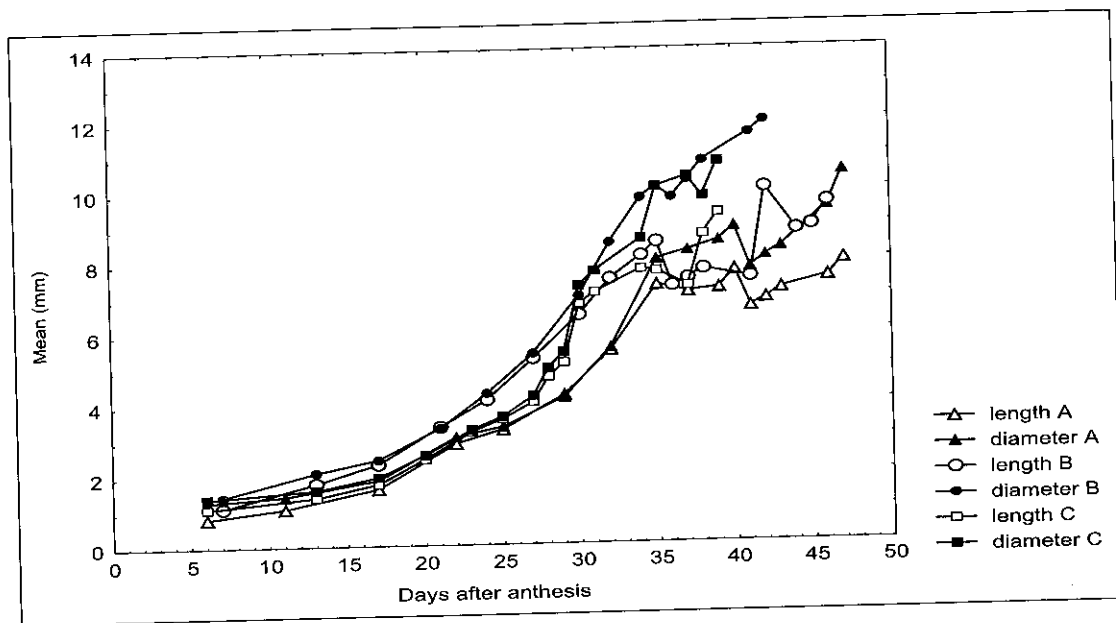


Figure 2. Mean growth in length and diameter of fruits from the day of anthesis. A reached anthesis on 24/5/02, B on 28/5/02 and C on 4/6/02

Table 1. Flower and fruit abortion in *G. pentaphylla*

INFLORESCENCE	NO. OF BUDS	NO. OF FLOWERS IN ANTHESIS	FLOWER ABORTION (%)	NO. OF MATURE FRUIT	FRUIT ABORTION (%)
1	47	45	4.3	3	93.3
2	33	9	72.7	0	100.0
3	285	217	23.9	3	98.6
4	200	200	0	9	95.5
5	43	9	79.1	1	88.9
6	51	46	9.8	3	93.5
7	25	25	0	2	92.0
Total	684	551		21	
Mean			27.1		94.5

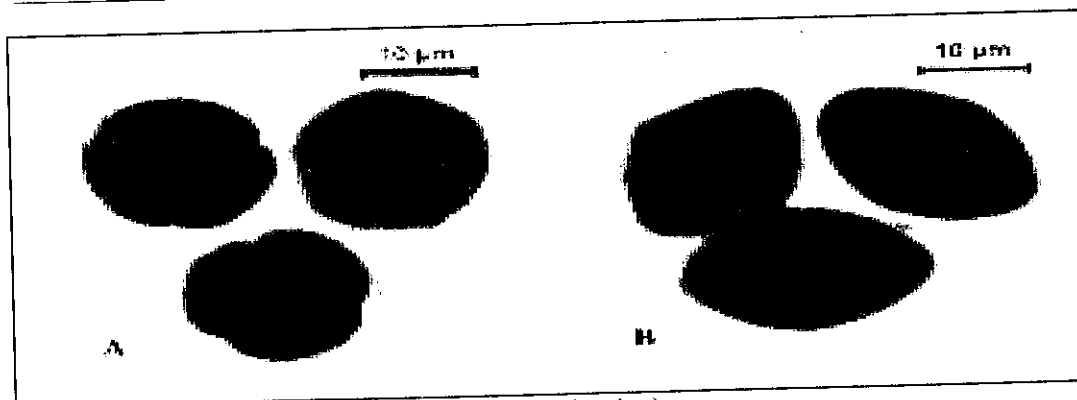


Figure 3 A (left). Pollen morphology (Pollen, polar view)

Figure 3 B (right). Pollen morphology (Pollen, equatorial view)

Table 2. Pollen germination and mean length of pollen tubes in six different sucrose concentrations

SUCROSE CONCENTRATION (%)	TOTAL NO. OF POLLEN	NO. OF POLLEN GERMINATED	GERMINATION (%)	MEAN LENGTH OF POLLEN TUBE (MM)	S. D. OF MEAN
0	394	27	6.9	0.21	0.06
2	550	146	26.5	0.30	0.12
4	389	129	33.2	0.42	0.08
6	672	196	29.2	0.34	0.07
8	406	124	30.5	0.34	0.12
10	438	128	29.2	0.46	0.13

Anther development

The young anther was a homogeneous mass of parenchymatous cells covered by a distinct epidermis. It later became four-lobed and a group of hypodermal archesporial cells differentiated in each lobe. The archesporial cells divided periclinally to form the primary parietal layer and sporogenous tissue. The development of the anther wall followed the basic type of Davis [11]. The primary parietal layer divided periclinally, producing two secondary parietal layers which further divided periclinally; the outer layer towards the epidermis became the endothecium and a middle layer, while the inner layer produced a second middle layer and tapetum (Figure 3C). The tapetum was initially uninucleate, later becoming multinucleate and secretory (Figure 3D). The tapetum started to degenerate during the maturation of pollen grains. At the same time, the middle layers were flattened or crushed. The cells of the endothecium were radially elongated. During maturation of the anther, when the microspores were at the uninucleate stage, the endothelial cells developed fibrous bands along the inner tangential wall and extending outward, terminating near the outer tangential wall (Figure 3E). The microspore mother cells underwent meiotic divisions asynchronously in the microsporangia. Cytokinesis was simultaneous by furrowing and the tetrads were tetrahedral. The pollen grains were shed at the two-celled stage.

Seed biology

Both extracted seeds and whole fruits sown started germinating after three weeks. Extracted seeds germinated faster and earlier than those sown intact as fruits; with a total of 39 seeds

germinated (97.5%). For the intact fruits, only 17 germinated (42.5%) with five viable seeds at the end of the test at day 55. Germination rate for the extracted seeds was 0.05 compared to 0.03 for the intact fruits. The CUG for the seeds was higher i.e. 0.04 than the fruits i.e. 0.01.

Glycosmis pentaphylla showed hypogeal germination. The radicle emerged first, followed by the plumule. The radicle was smooth and white, while the plumule was green and minutely hairy. The emergence of the plumule varied from 16 to 25 days after sowing. The first pair of leaves was formed 5 - 7 days after germination in the garden soil and 3 - 4 days after germination in the sand. The cotyledons shrank and degenerated after the first leaves were formed. The secondary roots were well formed when simple leaves were produced.

Three types of seedling development were observed: 1) normal seedling development producing only one primary shoot; 2) fasciation where stunting of the primary shoot was accompanied by development of one or more higher orders of shoot axes (although only one radicle formed) and 3) polyembryony with two seedlings formed in a single seed.

For the seed storage experiments, the control showed 100% germination. Percentage germination was very low for all the three treatments (5°C, 15°C and 28°C), i.e. 3.3% to 10% (Table 3). It was the lowest for seeds stored under 15°C and highest at 28°C. Although percentage germination was very low, the seeds had very high percentage viability, i.e., 96.7% when tested with 1% tetrazolium chloride solution.

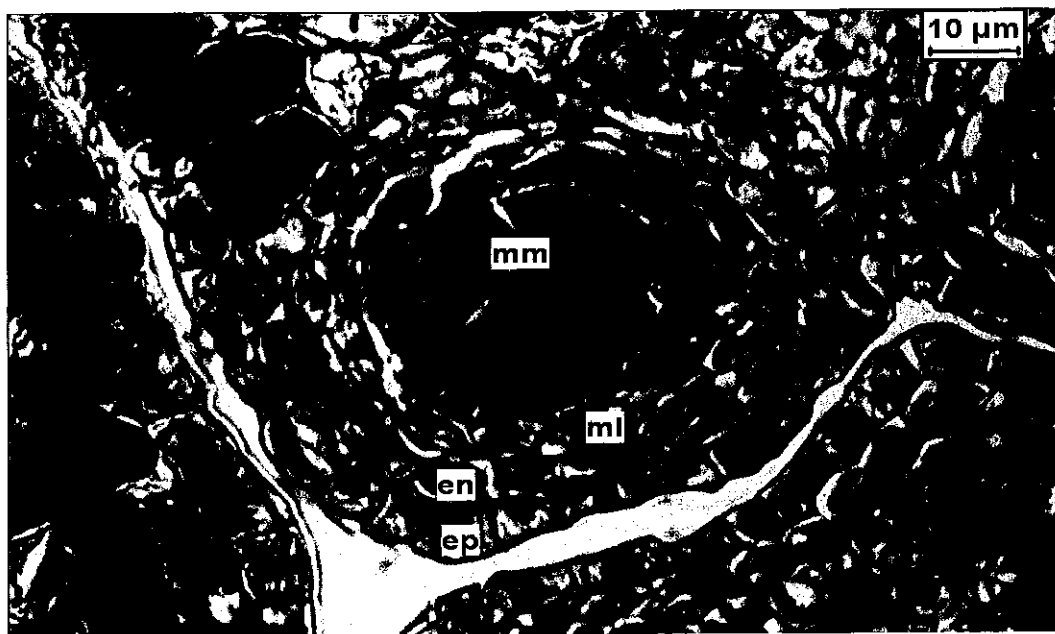


Figure 3C. Anther wall development (Anther wall at microspore mother cell stage)
(en – endothecium; ep – epidermis; ml – middle layer; mm – microspore mother cell)

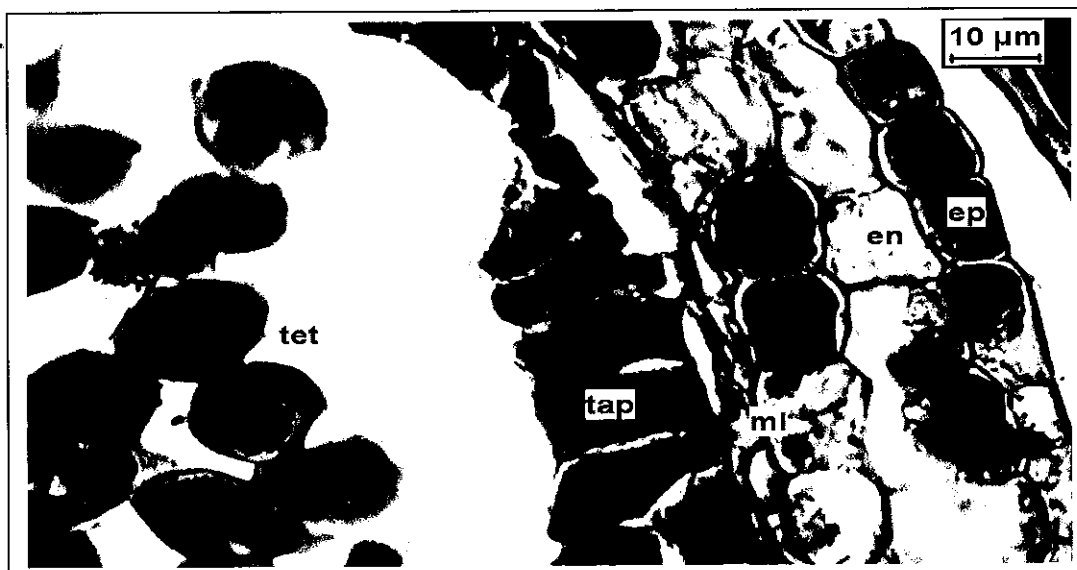


Figure 3D. Pollen morphology and anther wall development (Anther wall at microspore tetrad stage showing multinucleate tapetum)
(en – endothecium; ep – epidermis; ml – middle layer; tap – tapetum; tet – microspore tetrad)

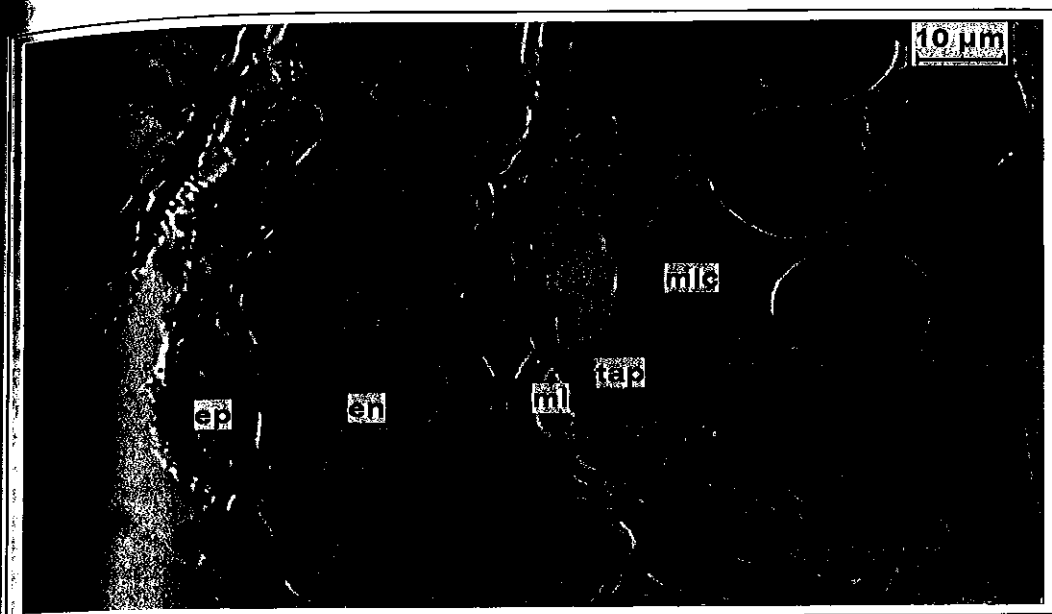


Figure 3E. Pollen morphology and anther wall development (Fibrous thickenings on endothecium of anther wall) (en – endothecium; ep – epidermis; mic – microspore; ml – middle layer; tap – tapetum)

Table 3. * Seed germination and viability after storage at different temperatures for 25 days without relative humidity control (30 seeds were stored at each temperature)

STORAGE TEMPERATURE (°C)	NO. OF GERMINATED SEEDS	GERMINATION (%)	NO. OF VIABLE SEEDS	SEED VIABILITY (%)
5	2	6.7	27	96.7
15	1	3.3	28	96.7
28	3	10.0	26	96.7

DISCUSSION

Flower and fruit development

Flower development from bud to flower anthesis was very slow in *G. pentaphylla*; it took about one month, and up to three months when compared to *Clausena harmandiana* [12] and *Glycosmis mauritiana* [13], which respectively took 17 days and 11 days.

Anthesis time for species in the Rutaceae is usually from 0730 - 1300 hours, as recorded for *Severinia buxifolia* [14], *G. mauritiana* [13], *Citrus grandis* [15] *C. harmandiana* [12] and *G. pentaphylla*. The anthers dehisce longitudinally and the stamens usually abscise three days after

anthesis. The peak hours of anthesis in *G. pentaphylla* at 0830 - 1000 hours, were indicated by the "mass abscission" of stamens and petals from time to time, due to the activities of insect visitors.

The flowers of *G. mauritiana*, *C. harmandiana* and *C. grandis* were observed wide open during anthesis, revealing the stigma and stamens, but not in the case of *G. pentaphylla*, where they remained closed with only very slight opening at the apex after the petals have loosened, until abscission. *G. pentaphylla* is not likely to be pollinator-specific as insects such as bees, wasps and butterflies can easily access nectar or pollen in the flowers. Stigma receptivity lasted three

days in all the species mentioned above. However, earlier reports noted that the stigma in *Citrus* was receptive for 6 - 8 days [16].

Generally, fruit development takes longer than flower development. Fruit maturity takes about 34 - 37 days in *G. pentaphylla*, 40 - 42 days in *G. mauritiana* and 56 - 63 days in *C. harmandiana*.

The Rutaceae family shows a low percentage of flower abortion varying from 4.9 to 42.9% in *C. harmandiana*, *G. pentaphylla*, *G. mauritiana* and *S. buxifolia*. Fruit abortion, however, is very high (71 - 94.5%) in *G. mauritiana*, *G. pentaphylla*, *C. grandis* and *Merrillia caloxylon* compared to that in other species such as *S. buxifolia*, *C. harmandiana* and *Murraya paniculata* [17] which is 15 - 36.2%. According to Janzen [18], a tree that flowers but does not set fruit, is not necessarily abnormal. Abortion of flowers and fruits may not be due to a failure in pollination, but may be the outcome of choice of parentage by the female genome [19]. Other important factors that affect flower and fruit abortions include soil and weather conditions especially rainfall.

Pollen studies

Rutaceae taxa have been reported to have tricolporate, tetracolporate or pentacolporate pollen [20, 21]. The pollen grains are shed at the two-celled stage, except for *Agathosma apiculata* and *Citrus grandis* [22], where they are three-celled. The pollen in the family is (2-)3-6(-8) colporate, subolate-perprolate [10]; and the pollen of *G. pentaphylla* is trizonocolporate and prolate spheroidal.

In the family members studied, maximum pollen germination was achieved in 4 - 9% sucrose solution [12, 13, 14, 17, 23], including *G. pentaphylla*. The pollen grains remain viable for up to 4 days after anthesis.

Anther development

Glycosmis pentaphylla has the basic type of anther wall development, similar to *Citrus grandis* [24], *Chloroxylon swietenia* and *Feronia elephantum* [5], *Severinia buxifolia* [14], *Murraya exotica*, *Murraya koenigii*, *Atalantia racemosa*, *Choisya ternata* and *Coleonema album* [4].

The tapetum is usually a single layer in species of this family and also in *G. pentaphylla*, but

tapetum with 2 - 3 layers has also been reported [22]. The endothelial cells of *G. pentaphylla* have helical thickenings, although this feature is more common in monocotyledons. The majority of dicotyledonous species studied have U-shaped and baseplate types of thickenings; U-shaped endothelial cell thickenings with basal anastomosis is probably basic and baseplate derived, and has been recorded for the Sapindales [25]. Asynchronous microsporogenesis in the microsporangia of *G. pentaphylla* is consistent with the findings for other species of the family [4, 22, 23]. In Rutaceae, tetrads are tetrahedral, decussate or isobilateral [11, 23, 26] although *G. pentaphylla* has only tetrahedral tetrads.

Seed biology

Germination in the Rutaceae is either hypogeal or epigeal [16, 23] but both types have been reported for *Murraya paniculata* [27]. Germination is hypogeal in *G. pentaphylla*. The percentage germination of fresh seeds varies from species to species. It is very high in *G. pentaphylla* (97.5%), *C. harmandiana* (95%), *S. buxifolia* (93%), and *G. mauritiana* (80%); while moderately low in *C. grandis* (60%), *Citrus hystrix* and *Citrus madurensis* (both 45%) [23]. *G. pentaphylla* seeds took 20 - 26 days to germinate but the other species mentioned germinated only 4 - 10 days after sowing, indicating generally shorter seed dormancy in those species. Impermeability of the seed coat to water could be a factor delaying germination, as it takes time for the seed coat to be softened or degraded by microorganisms in the soil.

The CUG of extracted seeds sown (without pericarps) is higher than that of seeds enclosed within pericarps, i.e. 0.04 and 0.01 respectively, in this study. This probably suggests a significant degree of chemically imposed dormancy operating, where fleshy fruits or juices inhibit seed germination. The fleshy coverings probably hinder exchanges of gases, i.e., intake of oxygen and escape of carbon dioxide from the embryo. Also, physiological changes following abscission of the fruit, as well as those that may be induced by contact with soil factors following degradation of the fruit wall may play a role in initiating germination.

Polyembryony and fasciation were noticed during seed germination of *G. pentaphylla*. Polyembryony is common in species of Rutaceae, such as *Citrus* spp. [28], although it is absent in *C. grandis* [16]. However, it is considered rare in

G. pentaphylla as only one seed out of all other seeds observed from the germination tests showed polyembryony with 2 seedlings. Fasciation occurred occasionally in *G. pentaphylla* but normal seedling development was the most common.

Hallé *et al.* [29] reported that there was evident correlation between germination type and shoot organization. In hypogeal germination the epicotyledonary axis (axis above the cotyledons) of the seedlings, usually developed scale leaves, with a gradual transition to foliage leaves distally. However, the seedlings of *G. pentaphylla* (grown from hypogeal germination) seem to follow the epigeal type, in which scale and transitional leaves are lacking, and the first leaves above the cotyledons are foliage leaves.

In the present study, the percentage germination of seeds was low after 25 days of storage but the viability was very high, i.e., 96.7%. Seed storage at 5°C gave a higher percentage germination than at 15°C but lower than at 28°C (Table 3). The results obtained show that seeds are able to germinate after 25 days of storage at different temperatures in high relative humidity (R. H). Ambient temperature and moisture content of seeds during storage are the prime factors responsible for the loss of viability in citrus seeds [30]. Seed storage in plastic bags at room temperature (28°C) without humidity control showed that *C. reticulata* and *Poncirus trifoliata* × *Citrus sinensis* lost their viability after 60 days; whereas *C. madurensis* and *G. pentaphylla* recorded 33.3% and 40% germination in petri dishes, respectively, after 60 days of storage [31]. Other factors that may affect seed longevity and deterioration include genetic factors (genetically and chemically disposed for longer storability), presence of fungi, mechanical damage and seed maturity.

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