Facultative Apomixis in *Garcinia atroviridis* (Clusiaceae) and Effects of Different Pollination Regimes on Reproductive Success

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Abstrak: Pelbagai aspek kejayaan dalam pembiakan *Garcinia atroviridis* Griff. telah dikaji. Eksperimen pendebungaan terkawal telah dijalankan di dalam sebuah kebun di wilayah Songkhla, selatan Thailand, dari Februari hingga Julai 2003. Kelanjutan usia bagi bunga, reseptiviti stigma dan keboleh hidupan debunga telah diperiksa sebelum eksperimen dijalankan. Tiada perbezaan yang signifikan dalam set buah awal antara tiga jenis rawatan pendebungaan, iaitu pendebungaan terbuka, pendebungaan dibeg dan manual, dan dibeg tanpa pendebungaan (apogami). Walau bagaimanapun, pendebungaan dibeg dan manual telah menghasilkan kadar jatuh buah yang jauh lebih signifikan daripada apogami dan pendebungaan semulajadi, yang dikesan satu minggu selepas bunga-bunga tersebut telah didebungakan. Rawatan apogami mempunyai kadar jatuh buah yang lebih besar berbanding pendebungaan semulajadi dan manual sebelum buah masak. Dalam pada itu, kebolehan membuah serta saiz buah didapati kurang dalam bunga-bunga yang dibeg tanpa pendebungaan, berbanding dengan bunga-bunga pendebungaan semulajadi dan pendebungaan silang. Walaupun buah daripada bunga-bunga yang tidak didebungakan boleh menjalani pembentukan benih secara aseksual, buah-buah ini menghasilkan nombor purata benih yang kurang, dan kualiti benih yang teruk dan kurang memuaskan (didefinasikan sebagai berat bersih purata dan kadar percambahan) berbanding dengan rawatan-rawatan yang lain. Pembiakan aseksual dan seksual juga dikaji menggunakan analisis *Random Amplification of Polymorphic DNA* (RAPD) dan melalui pembezaan corak-corak jalur yang dihasilkan daripada DNA yang diekstrak daripada baka buah yang melakukan pendebungaan silang secara semula jadi. Secara purata, 58% daripada baka tersebut mempunyai konstitusi genetik yang sama dengan induk (dari 36%–87%), yang menunjukkan ada di antara baka dihasilkan tanpa penyuburan. Penemuan ini mencadangkan bahawa apomisis fakultatif telah berlaku dalam populasi yang dikaji. Walaubagaimanapun, suatu residual seksual adalah penting untuk penghasilan buah, saiz buah, set biji benih normal dan kualiti benih.

Kata kunci: Apomisis Fakultatif, Kejayaan dalam Pembiakan, RAPD

Abstract: Various aspects of the reproductive success of *Garcinia atroviridis* Griff. were studied. Controlled pollination experiments were carried out in an orchard located in Songkhla province, southern Thailand, from February to July 2003. Floral longevity, stigma receptivity, and pollen viability were examined before carrying out the experiments. Three pollination treatments were compared: open pollination, manual pollination with bags, and bags without pollination (apogamy). Although there was no significant difference in the initial fruit set, bagged and manual pollination produced a significantly greater fruit drop rate than apogamy or natural pollination at one week after the flowers had been pollinated. On the other hand, the apogamy treatment had a greater fruit drop rate than natural and

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manual pollination treatments before fruit maturation. In addition, unpollinated bagged flowers bore fewer and smaller fruit than naturally and manually cross-pollinated flowers. Although the fruits from unpollinated flowers were capable of asexual seed formation, they produced fewer seeds and had poorer seed quality (defined as average fresh weight and germination rate) than those from the other treatments. The occurrence of asexual and sexual reproduction was also studied using Random Amplification of Polymorphic DNA (RAPD) analysis and by comparing the patterns of bands produced from DNA extracted from the offspring of the naturally cross-pollinated fruits. On average, 58% of the offspring had a genetic constitution identical to that of the maternal parent (ranging from 36% to 87%), indicating that some offspring were produced without prior fertilisation. However, the remainder showed polymorphism, demonstrating the occurrence of sexual reproduction. These findings indicate that facultative apomixis occurred in the study population. However, a residual sexuality was important for fruit production, fruit size, normal seed set and seed quality.

Keywords: Facultative Apomixis, Reproductive Success, RAPD

INTRODUCTION

The Clusiaceae (Guttiferae) family of trees is naturally distributed in tropical zones between the latitudes of 10° north and 10° south (Cox 1976). *Garcinia* is the biggest genus in this family, with over 400 species. *G. atroviridis* Griff. ex T. Anders is one of the best known fruit trees of this genus. This is an endemic species in peninsular Malaysia, and preparations from it have been widely used by ethnobotanists and ethnopharmacists as a preservative, for seasoning and for medicinal purposes. Its dried fruit has been used to improve blood circulation, as an expectorant, for treatment of coughs, and as a laxative (Yapwattanaphun *et al.* 2002). In addition, phytochemical studies of the fruit's rind have established the presence of a commercial substance ((-)-hydroxycitric acid (HCA)) that inhibits ATP-dependent citrate lyase, a key enzyme that diverts carbohydrate metabolites to fatty acid and cholesterol synthesis (Lewis & Neelakantan 1965). HCA is used in many popular over-the-counter weight loss formulations, and the demand for the fruit is thus increasing. However its horticultural potential has only been poorly developed (Subhadrabandhu 2001). In particular, its reproductive biology is still not well understood. A deeper knowledge of this could reveal barriers to seed and fruit set, increase fruit production, accelerate breeding programs, and promote its conservation.

G. atroviridis is gynodioecious (Pangsuban *et al.* 2007) and is commonly propagated by seeds. The trees begin to flower 5–6 years after planting, and maximum fruit yield is usually attained after 6–7 years. The female trees produce fruits, while the hermaphrodite trees sometimes bear only a few fruits or have no fruit-set (Pangsuban *et al.* 2007). Hence, the growers prefer to plant female trees rather than hermaphrodite trees to optimise fruit production. However, the gender selection of the trees poses a serious problem to the growers as the gender cannot be determined until the trees flower (Subhadrabandhu 2001). The growers usually resort to grafting or they inarch bud wood of female trees onto seedlings or existing hermaphrodite trees to ensure fruit production. By this approach, hermaphrodite trees, and hence paternity, tend to decline and be limited in

numbers in the population. Thus, knowledge of *G. atroviridis'* reproductive behaviour is urgently required because it is important for growers to design suitable breeding strategies.

Theoretically, the majority of dioecious tropical trees should exhibit male-biased secondary sex ratios, given the reduced allocation of resources required for successful reproduction by males (Thomas 1996; Opler & Bawa 1978). However, many *Garcinia* species have female-biased sex ratios, especially in southeast Asian forests (Thomas 1997). One explanation for the existence of the female-biased sex ratio is their reproductive behaviour, which has been proposed to be by agamospermy (apomixis by seed) (Thomas 1997; Richards 1997). More than 300 species (from more than 35 families) of angiosperms have been described as apomictic (Koltunow *et al.* 1995). Many species of *Garcinia* are in this category, including *G. livingstonii* (Puri 1939), *G. mangostana* (Kaur *et al.* 1978), *G. parviflora* (Ha *et al.* 1988), *G. scortechinii* (Thomas 1997), *G. hombroniana* (Richards 1990a), and *G. gummi-gatta* (Rai 2003). However, *G. cantleyana* is not an apomict. Hence, not all species of *Garcinia* are apomictic (Richards 1997). Likewise, Richards (1990b) has suggested that *G. atroviridis* might be a facultative apomict on the basis of its sex ratio. However, his suggestion has not been confirmed.

Here we present the results of the first comprehensive investigation of the reproductive behaviour of *G. atroviridis.* The first issue investigated was whether this species, which is known to reproduce by apomixis, could set unfertilised seed in unpollinated flowers under controlled pollination conditions. In order to ascertain the occurrence of asexual versus sexual reproduction, we also tested whether offspring would express an identical DNA fingerprint to the maternal parent in a RAPD marker survey. Any non-maternal RAPD fingerprint in the offspring would indicate that zygotic offspring were formed via fertilisation. These investigations also had the potential to reveal influences of reproductive behaviour on other important measures of reproductive success, such as fruit setting, fruit growth, seediness and seed quality.

MATERIALS AND METHODS

Study Site and Plant Materials

The study site for this work was an orchard located in Songkhla province, Tambon Pien in the south of Thailand (N7.13 E100.53) at an altitude of approximately 200 m. Annual rainfall in this region ranged from 29.2 mm in January to 360.2 mm in November. The average temperature varied from 22.5°C to 24.6°C, and relative humidity varied from 50.3% to 97.3% (Pattani Meteorological Station 2003). The trees bear fruit annually, with flowering and fruiting occurring once a year in the summer (February to June). The conspicuous flower has a long pedicel with four warm yellow sepals. Its petals are large, bright crimson, orbicular and/or obovate. The stigmas seem to be convex. There is no clear separation between stigma and style. The female flowers are usually solitary, but the hermaphrodite flowers are present in compound cymes (Whitmore 1973). Most female flowers have 10–16 ovules per flower, whereas the hermaphrodite flowers have 9–12 ovules per

flower (Pangsuban *et al.* 2007). The trees used for determining flowering performance in this study were randomly selected according to accessibility.

Floral Longevity

The female trees flower synchronously in February. Floral longevity was estimated on three selected female trees. In order to categorise the developmental stages of the flowers, 10 floral buds per tree were randomly chosen and individually tagged. The buds are concealed at the bases of the uppermost leaf stalks. No external features distinguish the floral buds from the vegetative buds until the two subtending bracts spread into a horizontal position due to the increasing size of the immature floral bud head. Developmental stages of the flowers were monitored daily from the initiation of the visible floral bud until the formation of fruit. Morphological changes and the time intervals between changes were recorded.

Stigma Receptivity

Stigma receptivity was assessed by two approaches; the appearance of exudate and detection of peroxidase activity on the stigmas under natural conditions (Kearns & Inouye 1993; Kalisz *et al.* 1999). Observations were made on the three selected female trees. Ten flowers at each different stage were tested for peroxidase activity by placing them into a 3% solution of hydrogen peroxide (H_2O_2) and watching for oxygen bubbling activity. Stigmas were scored as positive for peroxidase activity only if vigorous bubbling was observed across the entire surface of the stigma within one minute. The stigmas showing weak bubbling after the application of the H_2O_2 were presumed to be false positives and were scored as unreceptive (modified from Kalisz *et al.* 1999). The stigmatic exudate was also evaluated for its viscosity and glistening appearance.

Pollen Germination

Pollen viability was determined by an *in vitro* germination test using a germination medium consisting of 10% sucrose + Brewbaker and Kwack's Medium (Shivanna & Rangaswamy 1992). Ten hermaphrodite flower buds were collected prior to flower opening. Before placing these selected flower buds in Petri dishes, their sepals and petals were removed. In this way, their anthers could dehisce during the next day without disturbance of their stigmatic exudate. Eventually, their pollen grains were pooled and maintained under room temperature to evaluate their *in vitro* germination daily. A suitable number of pollen grains were dispersed in 200 μl of culture medium in Eppendorf tubes and made into a homogeneous suspension with a needle. The cultures were incubated under ambient conditions (~25%) for 48 h. This suspension was then drawn into a Pasteur pipette and transferred onto a clean microslide. A drop of 2% acetocarmine was added to terminate pollen development. The germination test was performed with three replicates. Three slides of each replicate were prepared for monitoring. A pollen grain was considered to have germinated when the length of its tube was greater than the grain's diameter (Shivanna & Rangaswamy 1992). For each slide, 100–200 grains from 9 randomly selected fields were counted at 100X magnification using a light microscope.

Controlled Pollination

Four female trees were selected (with diameters at breast height of 9.64, 7.96, 8.28 and 8.76 cm). The experiment was carried out under a Completely Randomised Design model. Controlled pollination was conducted during the flowering season (February 2003). For the purposes of the study, it was assumed that the position of the flower in the tree and the parthenocarpic ability of the fruit did not significantly influence the result. Female flowers selected for the bagging treatments were located on lower branches that could be reached from the ground. In a preliminary study, some flowers were bagged with cellophane bags to ensure that this process did not harm the flowers or alter fruit development. The weather was dry during the day of the experimental pollination. The female flowers of *G. atroviridis* did not require emasculation (Pangsuban *et al.* 2007). Ten healthy floral buds per treatment were labelled. Three types of treatment were applied to each selected tree, based on Dafni (1992). For "natural pollination," the floral buds were left naturally exposed. For "manual pollination," each bud was protected from natural pollination by a cellophane bag. When the buds reached a suitable stage, the bags were carefully opened to allow manual pollination with the picked hermaphrodite flowers, after which the pollinated flowers were rebagged. For "apogamy," the floral buds were bagged without pollination. To minimise the effects of pollen source, the pollen used for manual pollination was collected from the hermaphrodite plant used in the above study. All bags for treatments (2) and (3) were retained until the flowers senesced or the initial fruit set was observed (i.e., when an ovary recommenced growth, *c.* 13.00–15.00 mm). Finally, developing fruits from treatments (2) and (3) were released from their bags to achieve similar conditions to those from treatment (1). Four replicates were used for each treatment.

Fruit setting was recorded. Developing fruits were counted daily throughout the period of heaviest fruit loss (the first seven days after pollination), and then at two-week intervals until the fruits were fully mature. The fruit drop rate (the number of fruits that abscised during that week) was calculated (Trueman & Wallace 1999). The diameters of the fruits retained were measured with a vernier clipper. Ripe fruits were recognised by the change in colour of their rind from green to orange. After harvest, the ripe fruits were immediately taken to the laboratory to determine their diameter and weight. The number of seeds per ripe fruit was counted, and the mean fresh weight of the seeds was also determined before they were placed in the greenhouse for germination (located at the Faculty of Natural Resources, PSU, Songkhla, Thailand). All fresh seeds were immediately planted in pots filled with a sand mixture and then placed in the shade. Seed germination rate (%) was calculated after one, two and three months.

DNA Extraction and Molecular Analysis

Mature seeds from naturally cross-pollinated fruits of three female trees were used in this analysis. The fruits were randomly harvested. Seeds from each ripe fruit were sown singly in the greenhouse (Faculty of Natural Resources, PSU, Songkhla, Thailand). The genetic analyses were carried out on 25 intact seedlings from each female tree. Genomic DNA was isolated from young fresh leaves

according to Doyle and Doyle (1987). Leaf samples (approximately 30.0–50.0 mg) were ground into a powder with liquid nitrogen in a mortar. The material was thoroughly mixed with 2.5 ml of pre-warmed (60°C) extraction buffer (20 mM Na_2EDTA pH 8.0, 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl ,1% PVP-40, 2% CTAB, 2% β-mercaptoethanol v/v) and ground further, then transferred to a 5 ml centrifuge tube and incubated for 5 h at 60°C. One volume of phenol: chloroform: isoamyl alcohol (25: 24: 1 v/v) was added to the tubes and mixed thoroughly. The mixture was centrifuged for 15 min at 4000 rpm. The top layer was transferred to a fresh tube and one volume of chloroform: isoamyl alcohol (24: 1 v/v) was added and mixed again. The mixture was again centrifuged for 15 min at 4000 rpm. The top layer was transferred to a 1.5 ml Eppendorf tube. One volume of ice-cold isopropanol was added to precipitate the DNA. This was pelleted by centrifugation for 10 min at 10,000 rpm, and the supernatant was carefully poured off. The DNA pellet was washed twice with 800 μl of 70% ethanol and centrifuged for 3 min at 10,000 rpm. The supernatant was discarded and the pellet air-dried. The DNA was then resuspended in 30 μl of autoclaved double-distilled water (DDW). The DNA concentration was checked, and the DNA was then separated by electrophoresis on an 0.7% (w/v) agarose gel in 1X Tris-acetic acid-EDTA (TAE) and stained in 1.5 μl/ml ethidium bromide solution before comparing the bands to a range of concentrations of lambda high-molecular-weight DNA (Sigma, USA).

In a previous study (Nakkuntod 1998), 12 primers from Operon Technologies Inc. (Alameda, California) were successfully used in a RAPD analysis for a taxonomic study of the genus *Garcinia*. Therefore, all these primers were tested in the present study. However, two of these primers failed to give reproducible bands. Thus, only the following 10 primers (OPAA17: 5′-GAGCCCGACT-3′, OPAD04: 5′-GTAGGCCTCA-3′, OPAD05: 5′-ACCGCATGGG-3′, OPAD06: 5′-AAGTGCACGG-3′, OPAD08: 5′-GGCAGGCAAG-3′, OPAD09: 5′-TCGCTTCTCC-3′, OPAD10: 5′-AAGAGGCCAG-3′, OPAD11: 5′-CAATCGGGTC-3′, OPAD12: 5′-AAGAGGGCGT-3′, OPAD15: 5′-TTTGCCCCGT-3′) were used in this RAPD analysis. The RAPD reactions were carried out with 15 μl of *Taq* polymerase (0.5 U/μl); *Taq* buffer X1 (20 mM Tris-HCl, 50 mM KCl, pH 8.4); dNTPs (2 mM each); $MgCl_2$ (2.5 mM); primer (5 pmole/μl) and 50 ng of template DNA. Polymerase Chain Reaction (PCR) conditions were as follows: 3 min at 94°C; 94 cycles of : 1 min at 94°C, 1 min at 35°C, 2 min at 72°C; and as a last step 5 min at 72°C. Amplifications were carried out using a PCR Sprint (Hybaid, USA). The amplification products were resolved on 1.5% (w/v) agarose gels in 0.5X Tris-boric acid-EDTA (TBE) and stained in 1.5 μl/ml ethidium bromide solution. After ethidium bromide staining, the banding patterns were recorded by photography (Gel-documentation) and analysed later. The reproducibility of the RAPD methodology used in this study was checked very carefully. Differences in RAPD fingerprints were only accepted if they could be confirmed in two replicates.

Data Analysis
The means and standard errors were calculated for all measurements. Pollen viability was calculated by dividing the number of germinated pollen grains by the total number of pollen grains in the field of view and expressed as a percentage. Data from the controlled pollination experiment were analysed using the non-

parametric Kruskal-Wallis Test, since the data that was not distributed normally even after log or arcsine square-root transformations and the variance differed significantly. Later, the post-hoc Duncan's New Multiple Range Test was used to assess comparisons among treatments (Samuels & Witmer 2003). Molecular data analysis for identifying the apomictic seedlings was performed by comparing the banding patterns between each maternal parent and their seedlings. The RAPD fingerprints were considered polymorphic if they showed either fewer or more bands than their maternal parent and monomorphic if the bands were identical. The results were expressed as the percentage of genotypically maternal seedlings. All statistical analyses were performed with SPSS v. 10.

RESULTS AND DISCUSSION

Floral Longevity

The inception of the flower primordia occurred at the end of January. Blooming commenced during late February, progressed through March and terminated in April, and occurred synchronously among individual trees. The flower buds opened at night, had attractive colours (pink to red), were odourless, and secreted a small amount of stigmatic exudate, like other *Garcinia* species. The period between the commencement of floral buds and fruit setting averaged about 22 days, whereas the floral longevity (defined as the time from opening to shrivelling of the petals) was about 3 days. The changes in floral colour and form are described in detail in Table 1.

The development of the flowers was determined on the basis of their morphology and exudate, and was divided into five distinct stages (S_0–S_4): (1) closed buds (S_0) = buds that are still enclosed by the outer sepals and without any exudate; (2) open buds (S_1) = expanded sepals and flower petals (defined as petals open to an angle of less than 45° with the pedicel) with a small amount of exudate appearing over the stigmas; (3) mature flowers (S_2) = half-opened flowers (defined as petals open to an angle of more than 45° and less than 90° with the pedicel) with stigmatic exudate covering the entire stigmatic surface; (4) post-anthesis (S_3) = fully opened flowers (defined as petals open to an angle of 90° or more with the pedicel) with dried exudate; and (5) fruit setting (S_4) = the ovary has recommenced growth, fruit development has begun, petals are shrivelled and the exudate has disappeared.

Table 1: Developmental stages of the female flower of *G. atroviridis*.

Stage*	Days	Description	External features
S_0	−1	pink stigma, exudate absent, swollen flower bud	
S_1	0	pink to red stigma, little exudate present, sepals and petals start opening	
S_2**	1	reddish stigma, exudate covers entire stigma surface, sepals and petals half open and extremely expanded	
S_3	2	crimson stigma, exudate begins to dry, flower fully expanded, ovary swollen	
S_4	3	black-red stigma, exudate absent, sepals and petals start shrivelling and colour has faded, fruit setting	
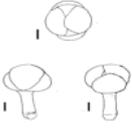
Notes: *See details in text.
   ** Floral stage selected for controlled pollination.
   Scale bar: 0.5 cm

Stigma Receptivity

On the basis of the peroxidase test, stigmas of the flower buds at S_0 were non-receptive, because vigorous bubbling was not observed. However, vigorous bubbling was observed to occur on the stigmas as flowers developed further (S_1 – S_4). Interestingly all stigmas in the half-opened flowers (S_2) were recognised as receptive (Fig. 1). Similarly, a stigmatic exudate was not observed in the S_0 stage (Table 1), while a small amount of exudate was first observed on the central region of the stigmas in the S_1 stage. Next, the viscous exudate gradually increased on the stigmatic surfaces (and started to glisten) until the entire stigmatic surface was covered in the half-opened flowers (S_2). Finally, the

stigmatic exudate began to dry in the fully opened flower (S_3) stage and toward the beginning of the initial fruit set stage (S_4). These results indicate that the receptivity of the stigmas (on the basis of the peroxidase test) was concomitant with the secretion of the exudate. The maximum amount of exudate in the S_2 stage coincides with the competence to support pollen hydration and germination during the pollination process. Thus, flowers in the S_2 stage were selected for the controlled pollination experiment.
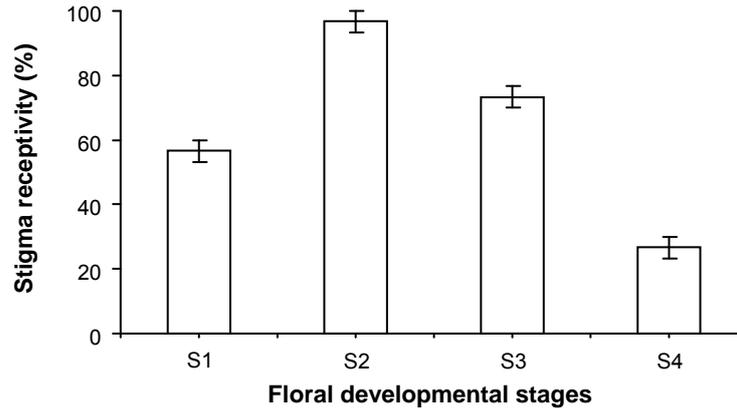
Figure 1: Frequencies of receptive stigmas of *G. atroviridis* female flowers (n = 30 flowers per stage). Each stage is described in the text. The vertical single lines represent the standard error (SE).

Pollen Germination

The hermaphrodite flowers were borne in compound cymes, in which a terminal central flower was first formed and lateral flowers developed sequentially. This sequential flowering in the hermaphrodite flowers meant that each flower cluster could present pollen over several days. The developmental features of the individual flowers were the same as in the female flower. However, each flower contained many anthers that randomly dehisced as soon as the floral bud broke. On the basis of the *in vitro* germination assay, pollen viability was high (79.5%) on the first day after anther dehiscence and had a half-life (50.0% loss of pollen viability) of 17 days (Fig. 2). By 25 days after anther dehiscence, none of the pollen grains could germinate. Therefore, to ensure efficient cross-pollination, pollen grains to be used as donors in the controlled pollination experiment were harvested on the first day after anther dehiscence.

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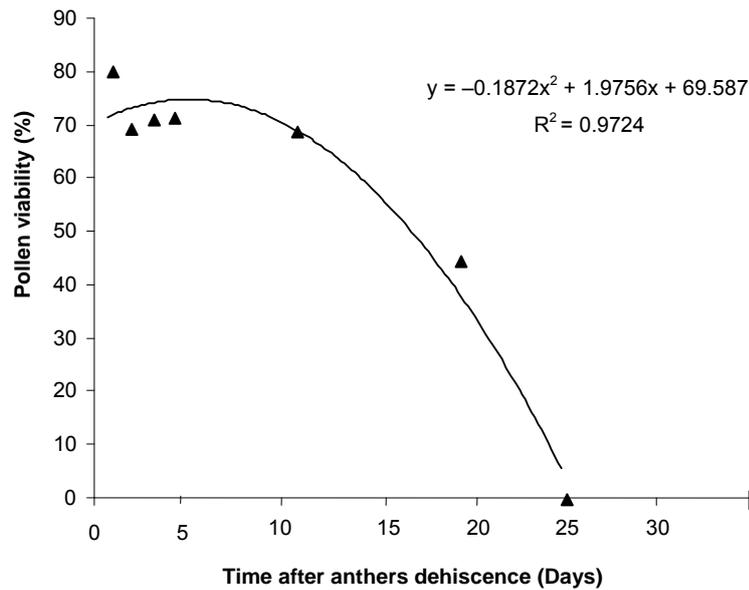
Figure 2: Viability of mature pollen grains from *G. atroviridis* hermaphrodite plants as assessed by *in vitro* germination test at various times after anther dehiscence (n = 10).

Controlled Pollination

The number of fruits obtained from the various controlled pollination treatments did not differ significantly (Kruskal Wallis, H = 1.494, d.f. = 2, P-value = 0.342) (Fig. 3). This result indicated that the initial fruit set was not dependent on pollination. Thus, it might be assumed that unpollinated flowers can continue into fruit set via asexual reproduction (apomixis). However, further molecular analysis was needed to test this assumption. One week after the flowers had been subjected to the different treatments, significant differences were observed in fruit drop (H = 6.870, d.f. = 2, P-value < 0.05). The heaviest abscissions occurred after manual pollination, while the naturally pollinated flowers did not shed fruits (Fig. 4). Possibly, the initial high fruit drop rate in the manually pollinated flowers relative to the other treatments was due to damage during manual pollination. Data on the fruit drop rate collected at the third and fifth weeks after fruit setting showed no significant differences (H = 4.094, d.f. = 2, P-value = 0.129 and H = 5.301, d.f. = 2, P- value = 0.071, respectively). Interestingly, the records taken 7 to 11 weeks before the fruit were ripe showed that a great number of unpollinated fruits had fallen from their trees (H = 6.488, d.f. = 2, P-value < 0.05). These results indicate that the natural thinning of premature fruits seems to selectively remove unpollinated fruits. This might be due to internal competition among fruits within the trees. As a result of this process, the average diameter of the fruits from unpollinated flowers was significantly lower than for the other treatments (Fig. 5). The retention of fruits growing from naturally and manually pollinated flowers was not significantly different (Table 2).
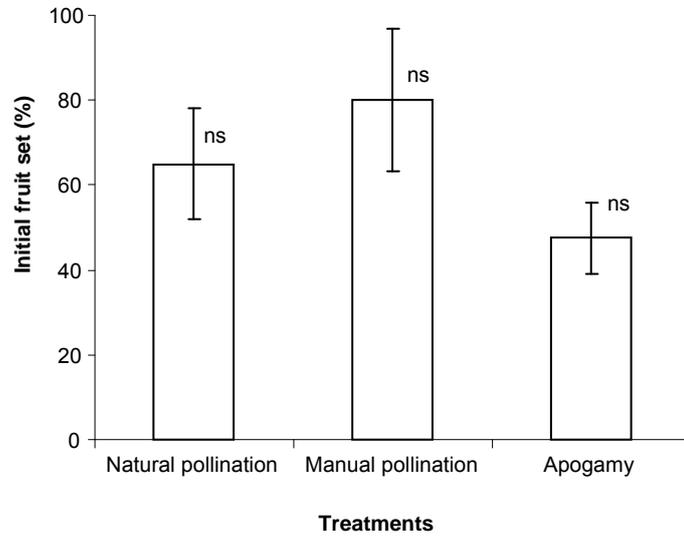
Figure 3: Mean (± SE) initial fruit set following three controlled pollination treatments in *G. atroviridis* (n = 40 treated flowers). Significant differences were assessed with the Kruskal-Wallis test. ns indicates no significant difference.
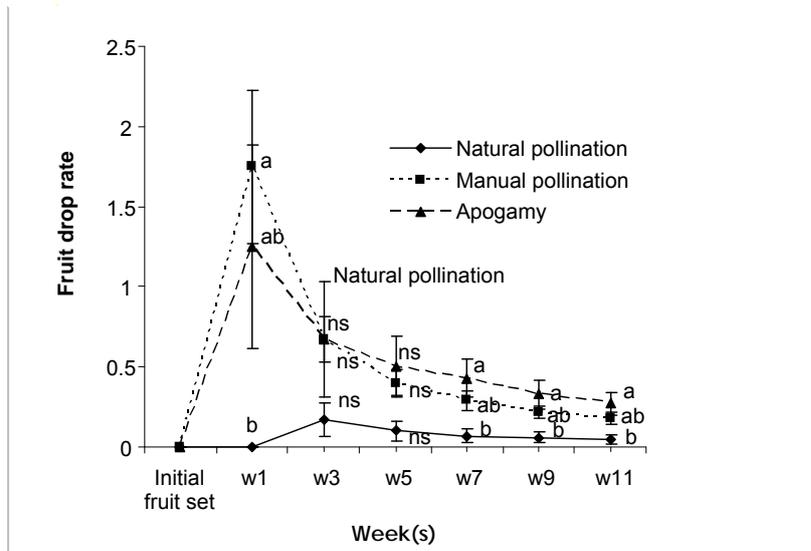
Figure 4: Mean (± SE) rates of fruit drop (number of fruits that abscised per week) in *G. atroviridis* at various stages of development. Significant differences were assessed with the Kruskal-Wallis test. Points with the different letters show significant differences at the 95% level. ns indicates no significant difference.
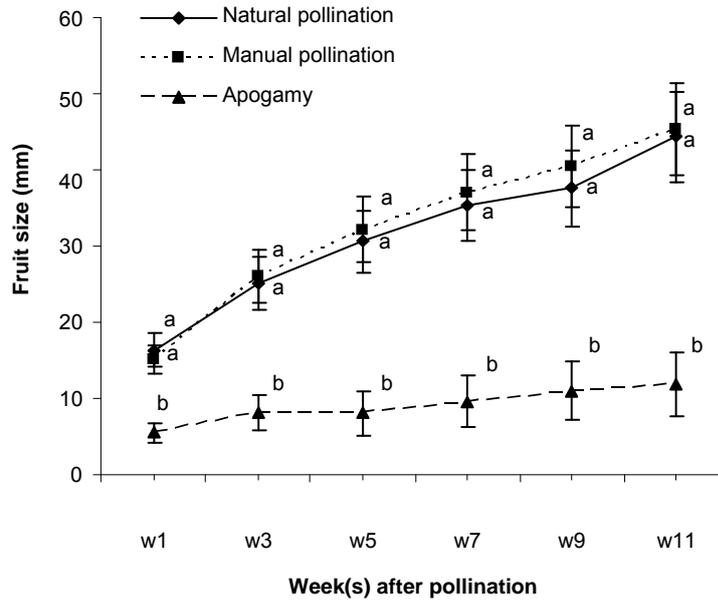
Figure 5: Mean (± SE) fruit enlargement in *G. atroviridis* at various stages of development. Significant differences were assessed with the Kruskal-Wallis test. Points with a different letter show significant differences at the 95% level.

Table 2: Kruskal-Wallis probability levels of differences in fruit diameters measured during development (from Fig. 5).

	Fruit development (weeks)					
	1	3	5	7	9	11
H	20.073	20.475	22.192	22.010	19.384	21.458
d.f.	2	2	2	2	2	2
P-value	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010

The fruits became ripe 11–12 weeks after fruit setting. Harvesting was done when their rinds showed a full orange colour. It is important to highlight the fact that unpollinated flowers were able to bear fruit (Table 3), and these fruits were able to set seeds. These results support the previous assumption that apomixis occurs in these flowers. Also, the seeds from the different pollination regimes were morphologically similar. Thus, it was not possible to visually distinguish whether they were produced sexually or via apomixis. The average number of seeds in ripe fruits following manual pollination was greater than the number produced by apogamic or natural pollination (*H* = 15.50, d.f. = 2, *P*-value < 0.05). This indicates that the seediness may have been regulated by the amount of pollination. Interestingly, the lower seed set in the naturally cross-pollinated fruits indicates that there either was a pollen limitation or a lack of

pollinators at the study site. However, the seeds from the unfertilised fruits had a lower average fresh weight than those from the other treatments (H = 8.733, d.f. = 2, P-value < 0.05). Possibly, the genetic quality of these unfertilised seeds was compromised. Consequently, they had a slower germination rate than the fertilised seeds from the naturally and manually cross-pollinated fruits (H = 6.338, d.f. = 2, P-value < 0.05) (Fig. 6). Thus, we speculate that fertilisation improved the genetic make-up of the seeds, giving them more vigour and fitness. Finally, seeds from all the three treatments had successfully germinated by three months. They followed the 'garcinia-type' of germination, in which the primary root and shoot emerged from opposite ends of the seed. This clearly indicates that apomixis occurred, since fertile seeds were produced without pollination or fertilisation.

Table 3: Fruit set and seed set in *G. atroviridis* after three controlled pollination treatments. Numbers in parentheses indicate the number of samples. Significant differences were assessed with the Kruskal-Wallis test. Numbers with different letters indicate significant differences at the 95% level.

Treatment	No. of treated flowers	Fruit set (%)	Average number of seeds per ripe fruit	Average seed fresh weight (mg)
Natural pollination	40	52.5	3.45 ± 0.71(21)b	29.99 ± 2.06(71)a
Manual pollination	40	60.0	6.70 ± 0.83(24)a	31.17 ± 2.42(81)a
Apogamy	40	17.5	2.82 ± 0.99(7)b	14.91 ± 4.48(23)b
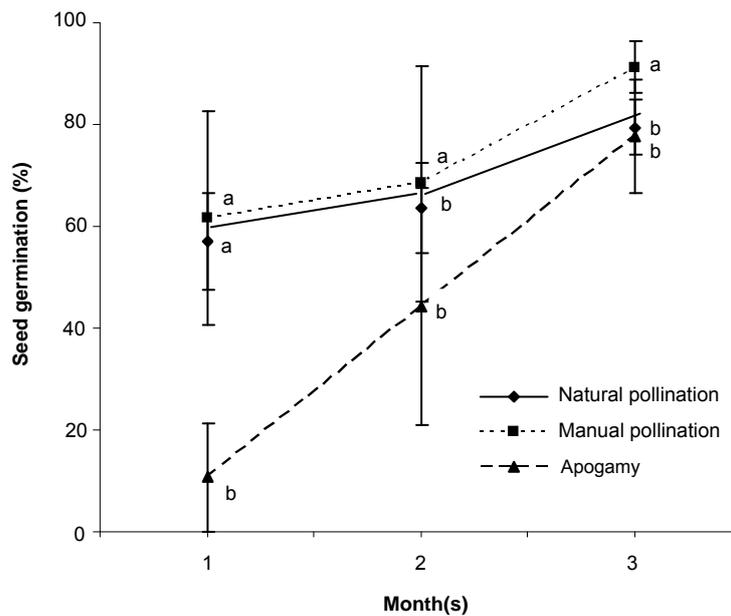
Figure 6: Seed germination (mean ± SE) following three controlled pollination treatments in *G. atroviridis*. Significant differences were assessed with the Kruskal-Wallis test. Points with different letters indicate significant differences at the 95% level.

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Molecular Markers Using RAPD Analysis

A total of ten 10 nt arbitrary primers were used to screen the 3 female trees, as well as a set of 25 offspring from each. From the entire set of primers, four (OPAD 04, OPAD 09, OPAD 10, and OPAD 12) failed to give polymorphic bands. Thus, these were omitted because the banding pattern did not help to discriminate between apomictic and zygotic seedlings. However, the other 6 primers did produce a total of 319 bands. The size of the polymorphic bands varied from 200 to 1000 bp, as shown in Figure 7. These bands were useful for screening apomictic and zygotic seedlings, because distinguishing these seedlings was made difficult by their very similar outward features. This use of RAPD fingerprints clearly demonstrates their utility for discriminating between apomictic and zygotic seedlings.

For the entire set of primers, the majority of the RAPD markers showed monomorphism (87.5%), whereas the remainder of the bands clearly showed polymorphisms (12.5%). The results indicated that 58% of the offspring were asexually derived, with a band pattern identical to that of the maternal parent. However, some seedlings came from mixed parentage, as their RAPD patterns were distinct from their maternal plants. These results indicate that under natural conditions, seedlings were produced both by apomixis and from zygotes after fertilisation. As a result, the natural reproductive behaviour ranged from 36% apomixis (a high amount of sexual reproduction) to 87% apomixis (a high amount of asexual reproduction) within the sampled plants. These results verified the occurrence of facultative apomixis in the study population.
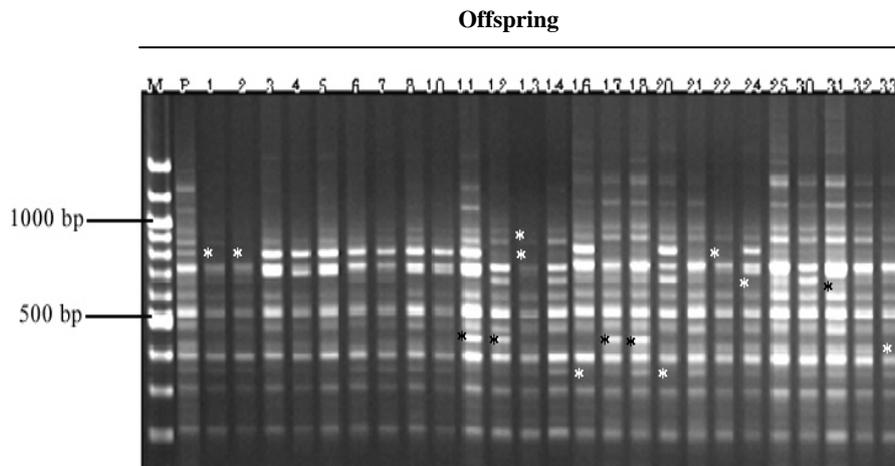
Offspring

Figure 7: RAPD profiles of an individual maternal plant and its offspring obtained by amplification of DNA samples with the primer OPAD 08. From left to right, the first lane is a 100 bp ladder (Operon, USA) fragment size marker (M), followed by the maternal parent (P) and 25 offspring. Numbers correspond to the code numbers of the offspring. The asterisks (∗) indicate diagnostic RAPD markers used for the discrimination of zygotic seedlings (Nos. 1, 2, 11–13, 16–18, 20, 22, 24, 31, 33) and apomictic seedlings (Nos. 3–8, 10, 14, 21, 25, 30, 32).

DISCUSSION

We conducted the first comprehensive study of the reproductive biology of *G. atroviridis*. The results provide evidential support for the suggestion of Richards (1990b, 1997) that this plant might be a facultative apomict. Although identifying an effective pollination period was not an objective of this study, successful fertilisation in sexual reproduction depends on three factors: stigma receptivity, pollen germination and pollen tube kinetics, and ovule longevity (Sanzol & Herrero 2001). Therefore, identifying the optimum conditions for stigma receptivity and pollen viability are important aspects of any evaluation of reproductive behaviour (Dafni 1992; Espinoza *et al.* 2002).

This work establishes that there is a correlation between stigma receptivity as measured by the peroxidase test and the timing of secretion of the stigma exudate. This provides optimum conditions allowing natural pollination to occur over the whole stigma. The stigmatic exudate has three important functions: the attachment of pollen at the stigmatic surface, the rehydration of pollen for germination and pollen tube entry into the style, and as a nectar reward to any visiting pollinator (Richards 1997). Little is known about pollen viability in *G. atroviridis*. According to the literature, harvested pollen grains of many *Garcinia* species have high viability as assessed by their stainability; e.g., 99.4% for *G. corymbosa*, 92.5% for *G. forbesii*, and 85% for *G.* cf. *forbesii* (Ha *et al.* 1988). Based on our *in vitro* germination test, we report a similar result for *G. atroviridis*. This characteristic might be a conserved property of this genus.

The results of the controlled pollination and RAPD analysis in this study indicate that facultative apomixis occurs in *G. atroviridis*. This reproductive behaviour is advantageous for the female trees, as it ensures reproduction and allows *G. atroviridis* to escape extinction even in the absence of hermaphrodite trees. However, Richards (2003) also stated that asexual seed formation provides opportunities for the accumulation of recessive mutants. Our findings lend support to this argument. In this study, hybridisation improved fruit production, fruit size, seediness and seed quality. It is of interest that manually pollinated flowers potentially produced more highly fertilised ovules than did unpollinated or open-pollinated flowers. Frequently, seeds in developing fruits produce hormones necessary for fruit development (Sedgley & Griffin 1989; Goldwin 1992). For instance, in *Arabidopsis*, ovaries without seeds exhibited normal fruit development, but the ovaries were smaller than those with seeds (Cox & Swain 2006). Fertilised *Arabidopsis* fruits with a larger number of seeds were bigger in size than those with a smaller number of seeds.

In our case, the fruits from unpollinated flowers were smaller in size and had a lower average number of seeds than fruits from pollinated flowers. A similar result was observed in *Rubus armeniacus*. Fruits from unpollinated flowers were smaller and the number of seeds was significantly lower relative to fruits from open-pollinated flowers (Kollmann *et al.* 2000). There are two possible explanations for this. First, the fruit size might be regulated by endogenous factors acting on unfertilised ovules. This idea is supported by several observations. For instance, less GA_1 was synthesised by unpollinated than by pollinated pericarps and/or ovules of *Pisum sativum* and *Juglans regia* (García-Martínez *et al.* 1991;

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Tadeo *et al.* 1994). Secondly, there were fewer unfertilised ovules in the multi-ovulate flowers that developed into mature seeds. This indicates that perhaps some growth-regulating hormones produced by the seeds were insufficient to induce fruit development. As a result of this process, the fruits with fewer developing seeds were likely to be smaller in size and dropped before reaching maturity (Ho 1992; Kollmann *et al.* 2000).

It seems reasonable to assume that the paternal genomes from pollen grains improved seed quality in this crop because the agamospermous seeds had lighter average fresh weights and required a longer incubation time for germination than did the fertilised seeds. These observations are similar to those reported in *G. mangostana,* in which the germination percentage was directly related to the weight of the agamospermous seeds (Morton 1987). The complex genetics of seeds should be taken into account when determining what it is that regulates seed weight. Variations in the ratio of maternal to paternal genomes could produce a spectrum of phenotypes in the embryo and endosperm, and could affect characters such as seed viability, seed weight, rate of mitosis in the endosperm, and timing of endosperm cellularisation (Scott *et al.* 1998). For example, pollen (as the paternal genome) in *R. armeniacus* caused positive heterosis during endosperm formation in cross-pollinated seeds, causing fertilised seeds to be heavier than unfertilised ones (Kollmann *et al.* 2000). By contrast, obligate apomixis gives rise to fertile seeds with embryos derived only from the maternal genome. However, facultative apomicts produce two types of fertile seeds: (1) apomictic embryos genetically identical to the maternal genotype, and (2) zygotic embryos genetically different from the maternal genotype. Consequently, their different genetic make-up may affect other physiological properties of these seedlings, as observed with *Opuntia* spp Cactaceae, in which zygotic embryos emerge earlier than apomictic seedlings (Jacobo 2001). There are two viewpoints to consider. First, there may be competition between the hybrid genomes in the developing embryos, resulting in more vigour and greater competition than occurs among the apomictic embryos (Koltunow *et al.* 2002). On the other hand, the apomictic progeny may compensate for their lower genetic variation by adopting a slower germination program that will ensure optimal growth. However, in order to confirm this suggestion, the ploidy levels of the progeny should first be determined (Jacobo 2001). Also, comparison of their RAPD markers will provide evidence of maternal inheritance even when the pollen source is unknown.

CONCLUSION

Female *G. atroviridis* trees do not normally outbreed because their flowers can produce unfertilised seeds without pollination, and both fertilised and unfertilised seeds can germinate into seedlings. Therefore, their reproductive behaviour is classified as facultative apomixis. This type of reproduction has the advantage that female plants successfully produce progeny even when they exist in a male-free population. However, fertilisation with a paternal genome does enhance fruit yield. Fruits produced by different pollination regimes showed a considerable

degree of physiological (fruit production, seediness, seed weight, and seed germination rate) and morphological (fruit size) variability. Thus, growers should consider growing hermaphroditic trees to facilitate the production of hybrid fruit. These results were obtained from one-year controlled pollination experiments carried out simultaneously within a single locality. Replication of this work would further enhance our knowledge of the reproductive strategies of facultative apomicts.

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