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Reproductive Biology of a tropical *Acacia* Hybrid (*Acacia mangium* Willd. x *A. auriculiformis* A. Cunn. ex Benth.)

by

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B.Sc., Kasetsart University, 1986

A Dissertation Submitted in Partial Fulfillment of the
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DOCTOR OF PHILOSOPHY

in the Department of Biology

We accept this dissertation as conforming
to the required standard

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ABSTRACT

The *Acacia* hybrid (*Acacia mangium* x *A. auriculiformis*, Leguminosae: Mimosoideae) has created considerable interest for plantations because of its adaptability and growth performance when compared to the parental species. This study concentrated on sexual reproduction, and seed and seedling quality using light and electron microscopy, histochemistry, and seed and seedling tests.

Two peak flowering periods in the hybrid appear to coincide with high rainfall and temperature, whereas two fruit-maturation periods occur during a windy dry season. The hybrid is andromonoecious. A floral spike consists of about 150 loosely arranged flowers. Flowers are cream colored and fragrant and have no floral nectaries. The pistil has a solid style with a smooth, wet stigma and amphitropous ovules with immature integuments at pollination. The flowers are weakly protogynous. Anthesis is complete at 0500-0600 h but peak female receptivity begins at 0200-0300 h and is completed that day. The stigmatic exudate is of the lipophilic type and is secreted from the stigmatic cells by a holocrine mechanism. Pollen is the main floral reward for the insect pollinators. There are several floral characteristics which facilitate pollen transfer from anthers to the stigmas. *Apis mellifera* and *Ceratina* sp. are the most effective pollinators. They are the most common insect visitors and carry a heavy load of hybrid polyads. However, their behavior in foraging for pollen in the same tree may promote self-pollination. The 16-pollen polyads have the highest viability at anthesis (over 80%) but lose viability within 3 days. *In vivo* pollen germination occurs within a few hours and pollen tubes grow up to 16 μm/min, reaching the ovarian chamber 7 to 8 hr after pollination. *In vivo* pollen tube growth is supported by the stylar secretion that may be stimulated by pollination

and an ovarian secretion which is independent of pollination. Abnormalities of pollen tube growth were observed and probably result from self-pollination. There is no evidence of pollen-tube competition and pollen tube penetration of the ovules appears to occur randomly.

Fertilization in the hybrid occurs within 3 days after pollination. One of the two synergids is the site of pollen tube penetration and its degeneration is triggered by the pollen tube penetration of the nucellus. Endoplasmic reticulum is likely involved in the polar nuclear fusion but not in the fusion of sperm nuclei with the egg and polar nuclei. Because no sperm-cytoplasmic fusion occurs during karyogamy, the hybrid, therefore, possesses maternal cytoplasmic inheritance. The hybrid zygote is metabolically inactive and has a two-month dormant period due to delays in embryo nutrition. Proembryo cell divisions are of the *Trifolium* variation of the Onagrad type without formation of a suspensor. Endosperm formation is of the nuclear type. The breakdown of stored products, abundant in the central cell and nucellus, provides nourishment to the developing endosperm through many nutrient pathways. The endosperm then becomes the main nutrient source for the embryo. Carbohydrates, lipids and proteins are the main seed storage products.

The hybrid has very low reproductive success (0.0054). Low fruit set in the hybrid (2%) was attributed primarily to insufficient pollination (65% of total) and early fruit abortion (33% of total). Low seed set (24%) is mainly caused by failure of pollen tube penetration of the ovules (over 70%). The seed treatment of soaking seeds in boiling water for 1 min gives high germination percentages (over 80%) and is practical. The F_2 hybrid seedlings possess features intermediate between the parental species. At 3 months, the F_2 seedlings have a high survival rate (90%) and their height and diameter growths vary significantly among parental trees but are superior to those of the parental species.

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Dedicated to the loving memory of my grandmother.

CHAPTER 1

Introduction

1.1 Background of species

1.1.1 History of genus

The genus *Acacia* belongs to the family Leguminosae (Fabaceae), subfamily Mimosoideae. *Acacia* species are widely distributed in tropical and subtropical regions, including all continents and Pacific Islands, except Europe and Antarctica (Atchinson, 1948). *Acacia* represents the largest genus of the angiosperms in which approximately 1,200 species of shrubs and trees are recorded, mainly in Australia and Africa. About 700 species are endemic to Australia which is believed to be a center of speciation and evolution (Guinet and Vassal, 1978; Pedley, 1978).

Acacia have been tentatively classified into three geographical groups based on chromosome number, i.e., Australian and Pacific Islands species (2n=26), American and West Indian species (2n=26), and Asiatic and African species (2n=52, 104, 208) (Atchison, 1948). The systematic classification of *Acacia* was first proposed by Bentham (1875); reviewed by Guinet and Vassal (1978); and Doran *et al.* (1983) in which there are six groups, the Filicinae, Vulgares, Botryocephalae, Phyllodineae, Pulchellae, and Gummiferae. However, Guinet and Vassal (1978) subdivided *Acacia* into three groups based on more recent studies of pollen, seeds and seedlings. These include Aculeiferum

Vassal (corresponding to Bentham's series, Filicinae and Vulgares), Heterophyllum Vassal (Botryocephalae, Phyllodineae, and Pulchellae), and Acacia Vassal (Gummiferae).

1.1.2 History of *A. mangium* and *A. auriculiformis*

Acacia mangium and *A. auriculiformis* belong to the sub-genus Phyllodineae, under section Heterophyllum, corresponding to section Juliflorae of Bentham's subseries (Tindale and Roux, 1969; Pettigrew and Watson, 1975). Both species have a chromosome number of 2n=26 (Ab. Shukor *et al.*, 1994). *A. mangium* is distributed naturally in northern Queensland, Australia and extends into western Papua New Guinea and Indonesia (Doran and Skelton, 1982). *A. auriculiformis* (former name, *A. auriculaeformis*) is native to Queensland, western and southern Papua New Guinea and extends into Irian Jaya and the Kei Islands of Indonesia (Turnbull *et al.*, 1986)

Acacia mangium and *A. auriculiformis* are fast-growing, multipurpose species and widely used for timber, fuelwood, tanning, agroforestry, ornamental horticulture, and soil improvement (Phillips *et al.*, 1979; Abdul Razak *et al.*, 1981; Turnbull *et al.*, 1986). Both species have been extensively introduced into several Southeast Asian countries, particularly Malaysia, Thailand and Indonesia (Pinyopusarerk and Puriyakorn, 1987; Ibrahim, 1991; Darmono and Dayanto, 1981) and some other countries, such as India, Fiji, Sudan, Bangladesh, Tanzania, and Nigeria (Basu *et al.*, 1987; Bell and Evo, 1983; Chaffey, 1984).

As early as 1935, *A. mangium* and *A. auriculiformis* had been introduced into Thailand as ornamentals and became widely distributed throughout the country as they

possess beautiful flowers and the ability to grow in a wide range of locations (Pinyopusarerk, 1984). Following their introduction to Thailand, both species have shown great potential for growth and adaptability as well as for ornamental purposes. Provenance trials of several *Acacia* species, including *A. mangium* and *A. auriculiformis* were established in 1985 as a collaborative research project between the Royal Forest Department (RFD) and the Australian Centre for International Agricultural Research (ACIAR) (Pinyopusarerk and Puriyakorn, 1987; Boontawee and Kuwalairat, 1991; Chittachumnonk and Sirilak, 1991). Both species have shown great growth performance and survival in nearly all trial sites throughout Thailand, suggesting that they likely possess great potential for plantation establishment in Thailand.

1.1.3 The occurrence of the *Acacia* hybrid

The hybrid between *A. mangium* and *A. auriculiformis* was first recognized in 1972 by Hepburn and Shim in roadside planting in Sook, Sabah, Malaysia (Pinso and Nasi, 1992). Tham (1976) also reported the occurrence of the natural hybrid which, later on, was officially confirmed in 1978 by Pedley. This natural hybrid is believed to occur in the area where *A. mangium* and *A. auriculiformis* grow in close proximity and overlap in flowering periods. The occurrence of natural hybrids was also possible in plantations at Ulu Sedili, Johor, Peninsular Malaysia (Darus and Rasip, 1989). The natural F_1 hybrid exhibits heterosis (hybrid vigour) in growth performance, form, and adaptability when compared to the parental species (Tham, 1979). This has increased interest in whether or not the hybrid may be an alternative fast-growing species for plantations. In Malaysia,

studies have been recently carried out on some aspects of the hybrid, such as a seedling morphology and identification (Rufelds, 1987, 1988; Gan and Liang, 1991), and isozyme analysis (Wickneswari and Norwati, 1992).

In Thailand, Korwanich (1982) has been interested in the hybrid for several years and suggested that the hybrid may be obtained through controlled pollination. The occurrence of the natural hybrid was first recognized in an *A. mangium* plantation located in close proximity with that of *A. auriculiformis* at the Thai/Japan International Cooperation Agency (JICA) Reforestation and Training Centre in Sakaerat, Nakhorn Ratchasima. A small experimental plot of the F_1 hybrid was established at the ASEAN Forest Tree Seed Centre, Muak-lek, Saraburi in 1988 by Pong-anant. These hybrid seedlings were obtained through seeds collected from *A. mangium* planted next to *A. auriculiformis* at a field station at Pak Chong, Nakhorn Ratchasima. At the age of two years, the hybrid averaged 7.2 m in height and 6.01 cm in stem diameter (maximum height, 10 m and diameter, 9.8 cm) which is superior to the parental species (Kijkar, 1992). Wongmanee *et al.* (1989) also reported possible vegetative propagation from shoot cuttings of the hybrid.

1.2 Purpose of the study

In Thailand, the forest area has been decreasing drastically during the past two decades due to population pressure and over-logging, resulting in a local shortages of timber and fuelwood and soil erosion. Reforestation, therefore, is one of the most important programs that has been encouraged by the government and private sectors to

alleviate these problems. In the past decade, several exotic species, particularly fast-growing trees, have been introduced to Thailand, including *Eucalyptus*, *Pinus*, and *Acacia* (Boontawee and Kuwalairat, 1991). These species have shown their abilities to meet the increasing demands for wood material and to be used for plantations. However, limitations of these fast-growing species have been encountered following establishment of field trials. For instance, *Eucalyptus* species have had adverse effects on the environment (Poore and Fries, 1985). *Acacia* species, which have been used extensively in the industrial plantations and reforestation programs, have been susceptible to pests, diseases, and heart-rot in *A. mangium*, and may have poor bole form in *A. auriculiformis* (Ibrahim, 1991). Because of the appearance of these undesired problems, the *Acacia* hybrid (*A. mangium* x *A. auriculiformis*) has generated considerable interest for plantations since it possesses adequate growth, adaptation to different types of soil, and resistance to some pests and diseases (Pinso and Nasi, 1992). Vegetative propagation of the hybrid may be possible by shoot cuttings (Wongmanee *et al.*, 1989) but sexual propagation using high quality seed is an alternative approach. However, as fruit and seed production in most tropical tree species is variable and often very low, biological constraints must be identified and we must understand the reproductive biology (Owens, 1994). Owens (1994) suggests a number of biological constraints to fruit and seed production of tropical tree species. Such constraints can occur during the pre- and/or postzygotic period and vary among species, sites and years. Major biological constraints may include:

1. Lack of pollination and fertilization

Several studies show that fruit and seed set are primarily limited by lack of pollination and fertilization (Wilson and Schemske, 1980; Gross and Werner, 1983; Rathcke, 1983). Pollination success may be affected by availability of pollinators as well as synchronization of pollinators and flowering phenology of the trees (Bernhardt *et al.*, 1984; Tybirk, 1993). Floral characteristics such as temporal and spatial separation of the female and male floral organs also play a key role in pollination (Sedgley and Griffin, 1989). Fertilization success may be limited by pollen quality, pollen-tube growth, and pollen-pistil interaction (Knox, 1984; Herrero, 1992; O'Brien, 1994).

2. Fruit and seed abortion

Major causes of fruit and seed abortion have been proposed in many studies (Stephenson, 1981, 1992; Harriss and Whelan, 1993; O'Donnel and Bawa, 1993; Guitian, 1994). Abscission of developing fruit and seeds may be caused by adverse climatic conditions such as low temperatures or heavy rainfall (Stephenson, 1981). Limitation of fruit and seed set by resource availability has been suggested for many plants (Stephenson, 1981; Martin and Lee, 1993; Tybirk, 1993). Competition among developing fruits and seeds for limited resources may occur by which the less vigorous, as influenced by pollen source or self-pollination, are more likely to abort (Harriss and Whelan, 1993; Vaughton and Carthew, 1993; Guitian, 1994).

Studies have been done on aspects, such as reproductive biology (Ibrahim, 1991; Josue, 1992; Sedgley *et al.*, 1992a,b,c,d), nursery techniques and vegetative propagation (Ahmad, 1992; Haines and Griffin, 1992; Wong and Haines, 1992), particularly in *A.*

mangium and *A. auriculiformis*,. However, little work has been done on reproductive biology of the *Acacia* hybrid in Thailand. Also, as the genus *Acacia* contains a large number of species which are widely distributed throughout tropical and subtropical regions, the reproductive process may be different among species and locations. The purpose of this study, therefore, is to provide information on reproductive biology, which will be related to the biological constraints to fruit and seed production in the *Acacia* hybrid. The study includes:

1. Reproductive phenology and reproductive success of the F_1 hybrid in relation to climatic factors.

2. The pattern of variability in F_1 flower, fruit, seed production, F_2 seed quality, and F_2 seedling growth performance and quality.

3. Pollination biology, including floral biology and pollen vectors in relation to pollination mechanisms.

4. Pollen-pistil interactions, including *in vivo* and *in vitro* pollen germination and tube growth.

5. Ultrastructure and histochemistry of the embryo sac pre- and post- fertilization.

6. Seed development, emphasizing time and causes of embryo and seed abortion and their relation to embryo sac and embryo nutrition.

CHAPTER 2

Literature Review

2.1 Reproductive phenology

Flowering and fruiting phenology in *Acacia* varies greatly among species and locations. In Australian *Acacia*, flowering is precocious and occurs in different periods of the year. For instance, in *A. monticalo* and *A. pycnantha* , flowering occurs throughout the year (Buttrose *et al.*, 1981; Turnbull, 1986), whereas in *A. baileyana* , floral buds are produced only once a year (Boden, 1969). However, in *Acacia* grown in the tropical regions, floral bud initiation and development usually occur once a year following the emergence of new leaves during the onset of the wet season (Radwanski and Wickens, 1967; Wickens, 1969; Khan, 1970).

Acacia mangium and *A. auriculiformis* have been reported to show remarkably different flowering and fruiting behaviors among locations. In the natural habitat of Papua New Guinea, both species flower twice a year in April and July, followed by seed ripening in late September (Skelton, 1980; Turnbull *et al.*, 1983), whereas in Queensland and the Northern Territory and in the southeast and southwest of Australia, peak flowering occurs in winter and in spring, respectively (Preece, 1971). When they were both introduced outside their natural ranges, as in Sabah, Malaysia, flowering appeared in two periods, January and July in *A. mangium* and July through August and December in *A.*

auriculiformis (Ibrahim and Awang, 1992). However, major pod production occurs in March through April in *A. mangium* and August through September in *A. auriculiformis*, resulting from only one flowering peak. Ibrahim (1991) also reported variation in flowering and fruiting in *A. mangium* and *A. auriculiformis* grown in Peninsular Malaysia where both species appear to flower throughout the year but with a single peak during the June and July period. Flowering synchrony is also observed within and among individuals. Complete flower and fruit development normally takes about 200 days in *A. mangium* and 160 days in *A. auriculiformis* similar to some *Acacia* in Australia (Sedgley and Griffin, 1989). In Thailand, *A. mangium* usually flowers only once a year, during August to October, whereas in *A. auriculiformis*, there are two flowering periods, June through July and October through November (Ngamkajornwiwat and Luangviriyasaeng, 1991; Sornsathapornkul and Tangmitchareon, 1992).

It is suggested that, as *Acacia* shows variation in reproductive phenology among locations, climatic factors such as rainfall, temperature, and photoperiod may play an important role in triggering flowering. Rainy seasons followed by dry periods seemingly trigger the flowering in some *Acacia* (Wickens, 1969; Davies, 1976; Skelton, 1980). The combination of low light intensity and high temperature is also reported to inhibit floral development in *A. pycnantha* (Sedgley, 1985). Several silvicultural treatments such as fertilizer application, hormone treatments or irrigation have been reported to increase flowering in many tree crops (Sedgley and Griffin, 1989). For instance, in *A. aneura*, several flowering periods per year are obtained using additional water supplied for a 12-month period (Preece, 1971).

2.2 Floral biology

The floral morphological features of *A. mangium* and *A. auriculiformis* have been described by Pedley (1978), Turnbull *et al.* (1986) and Sedgley *et al.* 1992b. Generally, floral morphology of *A. mangium* and *A. auriculiformis* is similar. According to Guinet and Vassal (1978), inflorescences of both species are classified into the spicate group in which the flowers are borne on spikes. *A. mangium* spikes are about 10 cm long and contain an average of 195 white or creamy flowers, whereas in *A. auriculiformis*, spikes are about 7 cm long and contain an average of 105 bright yellow flowers (Ibrahim, 1991). Spikes of the *Acacia* hybrid (*A. mangium* x *A. auriculiformis*) grown in Thailand are morphologically similar to those of *A. mangium* in which spikes are 8 to 10 cm in length and made up of creamy to white flowers (Kijkar, 1992).

The flower of *A. mangium* and *A. auriculiformis* is small, usually hermaphroditic, symmetrical, and has five sepals and five petals (Ibrahim, 1991; Ngamkajornwiwat and Luangviriyasaeng, 1991; Sornsathapornkul and Tangmitchareon, 1992). The flowers, which open in the early morning, usually have with a distinctive fragrance. Neutral red tests of *A. retinodes* flowers suggest that the scent originates from the stigma and from anther epidermal cells (Bernhardt *et al.*, 1984). No floral nectar is observed in either species but extrafloral nectaries are present on the adaxial edge of the phyllode (Ibrahim, 1991). This feature is common in most phyllodinous species of *Acacia* (Boughton , 1981)

Like most *Acacia* flowers produce numerous stamens (Newman, 1934a; Buttrose *et al.*, 1981), the number averaging 113.4 in *A. mangium* and 108.9 in *A. auriculiformis*

(Ibrahim, 1991). The anthers of both species resemble those of most Australian *Acacia* species in being bilobed and terminally located on filaments (Newman, 1934a; Kenrick and Knox, 1979). Each lobe has four separate loculi, each containing one 16-grain polyad (compound pollen). Kenrick and Knox (1982) concluded that the number of pollen grains per polyad varies among *Acacia* species, i.e. four, eight, 12, 16 in Australian species (most commonly, 16 grains per polyad), and 16 and 32 in African species. The polyad usually forms from a single sporogenous cell but the polyad grain number depends on the variation in the number of mitosis of the sporogenous cell (Newman, 1933; Kenrick and Knox, 1979). Ultrastructural studies of *A. paradoxa* polyads elucidated that mature grains are held together by endexine wall bridges (crosswall cohesion) which form following meiosis in the contact site between microspores adjacent to germinal apertures (Fitzgerald *et al.*, 1993). It is postulated that the polyad provides an efficient method of pollen transfer onto the stigma of *Acacia* species (Kenrick and Knox, 1982; Knox and Kenrick, 1983). Anther dehiscence is of the Papaver type in which pollen still remains in the anther locules after anther opening (Vogel, 1978).

The *Acacia* ovary is usually sessile and covered with minute hairs (Turnbull, 1986). Ovule number is variable within- and among-species, ranging from 1 to 14 in Australian species but usually it is less than the polyad grain number (Kenrick and Knox, 1982). This is also true in *A. mangium* and *A. auriculiformis*, although the number of pollen grains per polyad is consistent (16), the ovule number ranges from 6 to 13 in *A. mangium* and 13 to 16 in *A. auriculiformis* due to variation in the ovule size (Ibrahim, 1991; Sedgley *et al.*, 1992b). In both species, the style is solid, surrounded by numerous

stamens and has almost the same length as filaments. The cup-like stigma is of the wet, non-papillate type (Heslop-Harrison and Shivanna, 1977).

The *Acacia* ovule is amphitropous, crassinucellate and bitegmic and has a unique feature in which the integuments cover only the chalazal half of the embryo sac, exposing 3 to 4 layers of micropylar nucellar cells, as in *A. retinodes* (Kenrick *et al.*, 1986). Ultrastructural studies of fabaceous embryo sacs show that the cellular organization of the embryo sac is similar to those of most angiosperms (Folsom and Peterson, 1984; Folsom and Cass, 1989) but formation of amyloplasts in the central cell and development of wall ingrowths of the embryo sac wall are distinct (Folsom and Cass, 1992).

Cruden (1977) suggested that the pollen-ovule ratio can be a useful parameter for predicting the breeding system of an angiosperm. The lower the pollen-ovule ratio, the more efficient the system of pollen transport. In *Acacia* species which possess compound pollen, the ratio of polyad grains to ovule is applied. The polyad/ovule ratio varies between 0.8 from 4.0 in most Australian species and 0.7 to 3.2 in African species (Kenrick and Knox, 1982). The polyad/ovule ratio of *A. mangium* and *A. auriculiformis* (1.23) indicates a moderate efficiency of pollen transfer (Ibrahim, 1991).

2.3 Pollination biology

2.3.1 Anthesis and floral receptivity

As *Acacia* flowers are grouped into different types of inflorescences, the patterns

of flower opening are usually determined based on the whole inflorescence and individual flowers. *Acacia* flowers live for only a few days during which the female phase normally precedes the male phase (Newman, 1934a; Philp and Sherry, 1949). In *A. retinodes* in which globose inflorescences are arranged in racemes, flowering is acropetal and florets open synchronously within an inflorescence (Bernhardt *et al.*, 1984; Knox *et al.*, 1989). Within individual flowers, development of the female phase is first complete, indicated by morphological features of the style and stigma, followed by male phase, indicated by full filament extension. However, different patterns of flower opening have been reported in *A. mangium* and *A. auriculiformis*. Within an inflorescence, flower opening can occur randomly, basipetally, or simultaneously and is completed within about eight hours (Ibrahim, 1991).

According to Heslop-Harrison and Shivanna (1977), the stigma surfaces of the angiosperms are papillate or smooth, and during the receptive period they can be dry or wet. The angiosperm stigmatic exudates are more complex than the largely sugar-containing pollination drops of gymnosperms. This may account for not only the promotion of pollen germination but also the specific stigma-pollen interaction to determine the success of self- and cross-pollination. Several methods have been employed to assess stigma receptivity (Dafni, 1992; review by Dumas and Gaude, 1993). Histochemical detection of stigmatic exudate components has been extensively used in many angiosperms (Tilton *et al.*, 1984b; Vithanage, 1984; Clifford and Sedgley, 1993).

In *Acacia*, the stigma is non-papillate and, during the receptive period, glistens with hydrophilic exudates, containing proteins, carbohydrates, and lipids (Kenrick and

Knox, 1981b). In *A. retinodes*, the first stigmatic secretion occurs prior to stylar elongation and is completed before anther dehiscence (Knox *et al.*, 1989). Ultrastructural studies also reveal that the secretory products are synthesized in the stigma cells, secreted into the intercellular space, and then to the stigma surface, as evidenced by abundant cytoplasmic organelles involved in secretory activities, i.e. ER, dictyosomes. By using cytoplasmic probes, the stigmatic exudate of *A. retinodes* has been shown to contain unsaturated and saturated lipids, free fatty acids, flavonoid aglycones, proteins, carbohydrates, and phenolic compounds. In *A. mangium* and *A. auriculiformis*, stigmatic receptivity based on the appearance of the stigmatic surface first occurs in the slightly opened flower and continues until the flower opens fully (Ibrahim, 1991).

2.3.2 Pollinators

The pollination process in angiosperms is complex because it is often associated with animals, mainly insects (entomophily), birds (ornithophily), and mammals (therophily). According to Smith (1970), the effective pollinators are characterized not only as regular visitors to the flowers of particular species over a wide range of weather conditions, but also as agents capable of carrying a pollen load to receptive female structures, clearly differentiating pollinators and mere accidental visitors. Of particular interest is how flowers advertise themselves to pollinators since there is great variation in floral structures among individual species. Fægri and van der Pijl (1979) and Yeo (1993) summarize the possible floral attractants present in most angiosperms as primary

attractants such as food rewards for visitors, including pollen, nectar, oil and protection and brood-places, whereas the floral odor, color, temperature and motion which more likely attract visitors from a distance are considered as secondary attractants. Individual species may possess only certain attractants.

In most *Acacia* species, including *A. mangium* and *A. auriculiformis*, a large number of hermaphroditic and staminate flowers were produced to provide an abundant pollen source for pollinators (Sedgley, 1987; Ibrahim, 1991; Sedgley *et al.*, 1992b). As *Acacia* species lack floral nectar, extrafloral nectaries may be an alternative reward to attract some types of pollinators. The floral colors, white in *A. mangium* and bright yellow in *A. auriculiformis*, are considered highly reflective and attract a variety of pollinators (Barth, 1985). In addition, the sweet fragrance of the flowers may increase attractiveness to pollinators.

Floral architecture plays an important role in facilitating both pollen transfer from anthers and deposition on stigmas (Sedgley and Griffin, 1989; Ohara and Higashi, 1994). A number of floral characteristics have been known to affect pollination success. These include size, spatial separation and position of male and female floral parts, and accessibility of primary attractants. In general, flowers with exposed anthers and pistils have a better chance for pollination, as in most *Acacia* flowers (Bernhardt *et al.*, 1984; Ibrahim, 1991; Sedgley *et al.*, 1992b), whereas members of the Papilionaceae have specialized flowers with enclosed anthers and pistils requiring specific pollinators.

In many angiosperms, a variety of insects, particularly from the Hymenoptera, Diptera, Coleoptera, and Lepidoptera, have been recognized as effective pollinators

(Bawa, 1990). Due to variations in floral architecture and blossom behaviour, certain pollinator types are required and show different size and foraging behaviour in collecting pollen or nectar (Fægri and van der Pijl, 1979; Laverty, 1994). Several studies have shown that some species appear to associate with many pollinator types (Cruden *et al.*, 1990; Sedgley *et al.*, 1992c; Carthew, 1993; Byragi-Reddy and Reddi, 1994), whereas in many species, pollination may rely on only a few effective pollinators (Aronne *et al.*, 1993; Heard, 1993; Armbruster *et al.*, 1994; Kearns and Inouye, 1994; Hodges, 1995).

Members of the Hymenoptera, especially bee species, play very important roles in pollination in many angiosperms (Sedgley and Griffin, 1989; Bawa, 1990; Roubik, 1993). Honey and bumble bees are the most important pollinators of many plants (Goulson, 1994; Harder and Barclay, 1994; Ohara and Higashi, 1994; Takahashi *et al.*, 1994; Willmer *et al.*, 1994). They have well-adapted nectar- or pollen-collecting organs as well as foraging behaviour and are considered the most effective pollen vectors. Normally, pollen and nectar are the main food rewards to attract most bee species. The evolution of the pollination system in neotropical species of *Dalechampia* (Euphrobiaceae) suggests that besides pollen source, both resin and fragrance also act as rewards to attract bees (Armbruster, 1993). Other animal pollinators include birds and mammals which appear mostly in subtropical and tropical regions (Sedgley and Griffin, 1989). Several bird pollinators have been reported (Knox *et al.*, 1985; Collins and Spice, 1986; Bawa, 1990; Bernardello *et al.*, 1994; Burd, 1994; Galetto *et al.*, 1994). A few mammals were found to be pollinators, including possums, bats, lemurs and monkeys (Nillson *et al.*, 1993; Gautier-Hion and Maisels, 1994; Hopkins, 1994; Sazima *et al.*, 1994).

A variety of insects from the Hymenoptera, Diptera, and Coleoptera are observed to visit *Acacia* flowers but bees (*Apis* and *Trigona*) have been reported as effective pollinators (Zapata and Arroyo, 1978; Bernhardt and Walker, 1984; Bernhardt *et al.*, 1984). In *A. mangium* and *A. auriculiformis* grown in peninsular Malaysia, the pollinators include *T. iridipennis*, *T. apicalis*, *T. atripes*. *A. javana*, and *A. dorsata* whereas, in Sabah, Malaysia, *T. apicalis*, *T. collina*, *T. canifrons*, *Phanerotoma* sp. are effective pollinators (Ibrahim, 1991). Ants are also reported to be common flower visitors but their role in pollination is still unclear (Beattice *et al.*, 1984). Several birds such as honeyeaters, silvereye, or thornbills have been observed to forage for extrafloral nectar or flowers in *A. terminalis* and *A. pycnantha* and may be possible pollinators (Ford and Forde, 1976; Knox *et al.*, 1985).

2.4 Fertilization process

2.4.1 Pollen germination and tube growth

After pollen adhesion on the stigmatic surface, pollen hydration occurs by taking up water from the stigmatic surface via the germination apertures due to the water potential gradient between stigma and pollen (vegetative cell) (Heslop-Harrison, 1987; Sedgley and Griffin, 1989). Kenrick and Knox (1982) suggested that polyads of most *Acacia* species usually fit well the stigmas. This is also the case of *A. mangium* and *A. auriculiformis* in which the polyad (30 to 40 μm) can fit neatly into the stigma cup (about

63 μm) (Ibrahim, 1991). As the polyad is a biconvex disc, two grains thick in the centre (Kenrick and Knox, 1979), only half of the polyad grains contacting the stigma are capable of producing pollen tubes. However, Kenrick and Knox (1981a) and Marginson *et al.* (1985a,b) reported post-pollination exudation on the stigma of some *Acacia* species. It is suggested that this exudate is triggered by either self- or cross-pollination, functions as a pollen germination medium to ensure the germination of all polyad grains.

Many methods have been developed to determine pollen quality, i.e. fluorochromatic reaction (FCR) test, enzymatic examination, tetrazolium chloride test, the application of nuclear magnetic resonance spectroscopy (NMR), and *in vitro* germination (Dumas *et al.*, 1984; Dafni, 1992). In *Acacia* species, pollen quality was examined using 2,3,5-triphenyltetrazolium chloride (TTC), 5-bromo-4-chloro-3-indole-beta-galactoside (X-gel), fluorescein diacetate (FDA), and Brewbaker' s solution (Kenrick and Knox, 1985; Sedgley *et al.*, 1992a, Sedgley and Harbard, 1993). However, only the fluorescein diacetate method seems to provide a reliable indication of pollen germinability (Sedgley and Harbard, 1993).

Pollen-pistil interactions in angiosperms have long been recognized as a critical mechanism determining the fate of pollen germination and tube growth (Clark *et al.*, 1990). Dumas *et al.* (1984), and Mascarenhas (1993) suggest that the requirement of specific proteins for pollen germination and tube growth is variable among plant species. In some species, the synthesized proteins required for pollen germination already exist in the mature pollen, whereas new mRNA synthesis is necessary for subsequent pollen-tube

growth. In general, the pollen tube grows between cells of the stigmas having either smooth or papillar surfaces, and enters the stylar tissues (transmitting tissues).

How pollen tubes navigate the route to the ovule may involve two signalling mechanisms, mechanical and chemotropic. The extracellular matrix (ECM) in the transmitting tissue may be involved in mechanical pollen-tube guidance (Herrero, 1992; Lord and Sanders, 1992). A cryo-SEM study in *Lilium longiflorum* has shown that pollen tubes appear to grow randomly on the stigmatic surface but the exudate from the style likely directs them into the style (Janson *et al.*, 1994). Similar studies which show the effect of stylar exudates or specific proteins have also been reported (Li *et al.*, 1994; Olson 1994). In some *Acacia* species which possess a solid style, the accumulation of proteins in the cytoplasm of transmitting cells is evident (Kenrick and Knox, 1981b), suggesting that the stylar secretions may affect pollen tubes, probably by guiding pollen tubes into the ovarian chamber (Gifford and Foster, 1989). Cytological studies showed that *in vivo* pollen tube growth in *Acacia* has two phases (Kenrick and Knox, 1989a). In the first phase, pollen tubes grow at 4.5 μ/min in the stigma and upper style, whereas in the later phase, they grow at less than half the first rate in the lower style and ovary and reach the ovary within 18 hours after pollination.

Chemotropic mechanisms have been suggested for many angiosperms (Chaubul and Rager, 1990, 1992; Huang and Russell, 1992; Franssen-Verheijen and Willemse, 1993; also see review by Dumas and Gaude, 1993). No specific chemotropic substances have been identified but calcium has been shown to have an important role in the influence of pollen-tube growth. Miller *et al.* (1992) revealed that, by using 5,5'-dibromo BAPTA,

intracellular free calcium gradients in *Lilium* pollen tubes significantly affected pollen-tube behaviour. The ovule appears to be a possible source of chemotropic substances, particularly calcium, which is secreted during the course of fertilization. Recent studies in *Arabidopsis* have also suggested that a mechanism of pollen-tube guidance may be partially governed by the specific genes of the gametophytic embryo sac (Hulskamp *et al.*, 1995).

In angiosperms, callose plays an important role in pollen-tube growth as well as in self-incompatibility. A classic technique using decolorized aniline blue dye which fluoresces using fluorescence microscopy was first developed by Martin (1959) and has been successfully employed or modified to visualize the occurrence of pollen-tube callose in many angiosperms (Beardsell *et al.*, 1993; Janson *et al.*, 1994; O'Brien, 1994; Hulskamp *et al.*, 1995). Read *et al.* (1992) noted that callose plugs usually form in normal pollen tubes in the second phase of tube growth which is considered to be a critical stage to determine self- or cross-pollen tubes. In most cases, irregular callose deposition has been found in some self-pollen tubes which are arrested in the stylar tissue (Scribailo and Barrett, 1991; Kuboyama *et al.*, 1994; Sarker and Hoque, 1994). However, in some gametophytic self-incompatible species such as *Acacia*, self-pollen tubes that are arrested within the nucellus show no or little difference from cross-pollen tubes (Kenrick and Knox, 1985; Kenrick *et al.*, 1986).

2.4.2 Fusion of female and male gametes

In angiosperms, at least three types of pollen-tube pathway into the ovules were observed, e.g. porogamy, chalazogamy and mesogamy (Bhojwani and Bhatnagar, 1975). In the most common type, porogamy, the micropyle facilitates the pollen-tube entrance into the embryo sac (Bhojwani and Bhatnagar, 1975). In *Paspalum longifolium*, abundant ER is observed in the inner integumentary cells adjacent to the micropyle and may synthesize and secrete substances to guide the pollen tubes into the micropyle (Chao, 1971, 1977). However, in *A. retinodes*, the pollen tube directly penetrates the micropylar nucellus (Kenrick *et al.*, 1986) and the mechanism of directional pollen-tube growth to the micropylar nucellus may be different.

Johri (1984) concluded that the entrance of the pollen tube into the embryo sac occurs along different pathways, i.e. between the egg apparatus, between the egg apparatus and the embryo sac wall or into a synergid cell. The penetration of the pollen tube through a synergid via the filiform apparatus has been extensively studied in angiosperms, including *A. retinodes* (Kenrick *et al.*, 1986; Sedgley and Griffin, 1989). Accordingly, the synergid is thought to play a key role in male gamete discharge (Russell, 1992). Normally, the pathway of both male gametes from the pollen-tube tip to the fusion site is the degenerated synergid (Tilton *et al.*, 1983; Johri, 1984; Dute *et al.*, 1989; Russell, 1992). The time of synergid degeneration in relation to the pollination period appears variable. Huang and Russell (1992, 1994) summarized that synergid degeneration in most angiosperms requires pollination. What signals trigger synergid degeneration is

still unknown as is the selection of the degenerated synergid. Jensen *et al.* (1983) proposed that GA and IAA may partly promote synergid degeneration in cotton. However, it still remains unclear since only one synergid degenerates *in vivo*. It is suggested that the synergid which is rich in cytoplasmic organelles, particularly mitochondria, dictyosomes, and ER, may contain some chemotropic substances which likely affect the pollen tube guidance and discharge (Johri, 1984; Brownlee, 1994). In *Nicotiana tabacum*, fluorochromatic and chlorotetracycline studies revealed a high membrane-bound calcium level in the degenerated synergid and the formation of a calcium gradient near the synergid. This may function in pollen tube guidance and male gamete release (Huang and Russell, 1992). A similar phenomenon was also reported in wheat (*Triticum aestivum*) (Chabaul and Reger, 1990).

Following the migration of both sperm cells into the embryo sac, double fertilization occurs by which one sperm fertilizes the egg cell and the other fuses with the polar nuclei or secondary nucleus. A recent study has shown that cytoskeletal organization is involved in the movement of sperm toward the egg cell and the central cell (Huang *et al.*, 1993; Huang and Russell, 1994; Kropf, 1994). Following pollen-tube discharge into the receptive synergid, abundant F-actin, labelled by rhodamine-phalloidin and anti-actin immunogold, forms two distinct bands between the synergids, egg and central cell; the so called corona which may mechanically facilitate the movement of male gametes to the egg and central cell (Huang *et al.*, 1993; Huang and Russell, 1994). In addition to the cytoskeleton organization, the most recent ultrastructural study in *L. longiflorum* has revealed a network of endoplasmic reticulum which occurs between the

central cell and the sperm nucleus and may be involved in transport of the sperm cell to the central cell (Janson and Willemse, 1995). How the sperm nucleus moves into the egg cell is less understood in seed plants, whereas the cytoskeletal organization has been well studied in seaweeds (Fucales) by Kropf (1994).

In most angiosperms, there are no reports of male gamete competition within the embryo sac which usually receives only one pollen tube with two sperms. Two hypotheses have been proposed regarding whether double fertilization is preferential or random (Knox *et al.*, 1993). The fusion of sperm and egg may occur randomly and fertilized eggs may possess some electrochemical mechanism to prevent polyspermy. This has recently been found in *in vitro* fertilization of maize (Faure *et al.*, 1994). The existence of such a mechanism could be involved in the selective fusion of compatible male gametes with the egg or central cell. It appears that sperm cells are preprogrammed to fuse with the egg or central cell. Sperm dimorphism has been reported based on either cytoplasmic heritable organelles (cytoplasmic heterospermy) or the proportion of B-chromosomes in the nucleus (Johri, 1984; Russell, 1993). In *Plumbago*, the plastid-rich sperm cell most often fuses with the egg. In maize, the sperm cell with extra B-chromosomes more likely fuses with the egg cell.

In most species, the fusion of two haploid polar nuclei occurs prior to fertilization, as in soybean (Sedgley and Griffin, 1989; Folsom and Cass, 1992). Polar nuclear fusion starts with the linkage of the nuclear envelope, followed by fusion of nucleoplasm of both nuclei, as explained by Jensen (1964). In addition, abundant ER observed in the fusion site may be involved in the fusion of the polar nuclei as suggested in cotton (Jensen, 1964;

van Went, 1970c). The diploid polar nucleus (secondary nucleus) then fuses with the male gamete to form the primary endosperm. In contrast to the polar nucleus fusion, karyogamy of the egg and sperm nucleus likely occurs by alignment and contact of both nuclei and is less associated with the ER (van Went, 1970c; Janson and Willemse, 1995).

2.4.3 Patterns of cytoplasmic inheritance

During fertilization, gametic fusion is accompanied by the recombination of the heritable cytoplasmic organelles, especially plastids and mitochondria, present in both sperms and eggs (Johri, 1984). The patterns of cytoplasmic inheritance are variable among seed plants (Sears, 1980). In the majority of angiosperms maternal inheritance of plastids and mitochondria is common with only rare instances of biparental inheritance (Sears, 1980; Mogensen, 1988, 1996; Russell, 1992). Hagemann and Schröder (1989) noted that, in uniparental inheritance, the exclusion of plastids from the male gametophyte occurs between male gametophyte development and fertilization. At pre-fertilization, the absence of plastids in the generative cell results from extremely unequal mitosis of haploid pollen (Lycopersicon type), and the disappearance of a small amount of plastids or diminution of the cytoplasm during the maturation of the generative cell (Solanum type) as in *Cymbidium goeringii* (Orchidaceae) (Yu *et al.*, 1992). In the Triticum type, the plastids or cytoplamic bodies are stripped off the sperm nucleus and remain in the degenerated synergids during the fertilization process as in *Lilium longiflorum* (Janson and Willemse, 1995). The absence of paternal plastids in progeny may occur by plastid

degeneration or exclusion (Mogensen and Rusche, 1985; Connett, 1987; Hagemann and Schröder, 1989). Biparental plastid inheritance (Pelargonium type), present in a small number of angiosperms, occurs when both plastids and mitochondria are transmitted into the embryo. However, occasional biparental inheritance has been reported in some angiosperms such as *Nicotiana tabacum* (Yu and Russell, 1994) which normally possesses maternal inheritance. This phenomenon has been attributed to the incomplete exclusion of plastids and mitochondria during generative cell development. Both male plastids and mitochondria are transmitted to the embryo, resulting in biparental inheritance.

2.5 Breeding system

Acacia species have shown high rates of outcrossing (Philp and Sherry, 1946; Moffett, 1956; Coaldrake, 1971). For instance, in *A. mearnsii*, the outcrossing rate is estimated between 67 to 89% (Moffett, 1956; Sherry, 1971). At least three floral mechanisms have been reported to promote outcrossing in *Acacia*, e.g. protogynous dichogamy, andromonoecy, and self-incompatibility (Sedgley, 1987).

Protogynous dichogamy in flowering plants refers to temporal separation of male and female organs in which the female phase precedes the male phase. In *A. retinodes*, the stigma becomes receptive before anther dehiscence (Bernhardt, *et al.*, 1884; Knox *et al.*, 1989). However, protogynous dichogamy is less distinct in *A. mangium* and *A. auriculiformis* as development of female and male phases are complete almost synchronously, suggesting that the flowers are more likely homogamous (Ibrahim, 1991).

Andromonoecy is the condition in which both hermaphrodite and staminate flowers are present in the same plant (Richards, 1986). It is suggested that andromonoecy may promote outcrossing in which the excess staminate flowers increase attractiveness to pollinators as well as pollen donation (Zapata and Arroyo, 1978; Guitian, 1993). Andromonoecy has been reported in several *Acacia* species, such as *A. baileyana* (Newman, 1933, 1934a), *A. macracantha* (Zapata and Arroyo, 1978), and *A. nilotica* (Tybirk, 1989). The number of staminate flowers with absent or small pistils is highly variable, ranging from 4.0 to 57.4% in *A. mangium* and 0.4 to 5.1% in *A. auriculiformis* (Ibrahim, 1991; Sedgley *et al.*, 1992b).

Self-incompatibility (SI) is one of the most common mechanisms that prevent seed set from self-pollination in flowering plants (de Nettancourt, 1977). More than 3000 species of angiosperms were reported to have SI (Franklin *et al.*, 1995). In the homomorphic system, two SI models have been recognized, sporophytic and gametophytic SI. In the sporophytic system, SI is determined by interaction between the diploid genotype of the pollen wall and pistil, resulting in pollen-tube arrest on the stigmatic surface (Franklin *et al.*, 1995). In the gametophytic system, SI is controlled by the S alleles of haploid pollen tube and diploid genotype of the pistil, resulting in the pollen-tube inhibition in the style or in the ovary (Franklin *et al.*, 1995). According to Seavey and Bawa (1986), pollen-tube inhibition in the ovary (ovarian SI) is considered late-acting SI, which is indicated by pollen-tube arrest in the nucellar tissue (Kenrick *et al.*, 1986), male gamete abortion (Cope, 1962) or abortion of zygote and/or endosperm (Williams *et al.*, 1984).

A large number of the Leguminosae have been reported to have homomorphic SI, perhaps with some having heteromorphic system (Arroyo, 1981). In Mimosoideae, SI has been established for the tribes Acacieae, Mimoseae, Ingeae and probably Parkieae (Arroyo, 1981). In the Acacieae, SI has been observed in several *Acacia* species (Simpson, 1977; Kenrick and Knox, 1985, 1989b; Bernhardt *et al.*, 1984). Based on the index of self-incompatibility (ISI), determined by the ratio of seed set following self-pollination to that from cross-pollination, different degrees of self-incompatibility in *Acacia* include high self-incompatibility, as in *A. mearnsii* and *A. retinodes*, partial self-incompatibility, as in *A. myrtifolia* and *A. terminalis*, and complete self-compatibility, as in *A. ulicifolia* (Kenrick and Knox, 1989b). ISI of 0.38 in *A. mangium* and 0.21 in *A. auriculiformis* suggest that both are outcrossing species with partial self-incompatibility (Ibrahim, 1991).

Cytological studies of *in vivo* pollen-tube growth in *A. retinodes* suggested that the form of self-incompatibility in *Acacia* is likely gametophytic based on pollen-tube arrest in the nucellus following self-pollination (Kenrick *et al.*, 1986). As *Acacia* species show different degrees of gametophytic SI, different mechanisms have been proposed to explain SI. These include the S-gene controlled system, gamete-gamete interactions at fertilization, and action of recessive postzygotic lethal genes (Kenrick and Knox, 1989b).

2.6 Seed development and maturation

2.6.1 Embryo and endosperm development

Following zygote formation, the zygote may remain dormant or undivided while undergoing cellular and cytoplasmic changes (van Went and Willemse, 1984). The first zygotic division results in two cells which are asymmetrically separated by a transverse wall, rarely a vertical or oblique wall (West and Harada, 1993). Different embryonic types have been classified based on the orientation of cell walls as well as the sequence of cell divisions of the proembryo (Johri, 1984). Embryonic types include Onagrad, Asterad, Solanad, Chenopodiad, Caryophyllad, and Piperad. Embryo development of mimosoid plants, including some *Acacia* species, is of the Onagrad type (Johri *et al.*, 1992).

The apical cell, usually containing more cytoplasm, contributes in a major way to the embryo, and the basal cell undergoes transverse divisions, contributing to most of the suspensor. However, suspensorless embryos are observed in several members of Mimosaceae, including Acacieae such as *A. baileyana*, *A. albida*, *A. retinodes*, *A. farnesiana*, and *A. auriculiformis* (Narasimhachar, 1948; Dnyansagar, 1958; Lersten, 1983; Johri *et al.*, 1992). Embryo development in *A. paniculata* reveals that, as the proembryo lacks a suspensor, the embryo appears attached to the remaining micropylar nucellus which remains until the late globular embryo stage (Souza, 1993).

In most angiosperms, development of the zygote or proembryo is delayed until after complete development of the endosperm. Three types of endosperms have been

reported; nuclear, cellular, and helobial (Johri, 1984). Johri *et al.* (1992) reported that development of the endosperm in Mimosoid plants is of the nuclear type. Wall formation is usually initiated at the micropylar end, proceeding towards the chalazal end, and occurs at different developmental embryo stages, i.e. at the four-celled proembryo in *Mimosa hamata* or at the dicotyledonous embryo stage in *Neptunia triquetra* and *Prosopis spicigera* (Dnyansagar, 1952, 1954).

Although there is variation among families in the developmental patterns of angiospermous proembryos, late embryonic development in dicots appears morphologically similar. In *Acacia*, following zygotic division, the developmental sequence of the embryo includes different distinct morphological embryonic stages, including globular, heart, and linear cotyledon shapes (Dnyansagar, 1958; Souza, 1993).

2.6.2 Nutrition of the embryo sac and embryo

As the growing embryo acts as a sink in the embryo sac, a number of modifications of the ovular tissues, embryo sac, and embryo are thought to either provide nourishment or to facilitate nutrient movement into the growing embryo (Vijayaraghavan *et al.*, 1988). After fertilization, in most angiosperms, the ovule consists of intact nucellar tissues and integuments and the included embryo sac contains the diploid zygote, haploid synergids, antipodal cells, and a triploid primary endosperm nucleus.

The suspensor, which differentiates from the basal cell of the proembryo, elongates and pushes the developing embryo into the nourishing endosperm tissues (Folsom and

Cass, 1992; Johri, *et al.*, 1992; Taylor and Vasil, 1995). The suspensor probably serves as a nutrient pathway from sporophytic maternal tissues to the embryo during the actively-dividing endosperm stage (Cionini, 1987; Vijayaraghavan *et al.*, 1988; Yeung and Meinke, 1993). As well, the suspensor may be involved in early embryo development (Yeung and Meinke, 1993).

At the early embryo stage many cells in the embryo sac play an important role in facilitating a nutrient movement from maternal tissues into the embryo sac. Synergids exhibit some characteristics of transfer cells, as indicated by finger-like wall of filiform apparatus, and probably assist in short distance transport of nutrients into the embryo sac (Gunning and Pate, 1969; Gunning, 1977). In some species, such as *Papaver somniferum*, the antipodal cells which persist and multiply after fertilization, develop wall ingrowths and give the appearance of transfer cells (Bhandari and Bhargava, 1983). This provides a major nutrient pathway from integuments into the embryo sac through the chalazal region where the vascular supply terminates (Bhojwani and Bhatnagar, 1975; Vijayaraghavan *et al.*, 1988). As the largest cell in the embryo sac, the central cell also plays a central role in supporting development of the endosperm and embryo. In most legume embryo sacs, as in broad bean and soybean, wall ingrowths form in the central-cell wall, suggesting movement of metabolites among the integuments, endosperm and embryo (Vijayaraghavan *et al.*, 1988; Folsom and Cass, 1992; Johanssan and Walles, 1993b; Chamberlin *et al.*, 1994).

Endosperm functions as a nutrient source for the developing embryo. In some legumes, the endosperm acts as a nutrient sink during its early developmental stage and

becomes an important nutrient source at later developmental stages of the embryo (Johri *et al.*, 1992). During embryo development, stored products from the endosperm are relocated into cotyledons which, later, have an active role during seed germination in exalbuminous seeds. Van Staden *et al.* (1987) suggest that cytokinins and gibberellins accumulate in the endosperm and are then transported to the embryo. In albuminous seeds, the endosperm may not be used up as food reserves, as in grasses, maize, and some members of Orchidaceae (Johri, 1984; Johri *et al.*, 1992). A more complex relationship between endosperm and cotyledons has been shown in recent studies of *Cercis siliquastrum* (Caesalpinioideae) (Baldan *et al.*, 1995).

Changes in ovular tissues in relation to embryo nutrition mainly involve development of the nucellus and integuments. Degenerate nucellar tissues observed before or after fertilization provide nutrition for the embryo and/or endosperm but the time of the degeneration is variable. Degeneration may occur after fertilization (Johanssan and Walles, 1993a, b), or the nucellus may persist for a certain period of time (Chamberlin *et al.*, 1994; Palupi, 1996) then completely degenerate during embryo sac development (Bhojwani and Bhatnagar, 1975). As a result, the embryo sac comes in direct contact with the integuments. In some cases, the inner integumentary cells abutting the embryo sac differentiate into a distinct layer, called the endothelium. This specialized layer is thought to facilitate nutrient movement into the embryo sac (Torosian, 1971; Vijayaraghavan *et al.*, 1988; Chamberlin *et al.*, 1994) as indicated by histochemical (Sehgal and Gifford, 1979) and autoradiographic studies (Chamberlin *et al.*, 1993).

Johri *et al.* (1992) suggest that development of haustoria in various types of cells of the ovule or embryo sac plays an important role in the nutritive function. Haustorial structures can occur in embryo sac, synergids, antipodal cells, pollen tubes, suspensor and endosperm (Bhojwani and Bhatnagar, 1975). The presence of free- nuclear or cellular endosperm haustoria is well known in many members of the Leguminoseae, Cucurbitaceae, and Campanulaceae. In *Lobelia dunnii*, the endosperm haustoria develop wall ingrowths of transfer cell type that are thought to increase the nutrient flow to the embryo sac from the surrounding tissues (Torosian, 1971).

2.6.3 Seed maturation

Seed maturation is considered to be the last stage in development of the fertilized ovule in which the embryo is then ready to germinate and then develop into a mature plant. Seed maturity can be indicated by physical, biochemical, morphological and physiological indices (Edwards, 1980; Willan, 1985; Sedgley and Griffin, 1989). In most tree seeds, the physical indices have been practically and widely used to determine the state of maturity, including color, size, moisture content and specific gravity.

In some tree species, color changes in fruit and seed, usually accompanied by fruit hardening or dehiscence, may provide a simple and reliable indicator of maturity (Willan, 1985). In *Acacia* species such as *A. mangium*, the fruit (pod) is of the dry dehiscent type and is ready for harvest when its color turns from dark brown to black or the seed

funiculus color becomes orange (Bowen and Eusebio, 1981). Similarly, brown opening pods and black seedcoats indicate maturity in *A. auriculiformis* (Pukittayacamee, 1988).

Fruit and seed moisture content plays an important role in seed development, as water loss usually occurs during maturation. In many tropical tree seeds, moisture content is high, between 40 to 60 % at maturation (Yue-Luan, 1993). Amata-archachai and Hellum (1984) reported that, in *A. catechu* grown in Thailand, pod moisture content was 30 to 50% at maturation. In *A. auriculiformis* grown in the same location, fruit and seed moisture content were about 35% and 27% respectively, at maturation (Pukittayacamee, 1988). However, as moisture content is affected by weather conditions and varies among locations, it is recommended that fruit and seed moisture content can be used as reliable indices to determine maturity only when combined with other indices (Liengsiri and Hellum, 1984; Pukittayacamee, 1988).

The use of specific gravity in determining fruit and seed maturity is common for temperate tree seeds but is less popular for tropical tree seeds (Tanaka, 1984; Willan, 1985). When compared to other maturity indices, fruit and seed size appear to be less reliable because the seed reaches its maximum size prior to maturity and becomes variable in size and a poor indicator of maturity (Edwards, 1980).

2.6.4 Seed abortion

The primary limitation to fruit and seed set is lack of pollination and fertilization, but postzygotic seed abortion occurs in many flowering plants even after successful

pollination and fertilization (Casper, 1988; Nakamura, 1988). Stephenson (1981) discussed possible factors causing fruit and seed abortion during development, including many abiotic and biotic factors, such as adverse climatic conditions and insect predators reported to damage fruits, cause early abortion in many tree species. Other studies also indicate that fruit and seed abortion may be affected by recessive lethal genes through self-pollination, as in *Banksia spinulosa*, *Grevillea barklyana* and *Stylidium* sp. (Burbidge and James, 1991; Vaughton, 1993; Harriss and Whelan, 1993) Abnormal development of the seed, including problems with endosperm and embryo sac, may result in early seed abortion (Palupi, 1996; Shuraki and Sedgley, 1996). Maternal plant resource availability plays an important role in limiting fruit and seed set in many plants in which fruits or seeds that are more vigorous or better genetically, may have a better chance to obtain limited sources, and the others that initiate later, will abort, as suggested in *Cassia fasciculata* and *Prunus mahaleb* (Martin and Lee, 1993; Guitian, 1994). Studies in several African *Acacia* species, such as *A. nilotica* and *A polyacantha*, reveal that limitations in resource availability probably cause high seed abortion rate varying from 6.4 to 48.0% (Tybirk, 1993). Selective seed abortion, in which seeds tend to abort in the basal end of the pods, as in many legumes has also been reported (Bawa and Buckley, 1989).

2.7. Seed structure and components

2.7.1 Seed structure

Seed structure in the Leguminosae has been described by several authors (Corner, 1951; Gunn, 1981; van Staden, 1989). Among subfamilies of the Leguminosae, structures of mimosoid seed appear to be more similar to those of caesalpinioid seed rather than those of papilionoid seed. In most mimosoid plants, the seed is bilaterally symmetrical and contains a large, straight embryo with a short radicle and thick cotyledons. The plumule is usually rudimentary to well developed. The hilum is contiguous with the tip of the radicle and the slit-like micropyle. The discolored lens located near the hilum varies in shape, i.e., oval, circular or flattened. The testa is smooth with the presence of a pleurogram and fracture lines. The unbranched vascular bundle forms a loop which extends almost completely around the seed.

In most mimosoid seeds, the seedcoat is formed only by the outer integument, whereas the inner integument and nucellus usually degenerate during seed development (van Staden, 1989). Anatomically, the seedcoat consists of distinct layers, from the outermost layer, including cuticle, macrosclereid (palisade), osteosclereid (hour glass), and parenchyma cells. In some species, macrosclereid cells are separated into two parts, the body and cap, by the light line (Manning and van Staden, 1985). Scott *et al.* (1962) suggest that the light line is an optical phenomenon which may be caused by change of microfibrillar orientation between the body and the cap.

2.7.2 Seed storage

Nearly all legume seeds have been known to contain storage products in the form of discrete protein bodies, lipids droplets, and starch grains or cell wall polysaccharides, mainly in the cotyledons (Smith, 1981). Mobilization begining in the central region of the cotyledons, is most common in legume seeds, as in *Acacia*, *Cassia*, *Sophora*, *Trifolium*, and *Vicia*. The other types include mobilization at the abaxial or adaxial side of the cotyledons, as in *Pisum arvense* or at both sides of the cotyledons, as in *Piptanthus* sp. (Smith and Flinn, 1967).

Accumulation of storage products differs between albuminous and exalbuminous seeds. In exalbuminous seeds in which the endosperm is consumed during seed development, most reserve products are stored in the cotyledons and/or embryo. In albuminous seeds, most reserve products are stored in the cotyledons, embryonic axis and endosperm. Ultrastructural studies of the mature seed of *Cercis siliquastrum* (Caesalpinioideae) revealed that only lipids and proteins are usually stored in the endosperm and embryo, whereas starch is absent (Baldan *et al.*, 1995). In mature *Trifolium repens* seed, only proteins and starch are stored in the embryo and endosperm (Jakobsen, *et al.*, 1994).

As legume seeds are considered to be an important protein source, much attention has been paid to the synthesis and deposition of storage proteins (Shotwell and Larkins, 1989). Proteins are usually synthesized during seed development and, during maturation, accumulate in small membrane-bounded organelles, called protein bodies (Zheng *et al.*,

1992). Different mechanisms of protein body formation have been proposed in several legume seeds. Firstly, protein bodies are derived from the vacuoles as evidence by ultrastructural studies in *Lupinus* sp. (Davey and van Staden,1978). Secondly, protein bodies may be developed from rough ER (Bain and Mercer, 1966) or from smooth ER (Craig *et al.*, 1979), as in *Vicia faba* (Adler and Muntz, 1983). However, both mechanisms of protein body formation have been observed in *Glycine max* (Zheng *et al.*, 1992).

2.8 Seed quality test

Seed quality tests are required to ensure vigor and germination potential of seeds used for plantations. In many temperate forest tree seeds, methods used to test seed quality have been standardized by the International Seed Testing Association (ISTA, 1976). In tropical forest tree seeds, several methods of seed quality test have been optimized from those established by ISTA, based on seed characteristics and structures (Yue-Luan, 1993). Seed quality tests mainly concern viability and germinability. Seed viability tests include the tetrazolium (TZ) test, excised embryo test, embryo culture test, the hydrogen peroxide test, and X-radiography test. However, as tropical tree seeds vary greatly in their characteristics and structures, only selected methods may be employed for certain tree species. The technique of X-radiography has been widely used as a quick and reliable test of seed quality in many tree species, including legumes (Kobmoo *et al.*, 1990;

Yue-Luan, 1993). Pukittayacamee (1988) reported that, in *A. auriculiformis* grown in Thailand, approximately 90% of mature seeds are viable based on the X-radiography test.

The seed germination test is one of the most common tests for tree species. For germination of hard seeds of all three subfamilies of the Leguminosae, pretreatments are required to break dormancy (Yue-Luan, 1993). Longman (1969) suggests that in tropical regions, seed dormancy in tropical regions appears to be related to survival during the dry period and seedling establishment during the raining season. Different types of seed dormancy have been discussed by Bewley and Black (1982) and van Staden *et al.* (1989). Seed dormancy in legume species is attributed to seedcoat impermeability, controlled by the degree of seed dehydration during maturation (van Staden *et al.*, 1989). For instance, in *Trifolium subterraneum*, the percentage of hard seed depends on the drying period on the plant (Aitken, 1939). According to Lebedeff (1943), degree of testa impermeability is controlled by the critical seed moisture content which is usually below 20% and may be as low as 6%. Several authors pointed out that hardseededness and impermeability of the legume testa are caused mainly by the presence of the macrosclereids and seedcoat cuticle (Corner, 1951; Bukovac *et al.*, 1981).

Numerous pretreatments have been employed to break dormancy and improve germination in hard seeds, including hand and mechanical scarification, boiling or hot water, and chemical treatments. However, results of such treatments vary among families, species, and locations. This is also the case for *Acacia* species growing in different regions. For example, in six Sudanese and five Australian acacias, seedcoat scarification significantly improve seed germination rate (Clemens *et al.*, 1977; Bebawi and Mohamed,

1985). For boiling and hot water treatments, different initial water temperatures have been reported to give high seed germination, i.e., 100 C for *A. falcata* or 80 C for *A. terminalis* and *A. suaveolens* (Clemens *et al.*, 1977; Auld, 1986). ISTA (1976) recommends concentrated sulfuric acid to break dormancy and improve germination in some *Acacia* seeds (Shaybany and Rouhani, 1976; Danthu *et al.*, 1992). The application of microwave energy at 2450 MHz is also used to break dormancy in *A. longifolia* and *A. sophorae* (Tran, 1979). In *A. mangium* grown in Malaysia, a number of methods have been used, such as mechanical seedcoat scarification or application of concentrated sulfuric acid but boiling water treatment alone is considered practical and reliable (Adjers and Srivastava, 1993). In *A. auriculiformis* grown in Thailand, treatment with 95 C water for 1 and 3 minutes provides high seed germination of 95.5%, whereas using hand scarification and concentrated sulfuric acid, germination varies between 76.5 to 85.5% (Pukittayacamee, 1988).

2.9 Seedling growth

According to de Vogel (1980), patterns of seedling development in legume plants can be classified into six types based mainly on development of cotyledons and arrangement of seedling leaves (eophylls). These include Macaranga, Sloanea, Heliciopsis, Horsfieldia, Endertia, and Cynometra types. Two basic types of cotyledons were observed during early stages of legume seedling development, namely, foliar cotyledons (phyllolobees) and storage cotyledons (sarcolobees) (Duke and Polhill, 1981).

Cotyledons also vary in their position during seed germination; epigeal germination with cotyledons spreading above or at soil-level, and hypogeal germination with cotyledons remaining covered by the soil (Duke and Polhill, 1981).

In members of Mimosoideae, the seedlings are of the phyllolobees type with the phaneroepigeal feature (cotyledons becoming positioned above soil level during seed germination and releasing from the testa during early seedling growth) (Duke and Polhill, 1981). The first eophyll (true leaf) appears to be pinnate, followed by the alternately-arranged eophylls of bipinnate with one pair of pinnae, which often emit fragrance. In general, *Acacia* seedlings possess some features like those described in subfamily Mimosoideae except that the first eophyll or pair of opposite eophylls appear bipinnate. Also, the cotyledons have different forms of stalk and shape, that is used for the classification of *Acacia* (Guinet *et al.*, 1980).

Rufelds (1987, 1988) provided detailed information on seedling development in *A. mangium*, *A. auriculiformis* and the F_1 hybrid grown in Malaysia. Like most phyllodinous species, the hybrid and its parental species have similar patterns of leaf development in which the first eophyll is pinnate, followed by bipinnate and/or compoundly bipinnate eophylls. During development, new bipinnate and/or compoundly bipinnate leaves gradually transform into complete phyllode form. Gan and Boon Liang (1992) simplified the method for identification of seedlings of *A. mangium*, *A. auriculiformis* and the hybrid by using the number and features of bipinnate and/or compoundly bipinnate leaves.

Growth performance of the hybrid in relation to its parental species varies from location to location. The height performance of the hybrid grown in Sabah, Malaysia

appears insignificantly different from that of *A. mangium* (Rufelds, 1987), whereas, in Peninsular Malaysia, the natural hybrid has a better growth rate than that of *A. mangium* (Darus and Rasip, 1989), probably due partly to environmental differences (Martin, 1989). Recent studies by Ibrahim (1991) also showed that the 7-month old hybrid seedlings height growth of either *A. mangium* or *A. auriculiformis* as female trees is significantly superior to that of their parental species. However, there is no significant difference in biomass production between the hybrid and its parental species.

CHAPTER 3

Variation in Flower and Fruit Production, and Seed and Seedling Quality of a Tropical *Acacia* Hybrid (*A. mangium* Willd. x *A. auriculiformis* A. Cunn. ex. Benth.)

3.1 Introduction

The rapid depletion of natural forests in Southeast Asia, resulting in shortage of wood materials, has intensified the search for suitable species for rehabilitation programs. Many exotic fast-growing trees that have been introduced to Southeast Asia, especially Indonesia, Malaysia, Thailand and The Philippines, show potential in adaptability and growth. Among these, *Acacia*, particularly *A. mangium* and *A. auriculiformis* (Mimosoideae: Leguminosae), have been used extensively in industrial plantations and reforestation programs (Turnbull, 1986; Chittachumnonk and Sirilak, 1991; Othman and Tan Get Seng, 1993). *Acacia mangium* is native to northern Queensland, western Papua New Guinea, and Irian Jaya and Maluku of Indonesia (Doran and Skelton, 1982), whereas *A. auriculiformis* is native to Queensland, western and southern Papua New Guinea, and Irian Jaya and the Kei Islands of Indonesia (Turnbull *et al.*, 1986). When introduced outside their natural ranges, both species exhibit satisfactory growth characteristics and adaptation and have been widely used as multipurpose species. However, *A. mangium*, which usually requires fertile soil and higher moisture is reported to be susceptible to pests and heart-rot while *A. auriculiformis* has shown poor bole form (Ibrahim, 1991). Due to

these disadvantageous characteristics, their interspecific hybrid which grows faster, adapts to different soil types, has attracted considerable interest (Pinso and Nasi, 1992).

The occurrence of natural hybrids between *A. mangium* and *A. auriculiformis* has been reported in sites where they are growing in close proximity and have overlapping flowering periods (Tham, 1976; Darus and Rasip, 1989; Kijkar, 1992). Also, the F_1 hybrid seed can be produced through artificial cross- pollination (Sedgley *et al.*, 1992a) or in biclonal seed orchards (Griffin *et al.*, 1992; Wickneswari and Norwati, 1992) but these methods may not be practical.

Several studies, particularly in Malaysia and Thailand, have emphasized reproductive biology (Ibrahim, 1991; Ibrahim and Awang, 1992; Sedgley *et al.*, 1992b; Sedgley *et al.*, 1992c), nursery techniques and vegetative propagation (Ngulube, 1990; Ahmad, 1992; Pinyopusarerk *et al.*, 1991; Wong and Haines, 1992). For the F_1 hybrid, some research has been done on nursery techniques, particularly vegetative propagation, and a comparative study of early seedling growth in order to identify the F_1 hybrid (Rufelds, 1987, 1988; Gan and Liang, 1991; Kijkar, 1992). Although the hybrid has shown promise as a plantation tree, relatively little is known about its reproduction, especially, phenology, and flower, fruit and seed production.

The purpose of this study was to determine the variation in the reproductive characteristics among hybrids. Quantitative estimation of reproductive growth and seedling performance were statistically assessed for within- and among- tree variation in both the F_1 and F_2 *Acacia* hybrid for the following: i) reproductive phenology of F_1 hybrid in relation to environment; 2) variability in F_1 flower, floral organs, fruit, and seed

production; 3) variability of F_2 seed quality; and, 4) F_2 seedling growth performance and quality.

3.2 Material and methods

3.2.1 Study Site

The study was carried out using an experimental plot of 4-year-old *Acacia* hybrids at the ASEAN- Forest Tree Seed Center, Muak-lek, Saraburi, Thailand, located at latitude N 14° 40' and longitude E 101° 17' and about 200 m elevation. The hybrid trees were planted in 1988 and first flowered in late 1990. The hybrid seeds were obtained from a plantation of *A. mangium* adjacent to a stand of *A. auriculiformis*. Parental species overlap in their flowering period in October-November and appear to have the same pollinators, therefore the hybrids resulted from natural hybridization. Twelve uniform trees were selected for this study. All were dominant to ensure their capability of flowering and fruiting and all had quite similar microenvironments. Climatic data were obtained from a weather station at the Thai-Danish Dairy Farm, located about 500 m north of the Centre (Fig. 1).

3.2.2 General reproductive phenology

Phenological observations were made during 1991 and 1992. Reproductive growth was monitored every two weeks and vegetative growth every month. Observations on flowering phenology were carried out at first flowering, peak flowering, and last flowering. Floral initiation was determined by emergence of visible floral buds. Microscopic observations were made of subsequent stages, including anther dehiscence and pollen transfer. Detailed observations of the fruiting period began approximately one week following anthesis of individual inflorescences, or when all unpollinated flowers completely abscised or fruit in pollinated flowers began to develop. Fruit and seed development were monitored until maturation as indicated by brown color and dehiscence of dry fruit. Phenological data were compared with climatic data.

3.2.3 Variation in flower, fruit and seed production

The flowers are grouped into spikes, usually borne along indeterminate shoots. Individual flowers may be perfect or staminate. The staminate flowers are characterized by absence of the pistils or having rudimentary pistils (small pistils lacking the ovules). A number of insects, particularly aphids and some larvae cause flowers to abort. Accordingly, the flowers were assigned into three morphological categories; perfect, staminate and aborted. To determine the variation in the number and categories of flowers per spike, eight flowering spikes per tree were collected, four from the upper crown and

four from the lower crown, representing the four quadrants of each crown level. Individual spikes were measured and divided into basal, middle, and distal regions in order to determine the distribution and variation of different flower types. To determine the variation of floral organs, 20 flowers were randomly sampled from eight spikes from each of 11 trees. Ten of these flowers were fixed in glacial acetic acid:ethanol (1:1), cleared and, stained with decolorized aniline blue, then viewed by fluorescence microscopy (Martin, 1959) to assess the number of ovules per flower. Another 10 flowers were used to determine the average number of anthers and pollen per flower. Pollen grains are grouped into a polyad. At least 20 anthers were sampled from each of ten flowers to determine the average number of pollen grains per polyad.

On each of 12 trees, about 40 fruit-bearing spikes were tagged in two crown levels (upper and lower) and in each quadrant of the crown. The number of pods per infructescence and the number of mature and aborted seeds per pod were counted.

3.2.4 Evaluation of reproductive success

In angiosperms, preemergent reproductive success (PERS) has been used to measure plant ability to produce flowers that develop into fruits, and ovules that develop into viable seed (Wiens *et al.*, 1987). For the *Acacia* hybrid, PERS is calculated by counting the mean number of flowers per inflorescence (Fl), fruits per infructescence (Fr), ovules per flower (O), and seeds per fruit (S), and PERS = (Fr/Fl ratio) / (S/O ratio).

Pollen to ovule (P/O) ratio is calculated by the grain number per polyad divided by the ovule number per ovary.

3.2.5 Seed quality test

Mature pods were collected from all 12 hybrid trees and the F_2 seeds were extracted, cleaned and used for four tests, including seed weight, water content, viability and germination.

3.2.5.1 Seed weight

Due to the small size of the F_2 seed, 1000-seed weight was used rather than 100-seed weight. For each parent tree, ten replicates of 1000 seeds each were used.

3.2.5.2 Seed water content

Samples of four replicates of 400 F_2 seeds from each parent tree were weighed (fresh weight), and dried at 103°C for 24 hours, then seed dry weight was determined in order to calculate the water content (WC).

3.2.5.3 Seed viability

The F_2 seeds were exposed for 45 sec. at 18 kV. For each parent tree, four replicates of 250 seeds were used and the number of viable seeds and empty seeds were recorded. Viable seeds were characterized by presence of normal embryoes, whereas empty seeds show developmental abnormality or lack of either cotyledons or embryonic axis.

3.2.5.4 Seed germination

Numerous pretreatments have been used to break seed dormancy in *Acacia*, but there is considerable variation in response among and within species. Pretreatments include: i) control using only distilled water; ii) immersing in boiling water for 2 min followed by cold water; iii) soaking in concentrated H_2SO_4 for 5 min followed by water rinse; and, iv) manual scarification by trimming the seed at the chalazal end.

For each parent tree, four replications of 50 F_2 seeds for each treatment were made. Tests were made in a germination room with constant temperature of 30°C and photoperiod of eight hours. Germinated seeds were recorded daily for about one month and germination percentages calculated. Based on the viable seed determined by X-radiography, germination percentages were recalculated based on the actual number of viable seeds.

3.2.6 Seedling quality test

Mature F_2 seeds were collected from each parent tree. In order to obtain an adequate number of seedlings, a large number of seeds were germinated using the pretreatment of soaking in boiling water for 2 min. Usually, the F_2 seedlings were ready for selection 7 to 10 days after sowing and only uniform seedlings 2 to 3 cm high with the first two leaves were chosen. Seedlings were transplanted into circular pots (2 inches in diameter x 4 inches in deep), containing a medium of topsoil and coconut husk (2:1). About 0.5 gm of slow-release fertilizer (Osmocotte 14-14-14) and fungicide were applied about one week after transplanting. All seedlings were raised in 50% shade in the nursery for three months. Watering was done every morning. To determine the variation in among-parent tree seedling growth, the experiment was applied in a randomized block design using four replications of eight seedlings from each parent tree. A sample of eight seedlings from the middle portion of each 24-pot tray represents one replication.

3.2.6.1 Seedling growth and survival

Seedling height and root-collar diameter were measured every two weeks for 3 months and the survival rates at 3 months were recorded based on the total number of seedlings per replication of each parent tree. Also, observations were made on the pattern of seedling development since hybrid seedlings at the early stages possess two leaf types; compound pinnate and a phyllode (a petiole which is modified into a leaf-like structure).

3.2.6.2 Three-month-old seedling quality test

In order to evaluate the quality of 3-month-old seedlings, leaf number and area, fresh and dry weight, water content, shoot to root ratio and seedling sturdiness were determined. Four replications of eight seedlings from each parent tree were established using a randomized block design. In cases where the number of seedlings of each parent tree did not meet the required number, the method of unequal sampling was applied (Zar, 1984). Based on the two types of seedling leaves during early development, the total number and area of each leaf type for each seedling were examined separately. Each seedling was washed to remove all growth media and divided into roots, stems and leaves. Fresh weight of each portion was determined separately. Dry weight was determined after drying to a constant weight at 80°C and water content was calculated based on fresh weight. Root to shoot ratios were calculated. Also, seedling sturdiness was calculated as the ratio of seedling height to root-collar diameter.

3.2.7 Statistical analysis

All data were subjected to analysis of variance (ANOVA). For flower number and variation in different flower types, two-way analyses were used. Three-way analyses were used to assess the variation of fruit and seed production. Variation in seed and seedling qualities, except for seed germination, were analysed using a one-way analysis of variance. Two-way analyses of variance were used to assess the effects of pretreatments and parent

trees on seed germination. The Duncan' s new multiple range test at $P < 0.01$ was used to compare their means if there were significant differences among these variables.

3.3 Results

3.3.1 General reproductive phenology

The reproductive phenology of the *Acacia* hybrid was studied in relation to the climatic change over the period during 1991-92 (Fig. 1 and Table 1). The relatively high temperature in the beginning of summer (March) coincided with leaf flushing that continued until early winter (November) when the mean temperature was lower. Leaf primordia were initiated from both terminal and lateral buds on each shoot. The F_1 hybrids began to flower when they were about 1.5 years old. Flowering occurred from June through November. The first emergence of floral buds was observed early in the wet season, approximately 2.5 months after leaf flushing. Floral primodia were initiated in pairs as axillary primodia just following the appearance of leaf primodia. Floral bud initiation continued for almost six months, ending during mid-November. The cessation of floral-bud initiation was observed for about one month during late August and early September. The complete development of flowers took 50 to 60 days.

The hybrid inflorescence is made up of many floral spikes on which individual flowers are loosely arranged. The hybrid had two peak flowering periods between July and the end of August and the other from October through mid-November. Most of the

F_1 trees flowered synchronously, however, some trees flowered sporadically. Anthesis began about midnight and was completed by 0500 to 0600 h. Based on visible changes in style and stigma, the most receptive period occurred a few hours prior to anther dehiscence and continued until the peak period of insect pollinator activities from 0700 to 1000. Most flowers on a spike started to wither and abscise about 3 days following anthesis. Due to the continuous floral-bud initiation and flower development, spikes continued to form at the distal end of the inflorescence. Floral buds which were initiated in mid-November, when the mean temperature become relatively low, developed no further and appeared to abscise soon after. Pollination occurred sporadically over several months, from July to early November.

Early fruit set was recognized by elongation of the ovary and withering of the calyx, corolla, and style. The earliest fruit set was observed in mid-July and continued until November. Only a small portion of flowers set fruits. Fruits rapidly developed in length during early stages but developing seeds remained small for 6 to 7 wks except for the remarkable development of the funiculus and outer integuments. Fruit expansion began about 7 to 8 wks after pollination. Approximately 2 wks prior to maturity, the hybrid fruit began to desiccate and turn dark brown. The seed coat also turned dark brown and became very hard. The mature fruit dehisced and exposed mature seeds in which the funiculus remained attached to the inner pericarp. It normally took 4 to 5 months for flowers to develop into mature fruits. The two peak periods of fruit and seed maturation were during December, and February through early March corresponding with

two peak flowering periods. In winter, wind was most likely responsible for dispersal of mature fruit and seed. However, small birds also fed upon hybrid seeds.

In winter, the relatively low temperature and low humidity resulted in cessation of both vegetative and reproductive growth. Leaf senescence and abscission began in late November through early December and continued until the beginning of summer in March.

3.3.2 Variation in flower production

There were significant differences in the mean number of flowers, perfect flowers, staminate flowers, and aborted flowers per spike and spike length among trees (Tables 2, 3 and Fig. 2A). The average number of flowers per spike ranged from 116 (tree 6) to 168 (tree 7). Although spike length differed significantly among trees, there was no correlation between flower number and spike length. The mean percentage of different types of flowers per spike varied significantly among trees, ranging from 60 to 99% for perfect flowers, 0 to 37% for staminate flowers, and 1 to 5 % for aborted flowers (Fig. 2A). The number of ovules, anthers and polyads per flower varied greatly among trees (Table 2, $P < 0.01$). The frequency distributions of ovary and anther numbers are shown in Fig. 3 A, B. Even though the mean number of ovules per flower among trees was similar (about 15), individual ovaries contained 11 to 19 ovules with ovaries containing 16 ovules most common. The mean number of anthers per flower ranged from 99 (tree 6) to 123 (tree 2) and most flowers contained 110 to 119 anthers. Among-tree variation in the

number of polyads per flower appeared similar to that of anther number per flower as polyad number per anther was consistent (8) in all trees.

There was no significant difference in percentage of perfect, staminate and aborted flowers per spike between crown levels and quadrants (Table 3 and Fig. 2B, C). Only flower number per spike varies significantly between crown levels (Table 3) in which spikes collected from upper crowns had more flowers than those from lower crowns.

The distribution of different flower types by spike region was significantly different in basal regions for perfect and staminate flowers but not for aborted flowers (Table 4 and Fig. 2D). Basal portion of spikes contained significantly lower percentages of perfect flowers and higher percentages of staminate flowers.

3.3.3 Variation in fruit and seed production

Fruit and seed production varied significantly among trees, crown levels and quadrants (Table 5 and Fig. 5). For all trees, the mean number of mature pods per spike ranged from 1.73 (tree 6) to 7.73 (tree 11) with an average of 3.35. The mean number of intact seeds per pod ranged from 1.93 (tree 5) to 5.23 (tree 6) with an average of 3.65 (Table 6). Pods usually contained a greater mean number of intact seeds than aborted seeds, except tree 4, 5, 11 (Fig. 5A). The frequency distribution of fruit and seed number showed that, for all trees, spikes possessing two pods presents the highest number (25%) and pods containing a total of six seeds were most common (Figs. 4A, B, C, D).

Pods collected from upper crowns contained significantly more intact seeds than pods from lower crowns but pods from south quadrants produced significantly more intact and fewer aborted seeds. (Fig. 5B, C).

3.3.4. Reproductive success (RS) and pollen to ovule (P/O) ratio

The RS was low for all trees, ranging from 0.0030 (tree 5) to 0.0094 (tree 11), with an average of 0.0054 (Table 6). When compared to S/O ratios, Fr/Fl ratios appeared much lower and was more important in the reproductive success than did S/O ratios. P/O ratios were close to 1.00 for all trees, ranging from 1.01 (tree 1) to 1.11 (tree 8).

3.3.5 Seed quality

3.3.5.1 Seed weight, water content and viability

There were significant differences among trees in 1000- seed weight, seed WC and viable seed percentage (Table 7). The mean 1000- seed weight was 14.95 gm and ranged from 13.59 (tree 11) to 17.08 (tree 3). Seed WC ranged from 5.44 % (tree 5) to 7.18 % (tree 3) and averaged 6.41%. Based on X-radiography, mean viable seed percentages were high for all trees, ranging from 85.6 (tree 8) to 98.8 (tree 10).

3.3.5.2 Seed germination

Trees showed great variation in seed germination percentage and germination rate (R50) using different seed pretreatments (Table 8). Without pretreatment seed germination percentage was low (20 %). All three pretreatments significantly improved both germination and germination rates (Fig. 6). The application of concentrated H_2SO_4 resulted in moderate improvement of seed germination (58%), whereas boiling water and trimming resulted in germination percentages over 90%. With no pretreatments, seeds required about 15 days to reach R50. With the trimming treatment, R50 was reached most quickly (about 5 days).

3.3.6 Seedling performance

3.3.6.1 Seedling growth and survival

The performance of *Acacia* F_2 seedling growth was observed over three months. Following germination, a once-pinnate leaf emerged between the cotyledons, followed by a bipinnate leaf. The transition from pinnate leaves to phyllodes was recognized when the petiole of the pinnate leaf gradually expanded and elongated to form a phyllode. The first phyllode was initiated following formation of three to four bipinnate leaves, and still had the small pinnately compound leaves at the tip. Following this transition, newly initiated

leaves developed directly into phyllodes, usually at the seventh or eighth position from the main shoot tip.

The survival rate of hybrid seedlings in the nursery was over 90% during the first two months, then was slightly reduced during month 3. No diseases or insects damaged the seedlings. Growth in height was fairly consistent throughout the 3 months but increase in root-collar diameter was most rapid during the first month. There was an increase in variance of both height and diameter as the seedlings became larger (Fig. 7).

3.3.6.2 Three-month-old seedling quality

F_2 seedling height and diameter growth varied significantly among parent trees (Table 9). Three months after germination, seedling height ranged from 20.16 to 26.73 cm with an average of 22.82 cm and the diameter ranged from 0.13 to 0.20 cm with an average of 0.16 cm (Table 9).

Leaf number and total leaf area were based on two leaf types, pinnate leaves and phyllodes, since both were present during early seedling development. At 3 months, the mean number of phyllodes per seedling was not significantly different among F_2 seedlings from different parent trees, ranging from 8 to 10 with an average of 8.7, whereas the number of pinnate leaves differed among F_2 seedlings from different parent trees, ranging from 0.58 to 8.06 with an average of 3.25 (Fig. 8A). Leaf area for both types of leaves was significantly different among F_2 seedlings from different parent trees (Table 9 and Fig.

8B). The mean leaf area of phyllodes averaged 52.83 cm^2 (range 47.51 - 67.51cm^2) and that for pinnate leaves averaged 5.02 cm^2 (range 0.90 - 12.49 cm^2).

There were significant differences in fresh and dry weight among F_2 seedlings from different parent trees. The total fresh weight averaged 2.31 gm (range 1.73 - 3.09 gm) and dry weight averaged 0.81 gm (range 0.65 - 1.03 gm) (Table 9). Fresh and dry weights of stems, roots and leaves also varied significantly (Fig. 9).

The F_2 seedling water content averaged 64.14% (range 58.07 to 68.23%) (Table 9). Leaves and roots had higher water content than stems (Fig. 9c). Shoot to root ratio and sturdiness significantly varied among F_2 seedlings from different parent trees (Table 9). Shoot to root ratio averaged 0.36 (range 0.27 to 0.47) and height to diameter (H/D) ratio averaged 148.30 (range 119.69 to 181.49).

Table 1. Reproductive phenology of the *Acacia* hybrid (*A. mangium* x *A. auriculiformis*)

Location: Muak-lek, Saraburi, Thailand. Duration : 1991-92

Stage of development	M	J	J	A	S	O	N	D	J	F	M	A
Buds dormant								—	—	—	—	
Shoot growth	—	—	—	—	—	—	—				—	—
Floral bud initiation		—	—	—	—	—	—					
Flower development		—	—	—	—	—	—					
Fruit and seed initiation			—	—	—	—	—					
Fruit and seed development			—	—	—	—	—	—	—	—	—	
Fruit and seed maturation								—	—	—	—	
Leaf abscision								—	—	—	—	

Table 2. Among-tree variation in the mean number of flowers per spike, spike length and floral organs of the *Acacia* hybrid (mean ± s.e.); n=8; trees are listed in order of increasing flower number per spike.

tree no.	no. of flowers per spike	spike length (cm)	no. of ovules per ovary	no. of anthers per flower	no. of polyads per flower
6	116.00 ± 5.74 c [1]	7.61 ± 0.28 d	15.37 ± 0.12 ab	98.60 ± 1.47 f	788.8 ± 11.8 f
5	138.13 ± 4.28 bc	9.85 ± 0.25 bc	15.37 ± 0.14 ab	117.70 ± 1.37 abc	941.6 ± 10.9 abc
11	141.50 ± 6.04 b	7.47 ± 0.28 d	15.45 ± 0.11 ab	108.00 ± 1.47 de	864.0 ± 11.7 de
9	146.37 ± 5.34 ab	7.56 ± 0.24 d	14.58 ± 0.21 cd	112.30 ± 1.68 cd	898.4 ± 13.4 cd
4	146.87 ± 6.61 ab	9.19 ± 0.23 c	15.92 ± 0.14 a	119.93 ± 1.63 ab	959.5 ± 13.1 ab
3	153.62 ± 7.05 ab	10.10 ± 0.23 abc	15.23 ± 0.16 b	105.06 ± 1.27 e	840.5 ± 10.2 e
2	153.87 ± 9.69 ab	10.78 ± 0.13 ab	15.52 ± 0.16 ab	123.03 ± 1.65 a	984.3 ± 13.2 a
10	156.62 ± 7.73 ab	9.56 ± 0.19 c	15.25 ± 0.16 b	113.43 ± 1.64 cd	907.5 ± 13.1 cd
8	158.25 ± 4.96 ab	9.15 ± 0.39 c	14.33 ± 0.17 d	111.90 ± 1.30 cd	895.2 ± 10.4 cd
1	158.75 ± 7.99 a	10.88 ± 0.18 a	15.77 ± 0.13 ab	115.33 ± 1.47 bc	922.7 ± 11.8 bc
7	168.50 ± 5.52 a	9.96 ± 0.25 abc	15.14 ± 0.16 bc	119.46 ± 1.56 ab	955.7 ± 12.5 ab
avg.	148.95	9.28	15.268	113.16	905.28
s.e.	2.36	0.15	0.05	0.59	4 .69
N	88	88	528	330	330

[1] The means in a column followed by the same letters do not differ significantly at $P < 0.01$, using Duncan's new multiple range test.

Table 3. ANOVA results for the comparison of the number of different types of flowers among trees, crown levels and quadrants of the *Acacia* hybrid.

Source of Variations	df	no. of flowers per spike	spike length	no. of perfect flowers per spike	no. of staminate flowers per spike	no. of aborted flowers per spike
		prob.	prob.	prob.	prob.	prob.
One-way ANOVA						
tree	10	0.0001	0.0001	0.0001	0.0001	0.0086
error	77					
Two-way ANOVA						
crown level (CL)	1	0.003	0.740	0.817	0.533	0.170
crown quadrant (CQ)	3	0.954	0.782	0.905	0.856	0.872
CL x CQ	3	0.821	0.987	0.985	0.951	0.955
error	80					

Tree variability is independently analysed due to limitation of sample number.
All percentage data are arcsine-transformed for Analysis of Variance.

Table 4. ANOVA results for the comparison of the mean percentages of perfect, staminate and aborted flowers among trees, crown levels and quadrants of the *Acacia* hybrid based on spike regions.

Source of Variations	df	% of perfect flowers	% of staminate flowers	% of aborted flowers
		prob.	prob.	prob.
Two-way ANOVA				
spike region (R)	2	0.0001	0.0001	0.0580
tree (T)	10	0.0001	0.0001	0.0001
R x T	20	0.6110	0.0590	0.2970
error	231			
Three-way ANOVA				
spike region (R)	2	0.0001	0.0001	0.0590
crown level (CL)	1	0.7760	0.4050	0.1670
crown quadrant (CQ)	3	0.6020	0.4990	0.8710
R x CL	2	0.6000	0.6720	0.9380
R x CQ	6	0.9820	0.9800	0.8730
CL x CQ	3	0.9550	0.9310	0.9520
R x CL x CQ	6	0.9900	0.9820	0.9480
error	240			

The effects of spike region and tree are statistically analysed by two-way ANOVA whereas the effects of spike region, crown level and crown quadrant are statistically analysed by three-way ANOVA.
All percentage data are arcsine-transformed for Analysis of Variance.

Table 5. ANOVA results for comparison of the number of total seeds, intact and aborted seeds per pod among trees, crown levels and quadrants of the *Acacia* hybrid.

Source of Variations	df	no. of seeds per pod	no. of intact seeds per pod	no. of aborted seeds per pod
		prob.	prob.	prob.
tree (T)	11	0.0001	0.0001	0.0001
crown level (CL)	1	0.0050	0.0001	0.0001
crown quadrant (CQ)	3	0.0020	0.0001	0.0001
T x CL	11	0.0001	0.0001	0.0001
T x CQ	33	0.0001	0.0001	0.0001
CL x CQ	3	0.7160	0.3480	0.3480
T x CL x CQ	33	0.0010	0.0001	0.0001
error	1520			

All percentage data are arcsine-transformed for ANOVA.

Table 6. Among- tree variation in mean number of flowers per spike, pods per spike, ovules per flower, seeds per pod, pollen to ovule (P/O) ratio and reproductive success (RS) of the *Acacia* hybrid .

tree no.	no. of flowers per spike (Fl)	no. of pods per spike (Fr)	Fr/Fl ratio	no. of ovules per flower (O)	no. of seeds per pod (S)	S/O ratio	P/O	RS
1	158.75	2.78	0.017	15.77	4.19	0.266	1.01	0.0045
2	153.88	4.28	0.028	15.52	3.94	0.254	1.03	0.0071
3	153.63	2.20	0.014	15.23	4.91	0.322	1.05	0.0045
4	146.88	3.68	0.025	15.92	3.59	0.226	1.00	0.0056
5	138.13	3.35	0.024	15.38	1.93	0.125	1.04	0.0030
6	116.00	1.73	0.015	15.38	5.23	0.340	1.04	0.0051
7	168.50	2.28	0.014	15.15	3.95	0.261	1.06	0.0037
8	158.25	2.28	0.014	14.33	4.67	0.326	1.11	0.0046
9	146.38	3.20	0.022	14.54	4.16	0.286	1.10	0.0063
10	156.63	3.35	0.021	15.25	4.32	0.283	1.04	0.0059
11	141.50	7.73	0.055	15.46	2.64	0.171	1.03	0.0094
avg.	148.96	3.35	0.023	15.27	3.65	0.259	1.05	0.0054
s.e.	2.36	0.11	0.003	0.05	0.06	0.019	0.03	0.0005
N	88	478	11	528	1616	11	11	11

Reproductive success (RS) is calculated as the fruit-flower ratio multiplied by seed-ovule ratio based on mean number of intact seeds. The pollen grains per polyad (P) is 16 for all trees.

Table 7. Among-tree variation in 1000-seed weight, water content (WC) and X- radiography of the *Acacia* hybrid (means ± s.e.); n=10 for 1000-seed weight and n=4 for other variables.

tree no.	1000-seed weight (gm)	WC (%) [2]	X-radiograph	
			viable seeds (%)	aborted seeds (%)
1	14.59 ± 0.17 defg [1]	5.69 ± 0.17 bcd	90.90 ± 1.18 ef	9.10 ± 1.18 ef
2	14.06 ± 0.11 gh	6.84 ± 0.21 a	89.10 ± 1.25 fg	10.90 ± 1.25 fg
3	17.08 ± 0.16 a	7.18 ± 0.39 a	94.90 ± 0.80 bcd	5.10 ± 0.80 bcd
4	14.50 ± 0.13 efg	5.47 ± 0.18 cd	92.00 ± 1.27 def	8.00 ± 1.27 def
5	15.83 ± 0.18 bc	5.44 ± 0.29 d	94.90 ± 0.91 bcd	5.10 ± 0.91 bcd
6	14.78 ± 0.23 def	6.16 ± 0.09 abc	95.00 ± 0.52 bcd	5.00 ± 0.52 bcd
7	16.10 ± 0.15 b	6.54 ± 0.06 ab	88.90 ± 0.91 fg	11.10 ± 0.91 fg
8	14.99 ± 0.17 de	7.03 ± 0.22 a	85.60 ± 1.30 g	14.40 ± 1.30 g
9	15.22 ± 0.18 cd	6.72 ± 0.09 a	94.00 ± 0.51 cde	6.00 ± 0.51 cde
10	14.14 ± 0.15 fgh	6.84 ± 0.06 a	98.80 ± 0.16 a	1.20 ± 0.16 a
11	13.59 ± 0.15 h	6.05 ± 0.17 abc	95.90 ± 0.94 bc	4.10 ± 0.94 bc
12	14.43 ± 0.18 efg	6.91 ± 0.14 a	97.20 ± 0.43 ab	2.80 ± 0.43 ab
avg.	14.95	6.41	93.10	6.90
s.e.	0.09	0.09	0.59	0.59
N	120	48	48	48

All percentage data are arcsine-tranformed for ANOVA.

[1] The means in a column followed by the same letter do not differ significantly at $P < 0.01$, using by Duncan's new multiple range test.

[2] Seed water content is calculated based on fresh weight.

Table 8. The comparison of mean germination (%) and germination rate (R50) among different treatments (means ± s.e.) ; n=48

treatments	germination (%)	adjusted germination (%) [2]	R50 (days)
control	18.92 ± 2.00 c [1]	20.18 ± 2.08 c	14.82 ± 0.84 a
conc. H_2SO_4	54.21 ± 2.70 b	58.07 ± 2.79 b	8.61 ± 0.25 b
cutting	85.45 ± 2.52 a	90.39 ± 2.53 a	4.86 ± 0.48 c
boiling water	87.87 ± 1.44 a	93.62 ± 0.94 a	9.55 ± 0.17 b

All percentage data are arcsine-tranformed for ANOVA.

[1] The means in a column followed by the same letter do not differ significantly at $p < 0.01$, calculated by Duncan's new multiple range test.

[2] Adjusted germination percentages are calculated based on viable seed percentage, evaluated by X-radiography.

Table 9. Variation in 3-month old seedlings from different parental trees based on growth, water content, root-shoot (R:S) ratio and sturdiness (H/D) of the *Acacia* hybrid.

Seedling from tree no.	Height (cm)	Diameter (cm)	Total leaf area (cm^2)	Total fresh weight (gm)	Total dry weight (gm)	WC (%)	R:S ratio	H/D [2]
1	23.7 ± 0.8 ab [1]	0.20 ± 0.047 a	75.6 ± 3.7 a	3.1 ± 0.17 a	1.03 ± 0.05 a	66.1 ± 0.6 ab	0.33 ±0.015 bcd	119.7 ± 4.1 e
2	22.5 ± 0.9 ab	0.18 ± 0.005 ab	62.1 ± 4.2 ab	2.4 ± 0.14 bc	0.86 ± 0.05 abc	64.5 ± 0.5 abc	0.27 ± 0.009de	124.0 ± 4.2 de
3	24.2 ± 1.1 ab	0.16 ± 0.004 bcd	55.4 ± 4.1 b	1.8 ± 0.13 cd	0.75 ± 0.05 c	58.1 ± 0.1 d	0.28 ± 0.023cde	150.0 ± 7.4 bcde
4	24.0 ± 1.1 ab	0.16 ± 0.005 bcd	58.6 ± 3.3 b	1.9 ± 0.12 cd	0.73 ± 0.04 c	62.4 ± 0.7 bc	0.25 ± 0.015 e	152.3 ± 7.5 bcd
5	22.7 ± 1.1 ab	0.16 ± 0.005 cd	54.9 ± 2.6 b	2.4 ± 0.14 bc	0.76 ± 0.04 c	68.2 ± 0.6 a	0.35 ± 0.011bc	144.2 ± 5.8 cde
6	20.2 ± 0.9 b	0.13 ± 0.006 e	52.0 ± 3.6 b	2.2 ± 0.17 cd	0.74 ± 0.06 c	65.9 ± 0.8 ab	0.44 ± 0.021a	170.2 ± 12.8 abc
7	21.6 ± 0.8 b	0.13 ± 0.005 e	54.8 ± 3.4 b	1.9 ± 0.12 cd	0.65 ± 0.04 c	65.0 ± 2.1 ab	0.32 ± 0.015 cde	175.8 ± 7.8 ab
8	24.0 ± 1.0 ab	0.14 ± 0.006 e	52.4 ± 3.6 b	1.7 ± 0.14 d	0.67 ± 0.05 c	61.1 ± 0.9 cd	0.40 ± 0.017ab	181.5 ± 8.3 a
9	21.1 ± 1.3 b	0.14 ± 0.006 de	61.9 ± 3.2 ab	2.3 ± 0.16 bcd	0.83 ± 0.06 abc	63.7 ± 0.7 bc	0.35 ± 0.016 bc	149.6 ± 5.9 bcde
10	22.0 ± 1.0 b	0.17 ± 0.007 bc	48.4 ± 3.5 b	2.3 ± 0.17 bcd	0.81 ± 0.06 bc	64.4 ± 0.8 abc	0.46 ± 0.024 a	136.8 ± 8.9 de
11	20.7 ± 0.9 b	0.16 ± 0.005 cd	57.3 ± 5.1 b	2.4 ± 0.17 bcd	0.81 ± 0.06 bc	65.7 ± 0.7 ab	0.40 ± 0.017ab	132.7 ± 6.2 de
12	26.7 ± 1.2 a	0.18 ± 0.006 abc	57.3 ± 4.4 b	2.9 ± 0.21 ab	0.99 ± 0.06 ab	64.2 ± 1.0 bc	0.47 ± 0.025 a	150.5 ± 6.2 bcd
avg.	22.8	0.16	57.9	2.3	0.81	64.14	0.36	148.30
s.e.	0.3	0.002	1.2	0.05	0.02	0.30	0.006	2.29
N	351	351	351	351	351	351	351	351

All percentage data are arcsine- tranformed for ANOVA.

[1] The means in a column followed by the same letter do not differ significantly at $P < 0.01$.

[2] Seedling sturdiness is estimated by the ratio of seedling height to root-collar diameter.

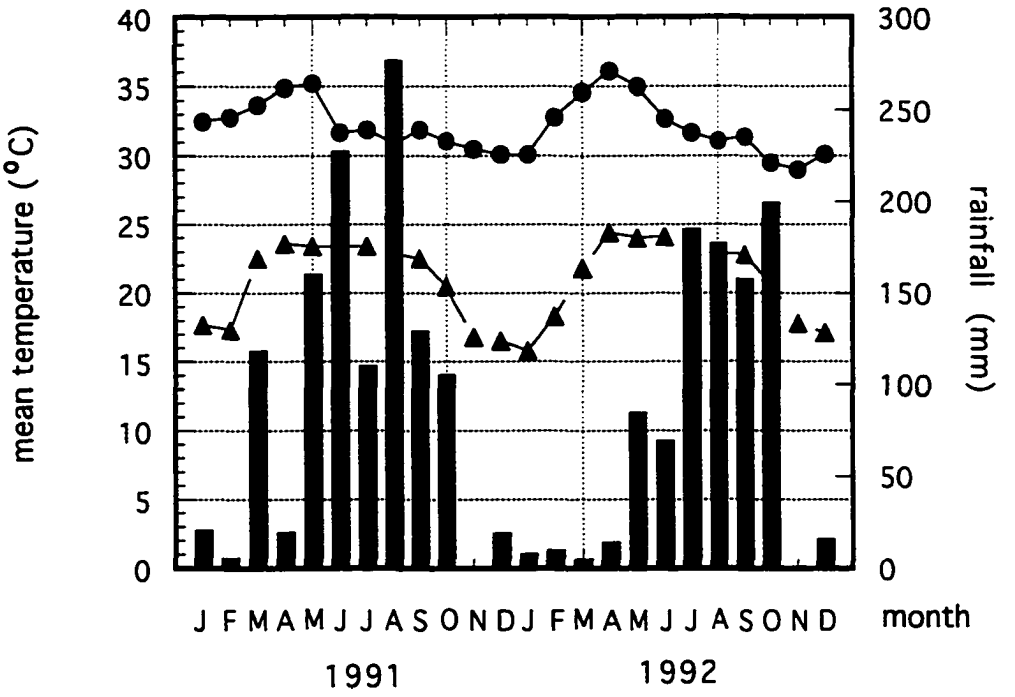


Figure 1. Mean monthly maximum and minimum temperatures and rainfall during 1991-92 at Thai-Danish Diary Farm, Muak-lek, Saraburi (—●— max.temp. —▲— min. temp. ■ rainfall).

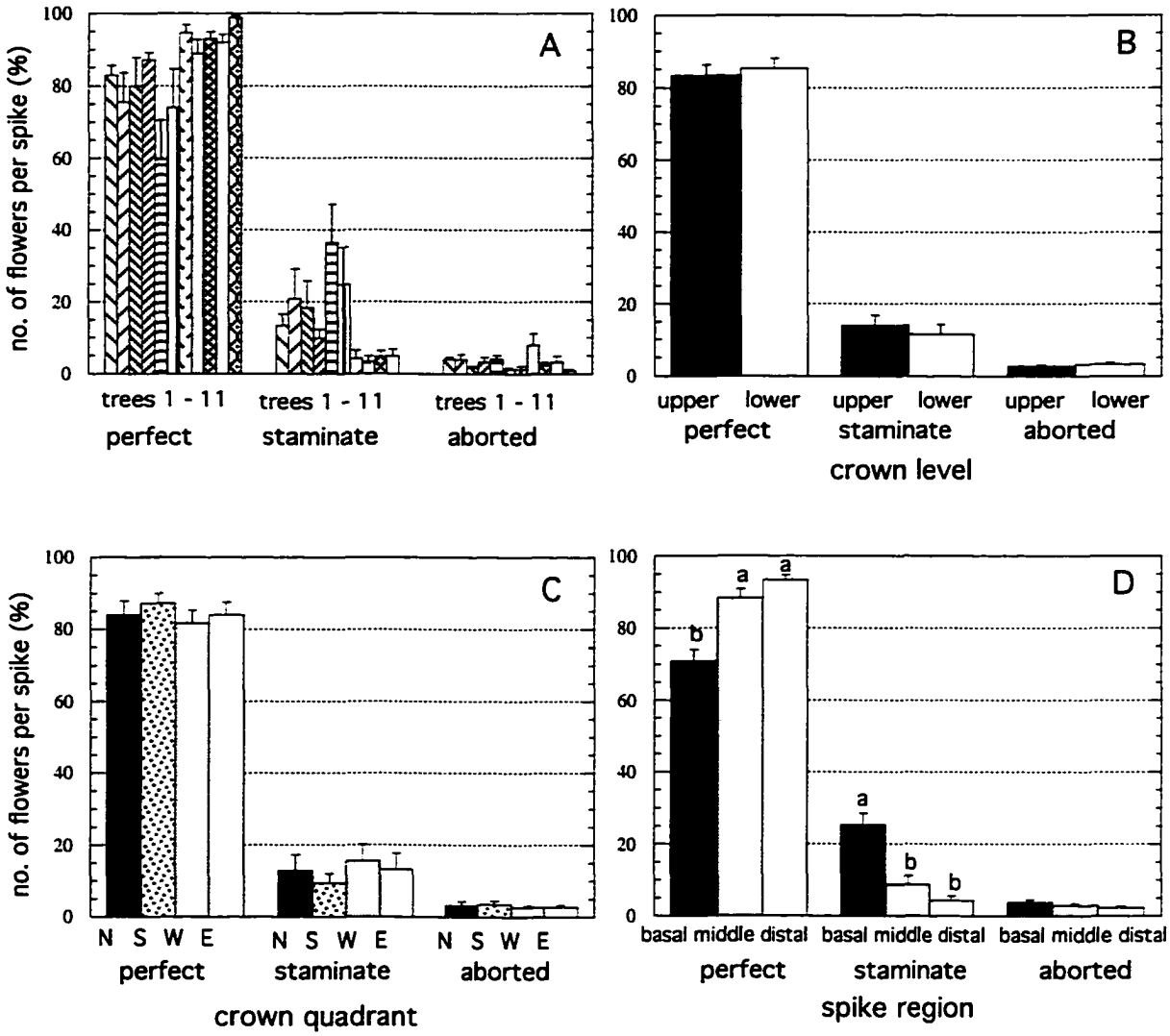


Figure 2. Variation in mean percentages of perfect, staminate and aborted flowers per spike in 11 *Acacia* hybrid trees, crown regions and spike regions. Means of each variable in the perfect and in the staminate categories shown by the same letter do not differ significantly at $p<0.1$. Vertical bars represent standard errors (n=8 for A, n=44 for B, n=22 for C and n=88 for D).

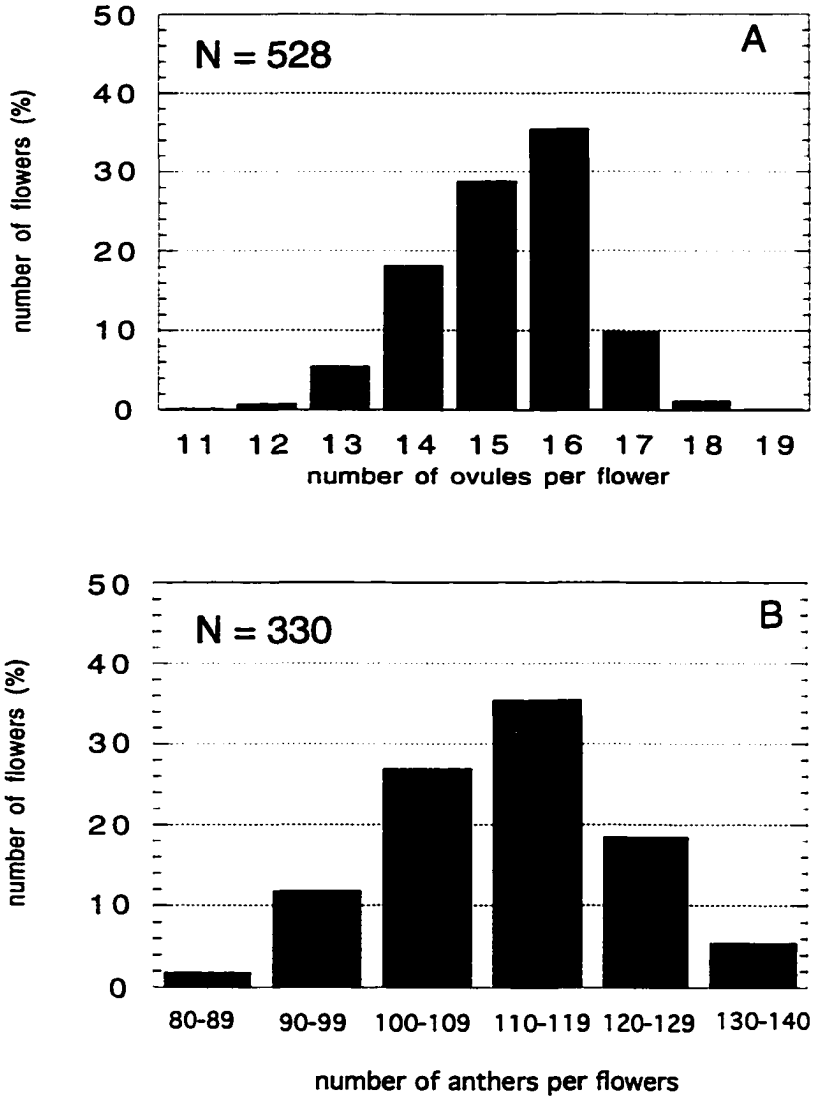


Figure 3. Frequency distribution of the number of ovules and anthers per flower of 11 *Acacia* hybrid trees.

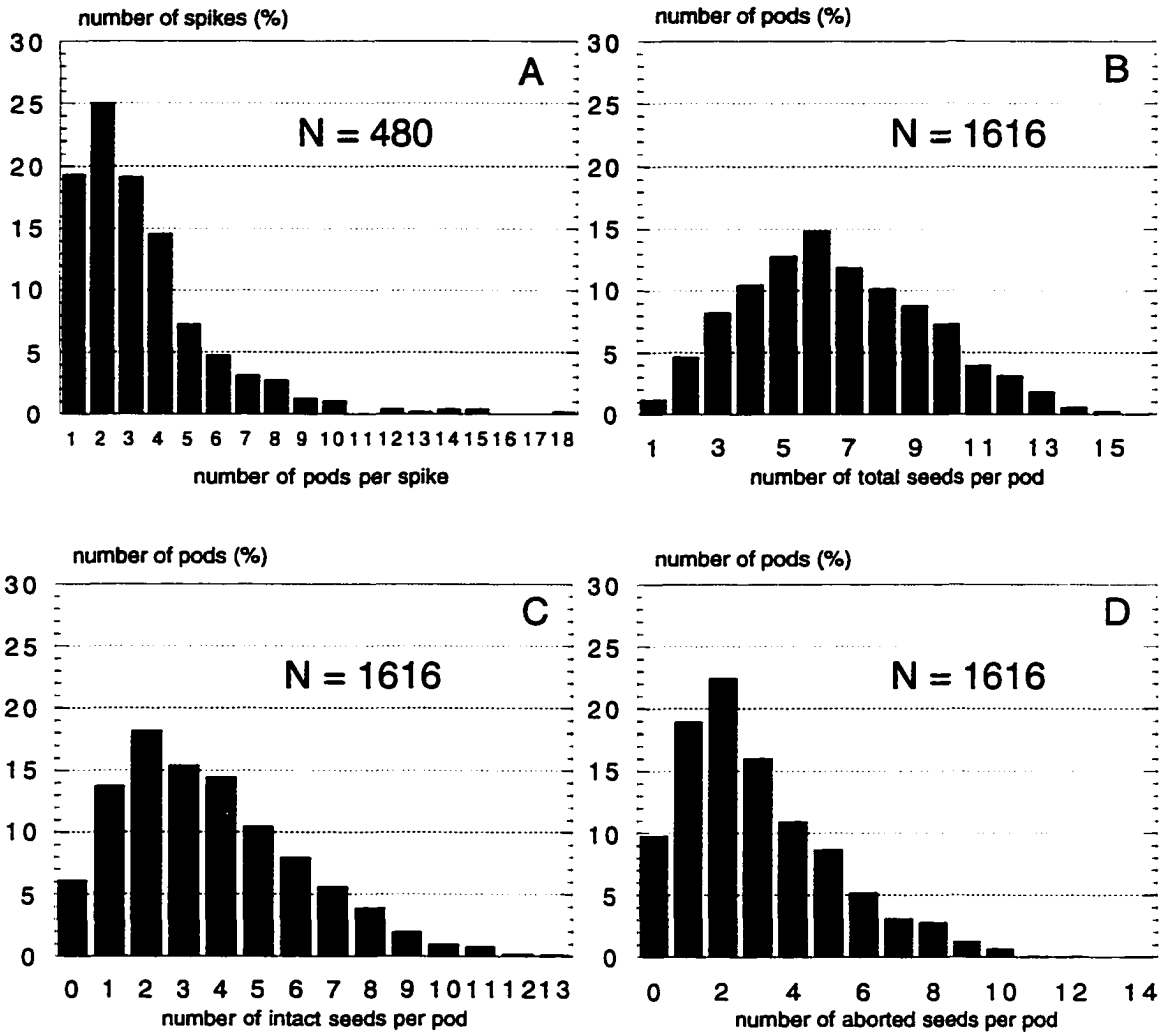


Figure 4. Frequency distribution of the number of pods per spike and the number of intact and aborted seeds per pod of 12 *Acacia* hybrid trees.

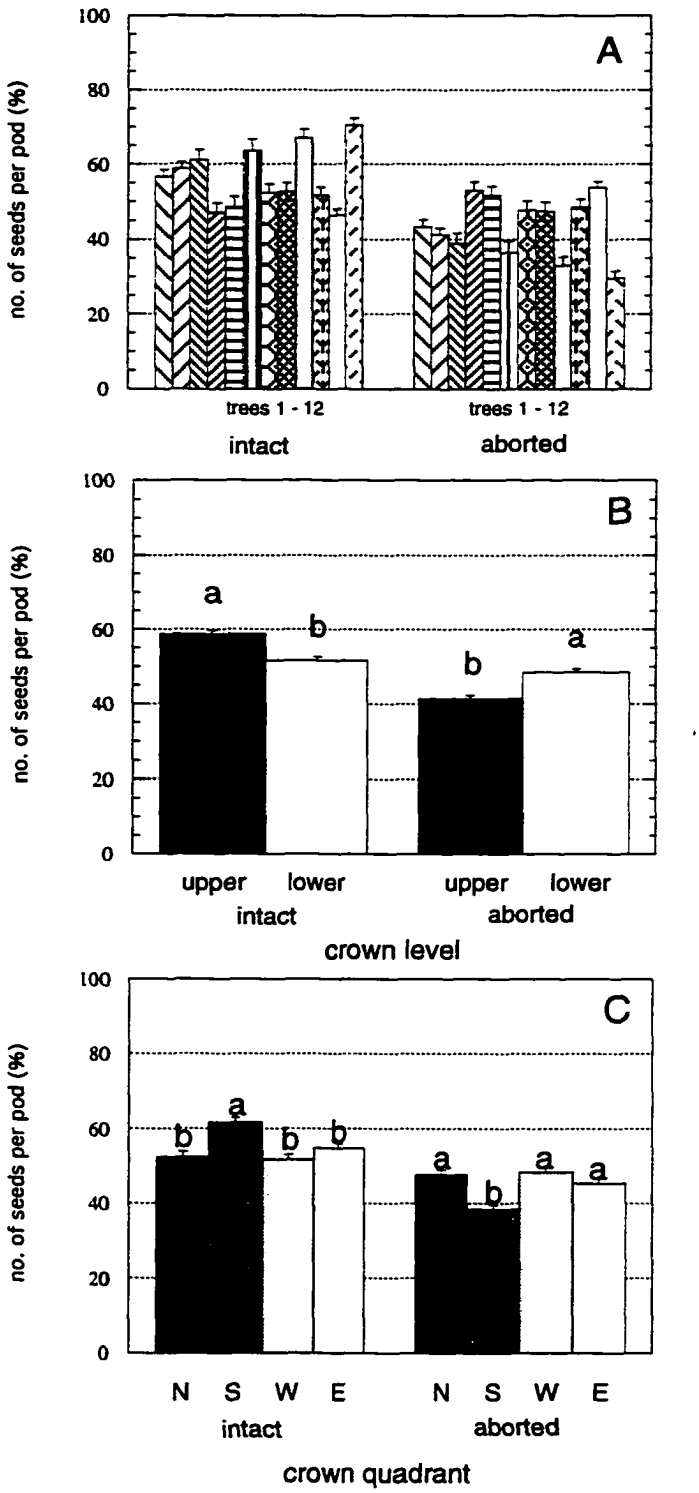


Figure 5. Mean percentages of intact and aborted seeds per pod of 12 *Acacia* hybrid trees and crown regions. Means of each variable in the intact and in the aborted categories shown by the same letter do not differ significantly at $p < 0.01$. Vertical bars represent standard errors (n=40 for A, n=240 for B and n=120 for C).

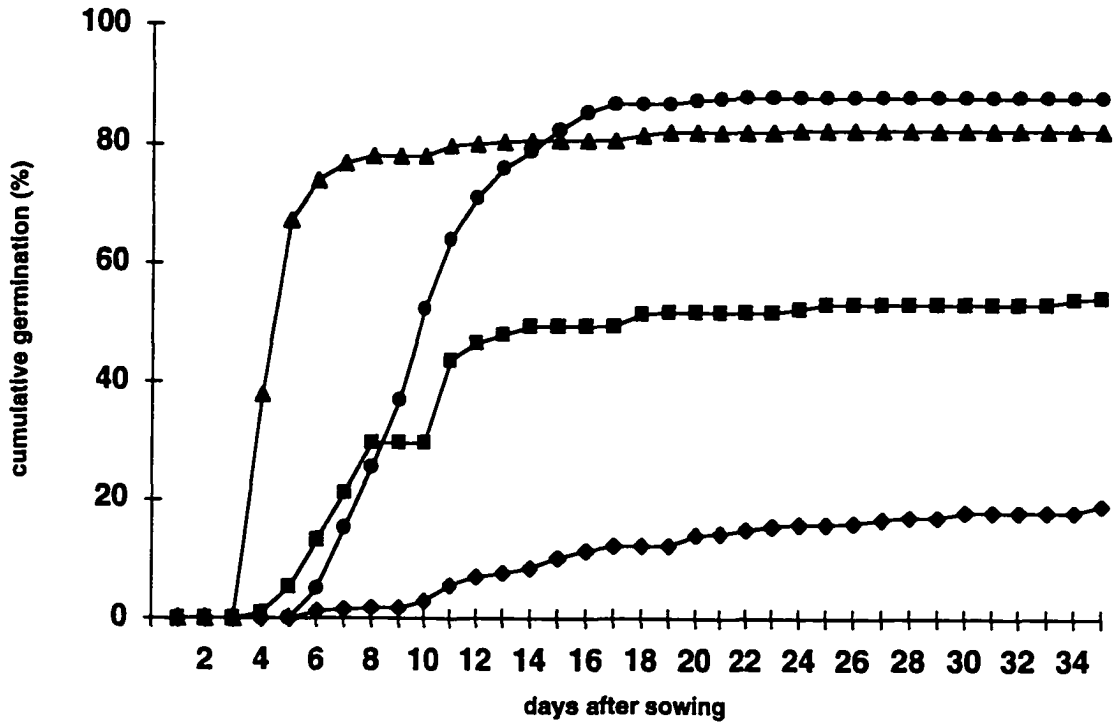


Figure 6. Cumulative germination of the *Acacia* hybrid seeds using different pretreatments (◆ control, ■ conc. H_2SO_4, ▲ cutting, ● boiling water).

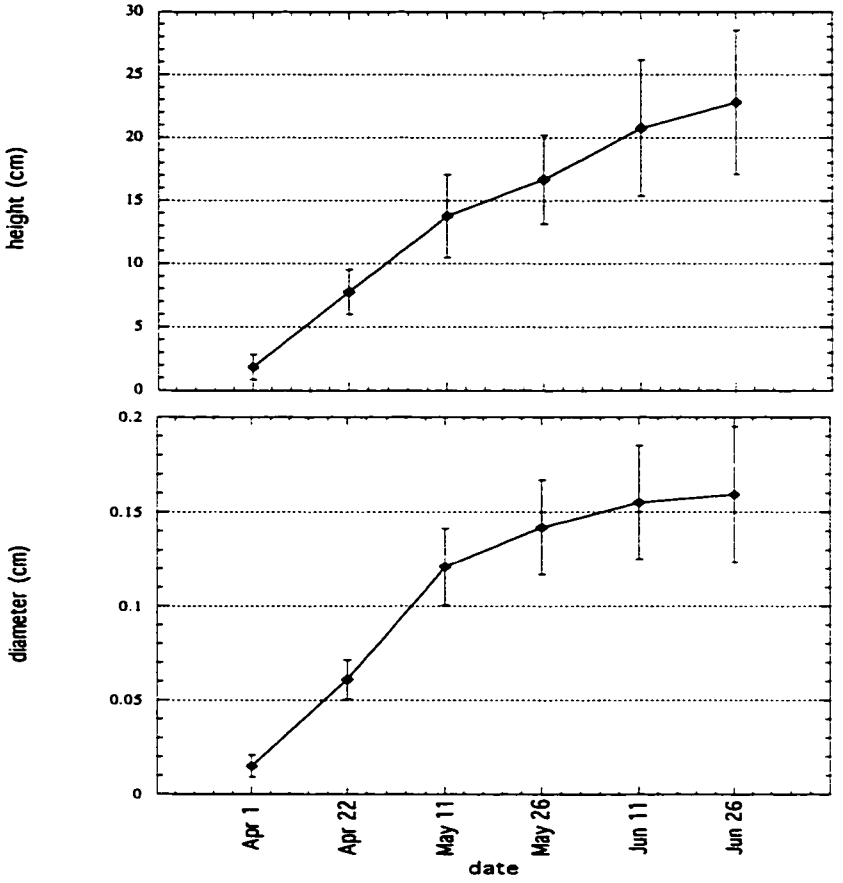


Figure 7. Mean height and root-collar diameter of the Acacia hybrid seedlings. Seedlings from 12 selected trees were raised in 50% shade under ambient condition for 3 months. Mean values are calculated based on a pooled sample number of four replications of all selected trees. Vertical bars represent standard deviations (n = 278 - 354).

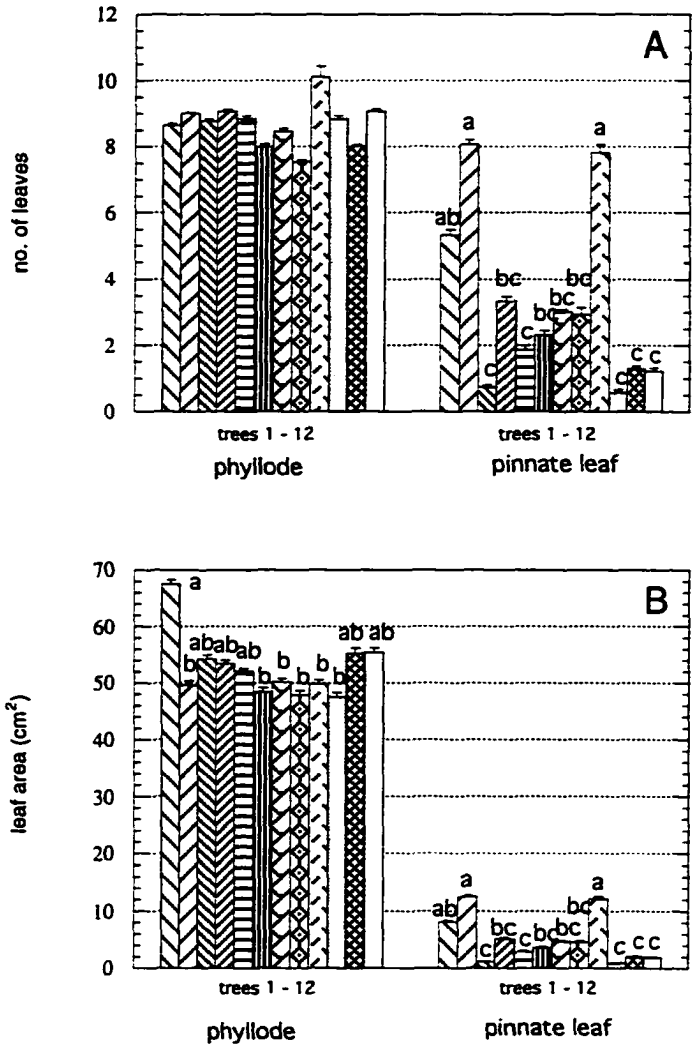


Figure 8. Among-tree variation in mean leaf number and area of the 3-month old *Acacia* F_2 seedlings from 12 hybrid parent trees. Means of each variable shown by the same letter do not differ significantly at $p<0.01$. Vertical bars represent standard errors (n=24-32).

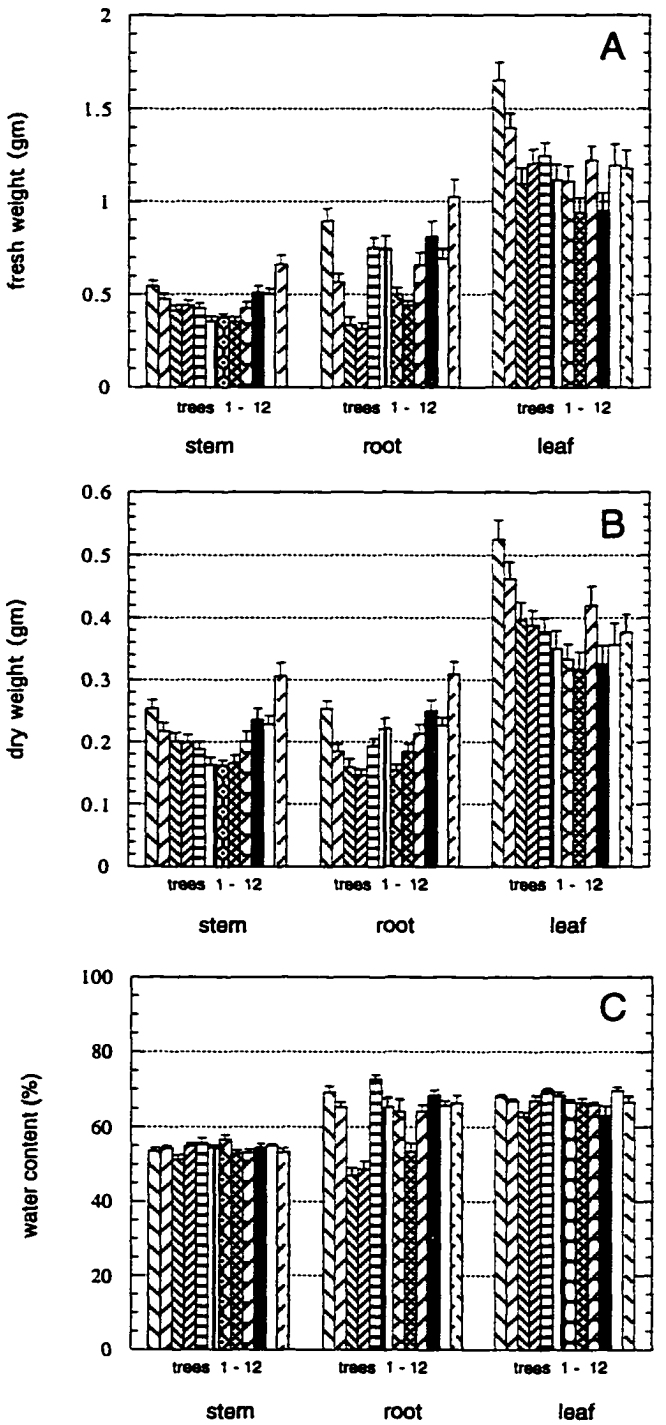


Figure 9. Among-tree variation in mean fresh and dry weight and percent water content of stems, leaves and roots of the 3- month old *Acacia* F_2 seedlings from 12 hybrid parent trees. Vertical bars represent standard errors (n=24-32).

3.4 Discussion

3.4.1 General reproductive phenology

Acacia species grown in their natural habitats show great variation in reproductive phenology among species and locations (Skelton, 1980; Buttrose *et al.*, 1981; Turnbull, 1983: Turnbull *et al.*, 1986; Sedgley *et al.*, 1992b; Tybirk,1993; Baranelli *et al.*, 1995). When they are introduced to tropical climates, their reproductive phenologies are also different (Ngamkajornwiwat and Luangviriyasaeng, 1991; Ibrahim 1991; Sedgley *et al.*, 1992b; Sornsathapornkul and Tangmitcharoen, 1992). It is suggested that reproductive phenology in many tropical woody plants, including *Acacia*, may respond to abiotic factors, such as rain, temperature and photoperiod (Bawa 1983; Bawa and Ng 1990; van Schaik *et al.*, 1993). This appears to be true in the *Acacia* hybrid for which neither parental species is native to Thailand. At Muak-lek, Thailand, there is a distinct dry season from November through April and a wet season from May through October. In the dry season, the mean temperature changes from cool to hot during February and March. Leaf flushing which begins in March and continues until November coincides with increasing temperature during the dry season; cessation in leafing coincides with the beginning of the cool season in November.

The first initiation of flowers occurred a few weeks following the beginning of the wet season after leaf flushing, when mean temperature and rainfall become much higher in May. Flower initiation continued through the wet season and terminated at the beginning

of the dry season, when temperature and rainfall decrease. These observations are in agreement with those for some other *Acacia* species in which floral-bud initiation and development usually occur following emergence of new leaves during the onset of the wet season (Wickens, 1969; Khan, 1970). A dry period followed by rain may trigger flowering in the *Acacia* hybrid, as has been suggested in some other tropical trees (Opler *et al.*, 1976; Heideman, 1989; Wright and van Schaik, 1994). However, long-term data on reproductive phenology of the hybrid in relation to climatic conditions are required to determine if it changes each year.

All sampled hybrid trees showed somewhat synchronous peak flowering. Compared to its parental species in Thailand, the flowering behavior of the *Acacia* hybrid, with the two peak flowering periods (July and August and October through mid-November) was similar to that of *A. auriculiformis* (June and July and October and November) (Ngamkajornwiwat and Luangviriyasaeng, 1991) rather than that of *A. mangium* which has only one peak flowering period (August through November) (Sornsathapornkul and Tangmitcharoen, 1992). However, in Peninsular Malaysia, *A. mangium* and *A. auriculiformis* have been reported to flower throughout the year with several peak flowering periods when mean monthly maximal and minimal temperatures as well as high total rainfall appear consistent (Ibrahim and Awang, 1992). It is possible that, besides internal control (Borchert, 1983), high temperature and soil moisture during the wet season may control flowering peaks, as proposed in some African and Australian *Acacia* species (Sedgley *et al*, 1992d; Tybrik, 1993). In *A. aneura*, several flowering

periods per year can be obtained using additional water supplied for the 12-month period (Preece, 1971).

There is a close interrelationship between flowering and fruiting. Each of the two peak periods of fruit and seed maturation occur 4 to 5 months following the two peak flowering periods. This pattern is like the parental species (Pukittayacamee and Hellum, 1988; Ibrahim, 1991). Following the flowering period, fruit and seed development occur during the late wet season and fruit and seed maturation and dispersal occur optimally during the windy dry season. Other studies have shown that peak fruiting of wind-dispersed species occurs during the windiest times of the year (Frankie *et al.*, 1974). Both peaks of the hybrid fruit and seed maturation occur at the beginning to mid-dry periods. This may ensure the optimal time of seed dispersal during windy conditions. Also, mature seeds can be induced to dormancy thus avoiding the high risk of seed mortality during unfavorable conditions for germination as suggested by van Schaik *et al.* (1993).

The opening of hybrid *Acacia* flowers took place early in the day when it could attract the most pollinators. Van Schaik *et al.* (1993) noted that the abundance of pollinators may vary seasonally with the number of insects being highest during the dry season. Accordingly, it may be disadvantageous for the hybrid to flower during the wet season since frequent rainfall may diminish pollinator activities.

At the beginning of dry and cool season in November, the decrease of rainfall and temperature may cause cessation of leafing and flowering due to the decrease of metabolitic activity and resources. Wright and Cornejo (1990) also suggested that atmospheric conditions such as relative humidity or temperature probably account for leaf

abscission during dry periods in some tropical species. The hybrid possesses growth characteristics of both deciduous and evergreen trees at the Muak-lek site. Some leaf abscission occurs implied. It is also observed that newly initiated flowers appear to abscise during this time as well. This may be due to the adjustment of maternal trees to maintain their resources for fruit and seed development which mature in mid-dry season.

3.4.2 Variation in flower, fruit and seed productions

The number of flowers per spike in the *Acacia* hybrid varied significantly among trees, ranging from 116 to 169 with an overall mean of 149. This appears intermediate between the parental species planted in Thailand and Malaysia, 195 to 256 in *A. mangium* (Ibrahim, 1991; Sornsathapornkul and Tangmitchareon, 1992) and 98 to 105 in *A. auriculiformis* (Ngamkhajornviwat and Luangviriyasaeng, 1991; Ibrahim, 1991). Also, the mean staminate flower number per spike in the hybrid varied considerably among trees, ranging from 0 to 37%, between that of *A. auriculiformis* (4.0 to 57.4%) and *A. mangium* (0.4 to 5.1%) (Ibrahim, 1991; Sedgley *et al.*, 1992b). It is likely that variation in total flowers and staminate flowers is a genetic difference attributable to variations in interspecific combinations of parental species. Variation in the number of aborted flowers caused by insect damage at pre-pollination is more likely influenced by a random distribution of insects pests.

In the hybrid, the presence of perfect and staminate flowers on the same tree suggests that the hybrid is andromonoecious. This has been reported in many *Acacia*

species (Zapata and Arroyo, 1978; Tybirk, 1989; Baranelli *et al.*, 1995), including *A. mangium* and *A. auriculiformis* (Ibrahim, 1991, Sedgley *et al.*, 1992b). It is suggested that, as the hybrid and most *Acacia* species lack floral nectaries, andromonoecy may promote outcrossing by which the excess staminate flowers increase pollinator attraction as well as pollen donation (male function) (Zapata and Arroyo, 1978; Sutherland and Delph, 1984; Bertin, 1988).

In *Acacia*, ovule and anther number per flower is greatly variable within- and among- species, and among sites (Buttrose *et al.*, 1981; Ibrahim, 1991; Ngamkhajornviwat and Luangviriyasaeng, 1991; Sedgley *et al.*, 1992b; Sornsathapornkul and Tangmitcharoen, 1992). This is also true in the hybrid in which the ovule and anther number per flower varied significantly among trees, ranging from 98.6 to 123.03 for the anthers and 14.58 to 15.26 for the ovules. In addition, the overall mean number of ovules (15.26) and anthers (113.16) are greater than those of *A. mangium* and *A. auriculiformis* grown in either Thailand or Malaysia (Ibrahim, 1991; Ngamkhajornviwat and Luangviriyasaeng, 1991; Sornsathapornkul and Tangmitcharoen, 1992).

Cruden (1977) suggested that P/O ratios can be used to determine the breeding system of angiosperms in which the lower pollen-ovule ratio, the more efficient the pollen transport system. Kenrick and Knox (1982) indicated that the P/O ratio varied between 0.8 to 4 in most Australian species and 0.7 to 3.2 in African species but most were close to one. The P/O ratio of the hybrid (1.05) suggests that, like most *Acacia* species, it behaves as 'facultative xenogamic' according to Cruden (1977). Also, this ratio indicates

moderate efficiency of pollen transfer in the hybrid and is slightly lower than that of its parental species (1.23) (Ibrahim, 1991).

A low RS (0.0054) in the hybrid indicates that only small numbers of flowers or ovules produce fruits or seeds, respectively. Compared to the S/O ratio, the Fr/Fl ratio was much lower for all trees, representing very high fruit abortion in the hybrid. A low Fr/Fl has been reported in many plant species and several explanations have been proposed (Stephenson, 1981; Willson and Burley, 1983; Bawa and Webb, 1984; Sutherland, 1987; Ehrlén, 1993). It has been concluded that the major cause of fruit and seed abortion during the prezygotic period was the lack of successful pollination and fertilization (Wilson and Schemske, 1980; Gross and Werner, 1983; Rathcke, 1983). This may occur in the hybrid in which fruit and seed set may be limited by pollen quality and availability, pollinator behavior, pollen-tube growth or gamete abortion.

Fruit and seed set in the hybrid may be limited during the postzygotic period by resource availability as suggested for many plants (Stephenson, 1981; Martin and Lee, 1993), including several African *Acacia* species (Tybirk, 1993). Since many young hybrid fruits abort during early development, this elimination may be the first step in which maternal trees determine the reasonable amount of developing fruits which are appropriate for their resources. Competition among developing fruits within the inflorescence or tree may result from the fact that early developing or more vigorous fruits obtain more of the limited resources (Stephenson, 1981, 1992; Harriss and Whelan, 1993; O'Donnel and Bawa, 1993; Guitian, 1994) and/or may produce growth regulators that inhibit other fruits from developing (Lee, 1988; Stephenson *et al.*, 1988). Seed abortion within pods may be

the second determination when resources become more limited during later stages of development. As a result, less vigorous or poorer quality seeds, as influenced by pollen source or self-pollination are more likely to be eliminated (Stephenson, 1981, 1992; Wiens, 1984; Vaughton and Carthew, 1993), resulting in a low S/O ratio (Bawa and Buckley, 1989). Therefore, variations in hybrid fruit and seed numbers at maturity may be attributed to degree of fruit and seed abortion which can occur during both pre- and postzygotic periods and vary within and among trees.

3.4.3 Seed quality

The 1000-seed weight averaged 14.96 gm for hybrid trees which is closer to that of *A. auriculiformis* (15.24) than that of *A. mangium* (10.18) (Yue-Luan *et al.*, 1993). However, some trees had a higher mean seed weight, ranging from 15.83 to 17.08 gm. This may be an advantage since several studies have shown that seedlings from heavier-seeded species tend to be more vigorous and survive longer due to the presence of more nutrient reserves than those of light-seeded species (Shepard *et al.*, 1989, Seiwa and Kikuzawa 1991, Jurado and Westoby, 1992).

In leguminous seeds, WC plays an important role in regulating water impermeability and seed dormancy. Reduction in water content increases the impermeability of seeds which then remain hard for a longer time. The critical water content below which seeds become impermeable varies among species but is usually below 20% and sometimes as low as 6% (Lebedeff 1943, cited in van Staden *et al.*, 1989).

Therefore, it is likely that the *Acacia* hybrid seeds have become impermeable since their water content at maturation averaged only 6.41%.

The degree of impermeability is usually attributed to relative humidity (van Staden *et al.*, 1989). Low relative humidity during the dry season causes the high degree of seed drying resulting in low WC which occurs during the period of maturation. This is also the case of the hybrid in which the seed maturation process usually occurs during the dry and cool season and seed WC was relatively low for all trees. It has been suggested that, in mimosoid legumes such as *Acacia*, the pleurogram may function as a hygroscopic control valve regulating the desiccation of seeds by opening and closing in response to changing relative humidity or outside moisture. (Gunn, 1981; van Staden *et al.*, 1989). Among-tree variation in WC of the hybrid seeds, therefore, may be partly attributed to such a mechanism which may vary from seed to seed and tree to tree.

X-radiography has been successfully employed to determine seed viability of several tropical species (Kamra, 1976: Liengsiri *et al.*, 1990, Yue-Luan *et al.*, 1993) including *A. auriculiformis* (Kobmoo *et al.*, 1990). In the *Acacia* hybrid, seed viability is high but varies significantly among trees. Most aborted seeds contain either abnormal cotyledons or embryo axes. Among-tree variation in seed abortion percentages may depend on several factors that cause seed abortion and vary within- and among trees.

Like most leguminous seeds, *Acacia* seeds become dormant during maturation or storage due to water impermeability of the seed coat (Kaul and Manohar, 1966; van Staden, 1989). Numerous treatments have been employed to overcome seed dormancy in *Acacia* (Clemens, 1977; Auld, 1986; Black and El Hadi, 1992; Kobmoo *et al.*, 1990;

Doran and Gunn, 1991) but no single treatment can be used to improve germination for all *Acacia* species. In the hybrid seed, all treatments enhanced germination when compared to the control. Without treatment, hybrid seeds can germinate but germination is very slow and variable. However, in *A. senegal* seeds, the untreated seeds germinate satisfactory (Danthu *et al.*, 1992). Treatment with concentrated H_2SO_4 has been successfully used for many *Acacia* seeds (Scifres, 1974; Shaybany and Rouhani, 1976) but may be harmful to hybrid seeds from over-immersion as only moderate germination percentages were obtained. It has been suggested that optimumized periods of concentrated H_2SO_4 treatment may help in breaking dormancy without seed damage but the exact time may vary among species, provenance and seed lots (Kemp, 1975). Cutting and soaking in boiling water for 1 min gave high germination percentages in the hybrid seeds. The former treatment provides faster germination but the latter treatment may be more practical in the nursery. Similarly, hot water treatment has been recommended for several *Acacia* species, including parental species of the hybrid (Clemens, 1977; Shea *et al.*, 1979; Pukittayacamee and Hellum, 1988; Adjers and Srivastava, 1993). However, the optimum water temperature and soaking time must be determined for each species. For instance, in *A. auriculiformis*, 95° C water for 1 to 3 min gave the highest germination percentage, compared with 80° C for *A. terminalis* and *A. suaveolens* (Clemens *et al.*, 1977).

3.4.4 Seedling performance

The *Acacia* hybrid seedlings are of the phyllolobees type with the phaneroepigeal feature (Duke and Polhill, 1981). Under nursery conditions, survival rate of F_2 seedlings is similar to the parental species, in which it was high during the first 1 or 2 months then slightly reduced during month three (Ngulube, 1988; Likitwunnawut, 1990).

Leaf development and arrangement of the F_2 hybrid seedling are intermediate between the parental species and are similar to that of the F_1 hybrid seedling as described by Rufelds (1987, 1988) and Gan and Linang (1992). The first phyllode with a compound pinnate leaf occurred at about the third position from the main shoot which is similar to that of *A. auriculiformis*, whereas the first complete phyllode occurred at about the eighth position from the shoot tip which is similar to that of *A. mangium* (Rufelds, 1987, 1988; Gan and Linang, 1992). Besides leaf development, F_2 seedlings from hybrid seed showed the other intermediate characteristics between parental species, such as leaf shape and color.

Growth performance of F_2 hybrid seedlings was inferior to both parental species at the age of one month but became greater during the next two months when compared to *A. mangium* but not when compared to *A. auriculiformis*. At 3 months, the F_2 hybrid seedlings showed superior growth performance compared to those of its parental species planted in Thailand and Malaysia (Likitwunnawut, 1990; Ibrahim, 1991). As in most leguminous plants, nodulation in *Acacia* roots plays an important role in promoting seedling growth (Ngulube, 1988, 1990; Jayasankar and Kumar, 1992). It is possible that,

when compared to its parental species, nodulation of the F_2 hybrid seedling roots may increase at later stages of seedling development and contribute to better growth at 3 month.

Biomass production in 3- month- old F_2 seedlings is low and varied among trees when compared to other *Acacia* species (Ngulube, 1988). Also, in 8-month-old seedlings, there was no significant difference in biomass between the F_1 hybrid and parental species but it tended to be higher at later growth (Ibrahim, 1991). Ibrahim (1991) also suggested that F_2 hybrid seedlings may exhibit greater biomass production than parental species during late growth. Several studies have shown that the F_1 hybrid seedlings maintain superiority in further growth over the parental species (Lapongan, 1987; Ibrahim, 1991; Kijkar, 1992). It is likely that the F_2 hybrid may also express greater potential in growth characteristics than its parental species at later stages of development or following outplanting.

The 3-month-old F_2 seedlings showed less among- tree variation in height growth than diameter growth. Mean height of F_2 seedling from individual trees ranged from 20.16 to 26.73 with overall mean of 22.82 cm. In *A. mangium*, seedlings are recommended for field transplanting when they are 25 to 40 cm in height (Adjers and Srivastava, 1993), whereas 20 cm seedlings are recommended in *A. auriculiformis* (Wiersum and Ramlan, 1982). Therefore, 3-month-old hybrid seedlings with an average of 22.82 cm high may be recommended for outplanting if the hybrid seedlings maintain survival ability in the field similar to that of *A. auriculiformis.*

F_2 Seedlings from individual trees are uniform in phyllode arrangement and number except total leaf area. Among-tree variation in leaf area results primarily from differences in phyllode size among trees. Generally, phyllodes of *A. mangium* are significantly larger than those of *A. auriculiformis*. It is likely that among-tree variation in phyllode size is attributed to the variation in phyllode size of F_1 trees which resulted from the variation in interspecific hybridization between the parental species. Also, significant differences in both pinnate leaf number and area among trees may result from variations among trees in the degree of development and appearance of pinnate leaves which can be once- or, bi-pinnate or other forms of compoundness at early stages of seedling growth.

There is a lack of correlation of root to shoot ratio with seedling height or diameter. Instead, the largest amount of above-ground growth is attributed to foliar area. The low biomass production of roots may not be limited by the size of container used as there was no difference in growth parameters related to pot size in 3-month-old *A. mangium* seedling (Adjers and Srivastava 1993). Seedlings appear to allocate a large proportion of biomass to shoots rather than roots during early growth. Ngulube (1988) suggested that the variation in root to shoot ratio is probably due to the difference in the morphological features of seedlings and may not necessarily be related to subsequent field performance. Also, the variability in seedling sturdiness among trees resulted from the significant differences in growth rate and pattern.

CHAPTER 4

Pollination Biology in a Tropical *Acacia* Hybrid (*A. mangium* Willd. x *A. auriculiformis* A. Cunn. ex. Benth.)

4.1 Introduction

Pollination is the first male-female interaction and recognition event to determine compatibility or incompatibility. In angiosperms, the pollination process is complex as it is often associated with behaviour of pollen vectors and floral characteristics to promote maximal pollination success (Smith, 1970; Fægri and van der Pijl, 1979; Meeuse and Morris, 1984; Barth, 1985; Sedgley and Griffin, 1989; Proctor *et al.*, 1996). As angiosperm flowers vary greatly in structure and morphology, different types of pollinators have been reported, mainly insects (entomophyly) (Cruden *et al.*, 1990; Carthew 1993; Tangmitcharoen and Owens, 1997), birds (ornithophyly) (Collins and Spice, 1986; Bernardello *et al.*, 1994; Galetto *et al.*, 1994), and mammals (therophyly) (Nillson *et al.*, 1993, Gautier-Hion and Maisels, 1994; Hopkins, 1994; Sazima *et al.*, 1994). Floral characteristics also play an important role in facilitating both pollen transfer from anthers and deposition on stigmas (Sedgley and Griffin, 1989; Ish-Am and Eisikowitch, 1993; Ohara and Higashi, 1994).

Acacia mangium (Leguminosae: Mimosoideae) is native to northern Queensland, western Papua New Guinea, and Irian Jaya and Maluku of Indonesia (Doran and Skelton, 1982), whereas *A. auriculiformis* is native to Queensland, western and southern Papua

New Guinea, and Irian Jaya and the Kei Islands of Indonesia (Turnbull *et al.*, 1986). They have been introduced outside their natural ranges, particularly in Indonesia, Malaysia, and Thailand. The occurrence of natural hybrids between these two species has been reported in Malaysia (Tham, 1976; Pedley, 1978) and Thailand (Kijkar, 1992). The hybrid is superior in growth, adaptation to different types of soil, and resistance to pests and diseases over the parental species. Several studies have investigated hybrid performance, including seedling morphology and identification (Rufelds, 1987, 1988; Gan and Liang, 1992), isozyme analysis to determine the F_1 hybrid (Wickneswari and Norwati, 1992), vegetative propagation (Wongmanee *et al.*, 1989, Ahmad, 1992; Haines and Griffin, 1992) and techniques to produce hybrid seed through artificial pollination (Sedgley *et al.*, 1992a) or in biclonal seed orchards (Griffin *et al.*, 1992).

In Thailand, the hybrid possesses very low fruit and seed set, even though abundant flowers are produced (Chapter 3). Several authors conclude that pollination limits fruit and seed set in many plants (Schemske, 1977; Wilson and Schemske, 1980; Bierzychudek, 1981; Gross and Werner, 1983; Rathcke, 1983, Spira *et al.*, 1992) but this has not been determined in the hybrid. Many aspects of reproductive biology have been studied in *Acacia* (Ford and Forde, 1976; Kenrick and Knox, 1979, 1981a, 1981b, 1982, 1985, 1989b; Buttrose *et al.*, 1981; Bernhardt *et al.*, 1984; Knox *et al.*, 1985, 1989; Kenrick *et al.*, 1986; Fitzgerald *et al.*, 1993) and the parental species of the hybrid (Turnbull, 1986; Ibrahim, 1991; Ngamkajornwiwat and Luangviriyasaeng, 1991; Sedgley *et al.*, 1992b, Sornsathapornkul and Tangmitchareon, 1992) but little information exists on pollination biology of the hybrid in Thailand.

This study addresses many aspects of pollination biology in the hybrid, including floral morphology, anthesis, and pistil receptivity and their implications to the breeding system. The study also concentrates on types and behaviour of insect visitors and their contributions to pollination. This information will increase our understanding of how pollination limits fruit and seed production in the hybrid, and provide further insight into variation in breeding systems of *Acacia*, the largest genus of angiosperms.

4.2 Materials and methods

4.2.1 Study site and plant materials

Plant materials were collected from three 4-year-old *Acacia* hybrid trees growing in an experimental plot at the ASEAN Forest Tree Seed Centre, Muak- lek, Saraburi, Thailand (latitude N 14° 40′, longitude E 101° 17′, and about 200 m elevation). Originally, the F_1 hybrid seeds were collected from *A. mangium* naturally crossed with *A. auriculiformis*. Each hybrid produced abundant flowers over several months (June-October), providing enough specimens for the experiments. Individual flowers are grouped into a spike.

4.2.2 Determination of floral morphology, anthesis and receptive periods

To determine anthesis and receptive periods, several spikes from each tree were tagged and individual flowers collected periodically. Due to the short period of anthesis,

flowers were collected and observed for 2 d before anthesis and 6 d after anthesis at one to three-hour intervals. The sequence of flower opening, style straightening, stigmatic secretion, and anther dehiscence were investigated using both fixed specimens for scanning electron microscopy (SEM), transmission electron microscopy (TEM) and histochemistry. To examine floral morphology, fully-open flowers were collected and observed using a dissecting microscope. Anthers and ovaries were dissected from mature flowers to expose pollen and ovules for SEM.

SEM - Anthers, stigmas, and ovules were dissected from flowers and fixed in formalin-acetic acid-alcohol (FAA 50% ethanol) (Johansen, 1940). Specimens were then dehydrated in a series of ethanol solutions, critical point dried, and mounted on aluminum stubs, sputter-coated with gold, and viewed using a JEOL JS M-35 SEM at 15 kV.

TEM - Stigmas were dissected from flowers and fixed in 2.5% glutaraldehyde in 0.075 M phosphate buffer (pH 7.2 to 7.4). Specimens were then rinsed in the same buffer and postfixed for 1 h in 1% osmium tetroxide in 0.075 M phosphate buffer (pH 7.2 to 7.4). Specimens were dehydrated in a series of acetone solutions, embedded in Spurr's resin (Spurr, 1969) and sectioned at 60 nm using an ultramicrotome. Ultrathin sections were collected on 200-mesh grids and stained with 2% aqueous uranyl acetate for 15 min and 0.2% Sato's lead citrate (Sato, 1968) for 15 min and viewed using Hitachi 7000 electron microscope at 75 kV.

Histochemistry - Stigmas fixed for TEM were used to localize lipids, proteins, and insoluble carbohydrates in the stigmatic secretion. Specimens were dehydrated in a series of acetone solutions, embedded in Spurr's resin as above. Semithin sections (about 1μm) were cut and stained with periodic acid-Schiff's reagent (PAS) for insoluble carbohydrates

(Jensen, 1962), Sudan black B for lipids (Bronner, 1975), and Amido black for proteins (Jensen, 1962).

4.2.3 Insect visitors to flowers

Insects observed visiting flowering branches were collected with a hand-held net from 0600 to 1800 h. over three consecutive days. Observations were made on the timing of visitation, foraging behavior, and the number of insect visitors. Insects were killed in a glass container containing cotton saturated with CCl_4 and sent to Entomology and Zoology Division, Royal Agricultural Research Department, Bangkok, Thailand and Biosystematics Research Centre, Agricultural and Agri-Food Canada for identification. In order to identify the hybrid pollen and other pollen grains, and pollen-collecting areas on insect bodies, insects with pollen loads were observed fresh using the dissecting microscope and gold coated using the SEM at 15 kV.

4.2.4 Pollination success

At least eight open- pollinated spikes from each of three hybrids were collected 3 d after anthesis but before flower abscission. Pollination success (PS) was determined by comparing the mean number of flowers and pollinated flowers per spike, and the number of polyads per stigma. To identify pollinated flowers and to determine the number of polyads, stigmas were dissected from flowers, fixed in FAA, stained with aniline blue and viewed using light microscopy.

4.2.5 Statistical analysis

The analysis of variance (ANOVA) was used to assess the among-tree variation in pollination success (pollinated flowers per spike and polyad number per stigma). All percent data were subjected to arc-sine transformation prior to being analysed by ANOVA. The Duncan New Multiple Range test at $P < 0.05$ was used to compare means if there were significant differences among variables (Zar, 1984).

4.3 Results

4.3.1 Floral morphology

The hybrid has an average of 150 flowers loosely arranged in spikes (Fig. 10). Two spikes are borne in the axil of a phyllode. Although most flowers are hermaphroditic, some are staminate, with the pistil absent or rudimentary. When compared to a normal pistil, the redimentary pistil is relatively small, made up of only ovarian wall and lacks style and ovules. Hermaphroditic flowers consist of five sepals, five petals and one style surrounded by an average of 113 anthers (Fig. 19). The flowers are cream in color, fragrant and possess no floral nectaries. As in most *Acacia* species, the hybrid possesses a compound pollen grains called a polyad, containing 16 pollen grains (Fig. 11). Anthers are bilobed and terminally located on the filaments (Fig. 12). Each lobe consists of four loculi, each bearing only one polyad (Figs. 13,14), therefore, the total pollen grains per anther is 128. The eight pollen grains in the center of the polyad are angular and identical,

and different from the surrounding eight pollen grains (Fig. 11). The mature pistil has a long, narrow style and a hairy ovary. The ovary has one locule, containing an average of 15 ovules (Fig. 15). The ovules are amphitropous and bitegmic. Unlike most angiosperms, the nucellus is only partly enclosed by the inner and outer integuments (Fig. 16). The stigma is non-papillate with a smooth surface at receptivity (Fig. 25).

4.3.2 Morphology of anthesis and pistil receptivity

Flower buds (Fig. 17) usually begin to open synchronously or basipetally within a spike between 2400 h and 0100 h. For each flower, the interval between style and filament extension is only a few hours and the time taken for all flowers on a spike to open is 5-6 hr. Flower opening results in considerable extension of the style and filaments (Figs. 18,19). Usually, the style is the first floral organ to emerge and extend, followed by extension of the filaments (Fig. 18). When the style has almost straightened, the anthers begin to dehisce along vertical lines (Figs. 13,14). Polyads are exposed (Fig. 14) when filaments have almost fully elongated and straightened. At the complete blooming stage, the straight style generally slightly exceeds the length of the stamens (Fig. 19) and most polyads usually remain in the loculi (Fig. 14). Abundant polyads and strong floral fragrance are present during the first half-day (0600-1200 h) after anthesis and then they decrease. Flowers remaining the days following anthesis, contain few polyads and have little fragrance. The hybrid flower lives for a short period with its organs starting to wilt 3 d after anthesis. Approximately 50% of flowers on each spike begin to abscise at the start of day 4 with only a few pollinated flowers remaining after day 6.

Female receptivity lasts for about 1 day and shows distinct morphological changes. SEM observations show that, at pre-anthesis, the stigma surface is smooth with a concave cup-shape (Fig. 23). As the style emerges from the floral bud (stage 1) (Fig. 20), the stigma slightly expands and the thin cuticular layer of stigmatic surface ruptures, showing the secretion (Figs. 21, 22). Within a few hours following style emergence, the stigma secretion remarkably increases in volume and spreads over the surface. The peak receptive period, indicated by copious stigma exudute (Fig. 24), appearing shiny under the dissecting microscope, usually precedes the completion of style straightening. However, there is variation among flowers on a spike. The stigma sometimes reaches peak receptivity after the style is completely straightened. The post- receptive period occurs the day following flower opening and is characterized by a reduction in the amount of stigma exudate, revealing the smooth stigmatic surface with only some exudate remnants (Fig. 25). Most stigmas turn brown within 48 hr of anthesis and collapse within 5-6 d of anthesis (Fig. 26).

4.3.3 Ultrastructure and histochemistry of the stigmatic secretions

The hybrid stigma is 3 to 4 cells deep and is connected to the transmitting tissues of the solid style (Fig. 33). A few hours before anthesis (flower stage 0), the stigmatic cells are columnar, closely packed, and perpendicular to the stigma surface (Fig. 27). They have thick cell walls and dense cytoplasm containing large nuclei, several vacuoles and abundant cytoplasmic organelles, particularly, mitochondria, rough endoplasmic reticulum (ER), golgi bodies (Figs. 29, 30) and plastids with starch grains.

At this stage, the stigmatic secretion consists of small osmiophilic droplets in the space between the cell walls and the thin cuticle (Fig. 28), and in the intercellular spaces between the longitudinal walls, causing the stigmatic cells to separate from each other (Figs. 27, 30). The stigmatic cells are connected with each other primarily via transverse walls (Fig. 27). Secretion droplets also occur in transverse and longitudinal cell walls of the stigmatic cells. The tiny droplets accumulate in the transverse cell walls (Figs. 28, 29), and large droplets accumulate along the longitudinal cell walls (Fig. 29). The homogeneous, electron-dense droplets have different shapes. The droplets observed between the stigmatic cells and the surface cuticle are small and spherical (Figs. 28, 29), and those in the intercellular spaces are large and irregular (Fig. 30).

For about 1 hr after flower opening (stage 1) the shape of the stigmatic cells remains unchanged but considerable exudate is secreted onto the stigmatic surface, causing the surface cuticle to lift or rupture (Fig. 31). Substantial increase in stigmatic secretion was observed at stage 3 (Fig. 32). The secretions on the stigmatic surface and in the intercellular spaces stained positive for lipids with Sudan black B (Fig. 35) but not for proteins with Amido black (Fig. 34) or for insoluble carbohydrate with PAS (Fig. 33). Generally, lipids localized in the fresh tissues can be examined using Sudan black B. However, they can be washed away if specimens are dehydrated through an acetone series during resin-embedding (Bronner, 1975). In case of the hybrid stigma, the specimens were dehydrated through an acetone series but the stigmatic exudate still stained positively with Sudan black B for lipids. It is suggested that the hybid stigma may produce very abundant lipidic exudate during peak receptivity and some still remains on the stigmatic surface after dehydration.

4.3.4 Insect visitors

A diverse array of insects, belonging to the orders Hymenoptera, Diptera, Coleoptera, and Lepidoptera were observed visiting hybrid flowering branches during three days (Table 10). Most insects had polyads on their bodies except the representatives from Formicidae and Lepidoptera. The number of polyads per insect was quite variable among insect groups. Hymenoptera and Diptera usually visited throughout the day except *Apis mellifara* which visited only in the morning between 0600-1000 h. Coleoptera and Lepidoptera generally visited during afternoon. Only two species, *A. mellifara* (Apidae) and *Ceratina* sp. (Anthophoridae) were common visitors and had heavy pollen loads on their bodies. All others occasionally visited flowering branches and carried few pollen.

The honey bee (*A. mellifera*) and carpenter bee (*Ceratina* sp.) had similar behavior in that they preferentially foraged on individual spikes which contained newly open flowers for 3-5 min and traveled between spikes in the same tree and then between trees. During their foraging, abundant polyads became randomly distributed over the ventral region of their bodies, especially the legs, thorax, and abdomen which had many long hairs.

Both species exhibited different pollen-collecting features. *Ceratina* sp. had special long hairs on both hind legs to which a large number of polyads attached (Figs. 36, 37), whereas *A. mellifera* had distinct pollen baskets and many long hairs on its hind legs in which a large number of polyads occurred (Figs. 38, 39). Both species carried mostly *Acacia* polyads. However, a few pollen grains from members of the Asteraceae were found in pollen baskets of some honey bees (Fig. 40). The SEM studies showed that there

were sticky substances among polyads or between polyads and leg hairs, mostly in the pollen-basket area of the honey bee (Fig. 41).

4.3.5 Pollination success

Following open- pollination in all sample trees, less than 20% of the flowers per spike were pollinated during the three days and the amount of polyads deposited on stigmas was similar (Table 11). There were no significant difference in the percentages of pollinated flowers per spike among trees ($P < 0.05$). Most flowers received only one polyad, rarely more (Fig. 42). Due to the relatively small size of the stigma (60-80 μm) and the polyad (30-40 μm), only one or two polyads can fit onto the stigma. The disc-like polyad results in to differences in landing patterns of pollen onto the stigmatic surface. In some cases, when there is only one polyad, it may contact the surface on its flat surface (Fig. 25). In such case, a maximum of 12 pollen grains of the 16-grain polyad can directly contact the stigmatic surface.

Table 10. Insects visitors to the *Acacia* hybrid flowers collected at ASEAN Forest Tree Seed Centre, Saraburi, Thailand during a 3- day period in July 1994.

Insect visitors	Relative [1] amount of polyads per insect	Types of pollen collected	Estimated [2] number of insects observed	Visiting time
Order Hymenoptera				
Family Apidae				
Apis mellifera	very high	*Acacia* and Asteraceae	high	0600-1000
Anthophoridae				
Ceratina sp.	very high	*Acacia*	high	0600-1600
Vespidae				
Polistes stigma Fabricius	low	*Acacia*	low	1000-1400
Vespa tropica Bequaert	low	*Acacia*	low	1000-1400
Formicidae	none	none	medium	1000-1600
Diptera				
Calliphoridae				
Stomorhina lunata Fabricius	very low	*Acacia*	low	1000-1600
Syrphidae				
Eristalinus arvorum Fabricius	very low	*Acacia*	low	1000-1600
3 unknown species	very low	*Acacia*	low	1000-1600
Coleoptera				
Curculionidae				
Polydrusus sp.	very low	*Acacia*	occasional	1200-1600
2 unknown species	very low	*Acacia*	occasional	1200-1600
Lepidoptera				
Acraeidae				
Acraea viloae Fabricius	none	none	occasional	1200-1600
Nymphalidae				
Hypolimnas bolina Linneus	none	none	occasional	1200-1600
Papilionidae				
Atrophaneura aristolochiae Fabricius	none	none	occasional	1200-1600
Pieridae				
Eurema sp.	none	none	occasional	1200-1600

[1] Relative amount of polyads per insect: very low = polyad number range 1- 10, low = 11 - 50, medium = 51- 100, high = 101- 200, very high = > 201.
[2] Estimated number of insects observed: occasional = number of insect visitors range 1 - 5, low = 6 - 10, medium = 11- 20, high = > 20.

Table 11. Mean percentage of pollinated flowers per spike with the variation in polyad deposition on stigmatic surface in the *Acacia* hybrid (mean ± s.e.).

tree #	mean number of flowers per spike	pollinated [1] flowers (%)	stigmas bearing different number of polyads			
			1	2	3	4
1	146.4 ± 4.9	16.1 ± 2.6	13.6 ± 2.2	2.3 ± 0.3	0.2 ± 0.1	0
2	156.6 ± 7.2	17.3 ± 2.1	14.7 ± 1.5	2.2 ± 0.4	0.4 ± 0.2	0
3	141.5 ± 5.7	18.3 ± 1.8	14.9 ± 1.3	2.3 ± 0.2	0.9 ± 0.3	0.2 ± 0.1

Pollination success was estimated based on open- pollination.
[1] There was no significant difference among trees in pollinated flower percentages.

Fig. 10. The *Acacia* hybrid inflorescences showing the spikes at fully blooming stage (arrow) and early developing stage (arrowhead). Bar = 10 cm.

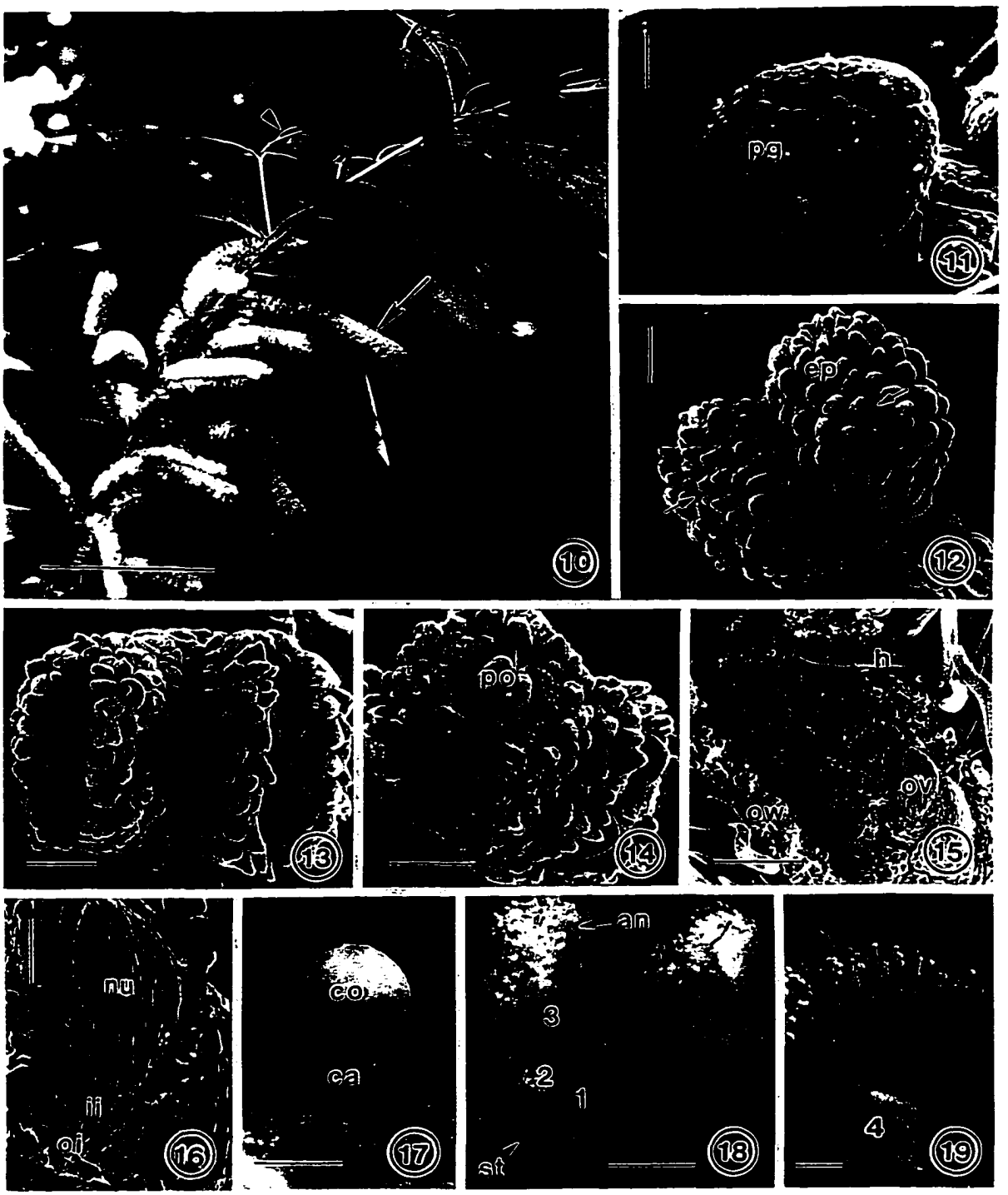
Fig. 11. Scanning electron micrograph (SEM) of the *Acacia* hybrid polyad, showing the arrangement of the pollen grains (pg). Bar = 5 μm.

Figs. 12-14. SEMs showing stages of anther dehiscence in the *Acacia* hybrid. Fig. 12. The mature anther excised from the flower at stage 0, showing two lobes, cone-shaped epidermal cells (ep), and dehiscent line (arrows). Fig. 13. Anther partly opened along the dehiscent line of the anther from a flower at stage 3. Fig. 14. Completely opened anther from a flower at stage 4, exposing the polyads (po). Bar = 40 μm in Figs. 12-14.

Fig. 15. SEM of the *Acacia* hybrid ovary with side removed showing hairs (h), ovary wall (ow) and arrangement of the ovules (ov). Bar = 100 μm.

Fig. 16. SEM of the mature *Acacia* hybrid ovule showing partial development of the inner (ii) and outer (oi) integuments, exposing the nucellus (nu). Bar = 25 μm.

Figs. 17-19. Anthesis of the *Acacia* hybrid flowers. Fig. 17. Floral buds at stage 0 (2200 - 2300 h or about 1-2 hr before anthesis), showing calyx (ca), corolla (co). Bar = 1 mm. Fig. 18. Flower opening at different stages, stage 1 = flower with slightly opened corolla and emerging folded style, stage 2 = flower with progressively opened corolla and straightening of the style (st), stage 3 = flower with partly opened corolla and straightening of the style and anthers (an). Bar = 2 mm. Fig. 19. Completely opened flower (stage 4) at 0500-0600 h, showing straightening style and anthers. Bar = 1 mm.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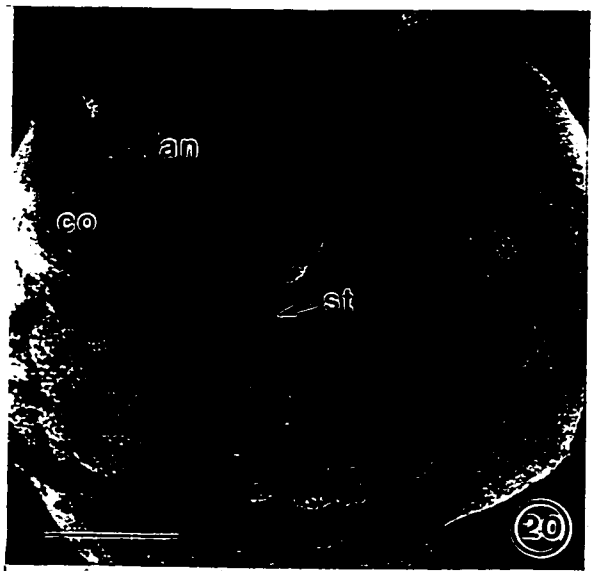
Figs. 20-22. Scanning electron micrographs (SEMs) of the *Acacia* hybrid flower at stage 1. Fig. 20. Slightly opened corolla (co) and emergence of the folded style (st) and anthers (an). Bar = 30 μm. Fig. 21. Stigmatic surface showing rupuring of the cuticular layer (cl), exposing secretion droplets (sd). Bar = 20 μm. Fig. 22. Higher magnification of the stigmatic secretion droplets as in Fig. 21. Bar = 4 μm.

Figs. 23-26. SEMs showing receptivity of the *Acacia* hybrid stigma. Fig. 23. The pre-receptive stigma of the flower at stage 0, showing the cup-shaped stigmatic surface (ss) and no secretion is observed. Fig. 24. The peak receptive stigma of the flower at stage 3, showing copious stigmatic exudate (se). Fig. 25. Early post-receptive stigma about 3 days after anthesis, showing remnants of the exudate (ex) and adhering polyad (po). Fig. 26. Late post-receptive stigma 5-6 days after anthesis, showing collapsing stigmatic surface (*). Bar = 20 μm in Figs. 23-26.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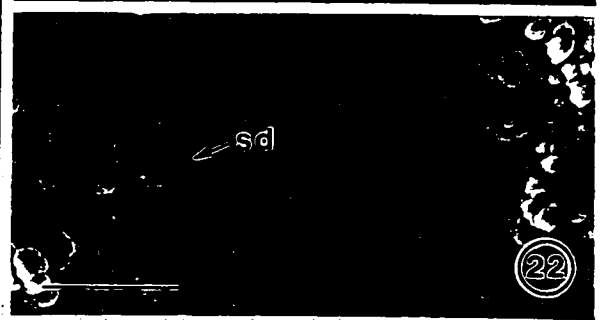
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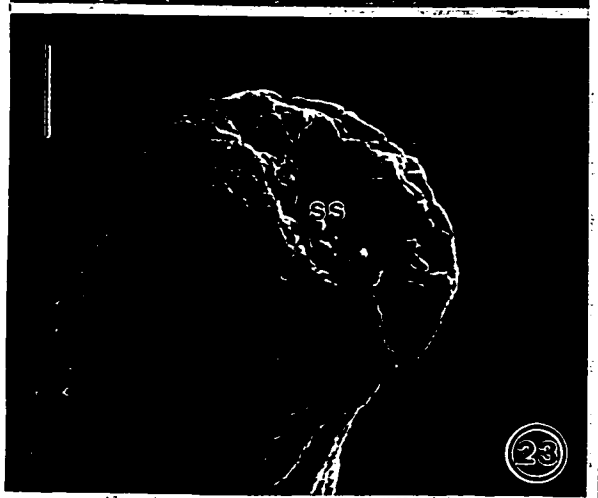
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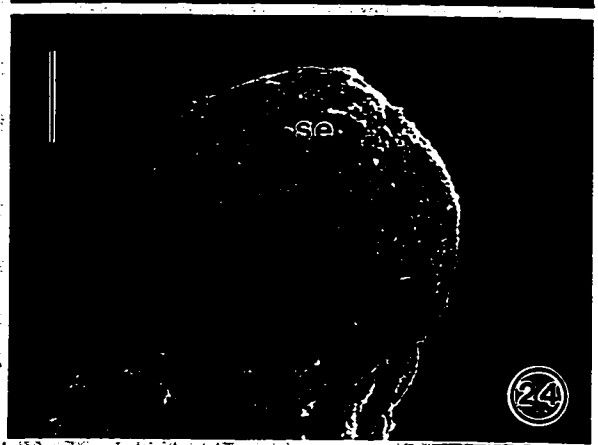
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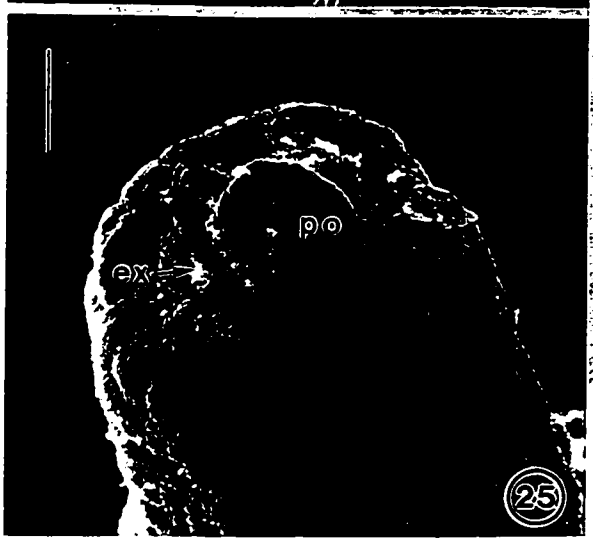
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Figs. 27-30. Transmission electron micrographs (TEMs) of the stigma of the *Acacia* hybrid flower at stage 0. Fig. 27. Longitudinal section of the stigmatic zone, showing columnar stigmatic cells (sc), each containing a large nucleus (n) and vacuoles (v). Secretions (*) accumulate in the intercellular space (arrow) and the space (arrowhead) between the stigmatic cells and the cuticular layer (cl); longitudinal cell wall (lcw); transverse cell wall (tcw). Bar = 4 μm. Fig. 28. The stigmatic surface zone from Fig. 27 showing spherical secretion droplets (sd) in the space between the cell walls (cw) and the cuticular layer, and tiny secretion droplets (arrow) in the transverse cell wall. Bar 1 μm. Fig. 29. Secretion droplets in tangential (gcw) and transverse cell walls of the stigmatic cell; plasmodesmata (pd). Bar = 1 μm. Fig. 30. Accumulation of the irregular-shaped secretion droplets in the intercellular space. Bar = 3 μm.

Fig. 31. TEM of the stigmatic surface of the *Acacia* hybrid flower at stage 1, showing the lifting of the cuticular layer resulting from the copious secretion of the droplets from the intercellular space. Bar = 2 μm.

Fig. 32. TEM showing abundant stigmatic exudate (se) observed in the *Acacia* hybrid flower at stage 3. Bar = 2 μm.


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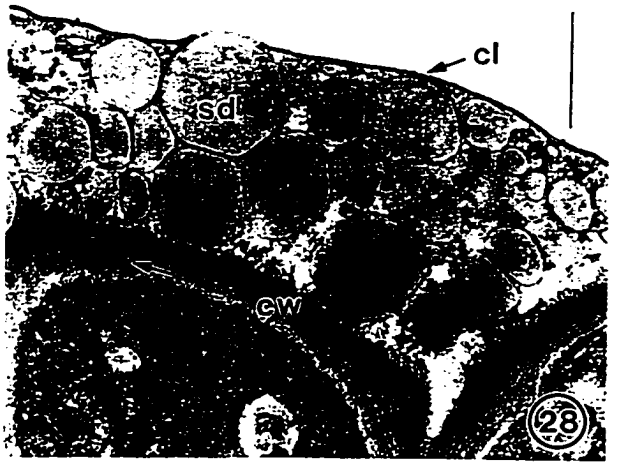
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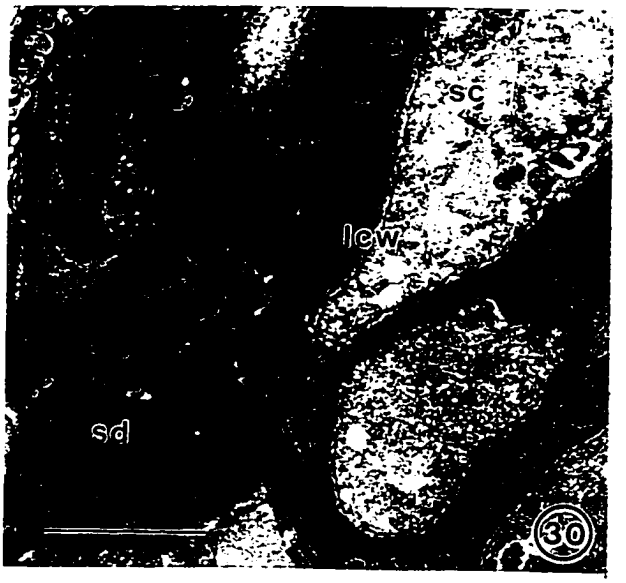
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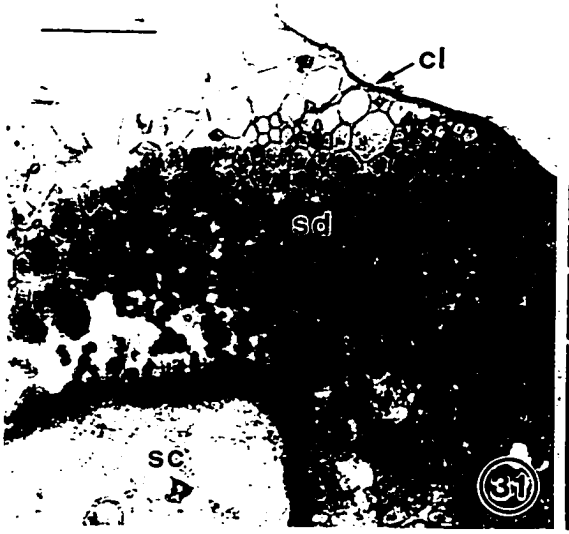
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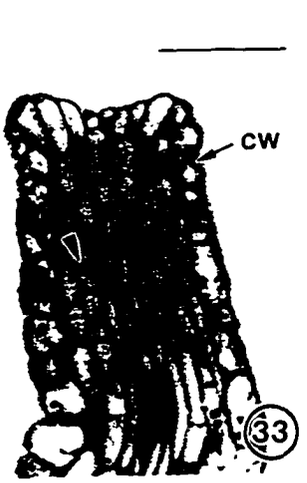
Figs. 33-35. Histochemical stains of 1 μm longitudinal sections of the *Acacia* hybrid stigma from a flower at stage 3. Fig. 33. Section stained with PAS. Cell wall (cw) and plastids containing starch grains (arrowhead) stained but stigmatic exudate did not. Fig. 34. Section stained with Amido Black. Only the cell wall and protein bodies (arrow) in the outermost epidermal cells (ep) stained. Fig. 35. Section stained with Sudan Black B. Copious stigmatic exudate (se) and secretions (sd) in the intercellular space stained intensely. Bar = 40 μm in Figs. 33-35.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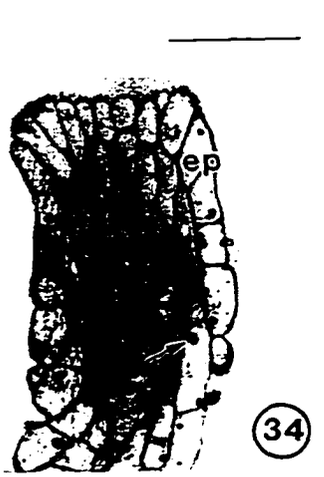
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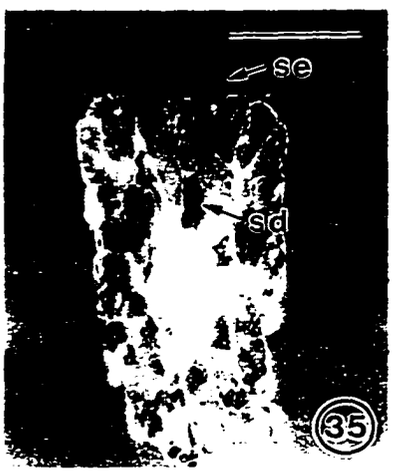
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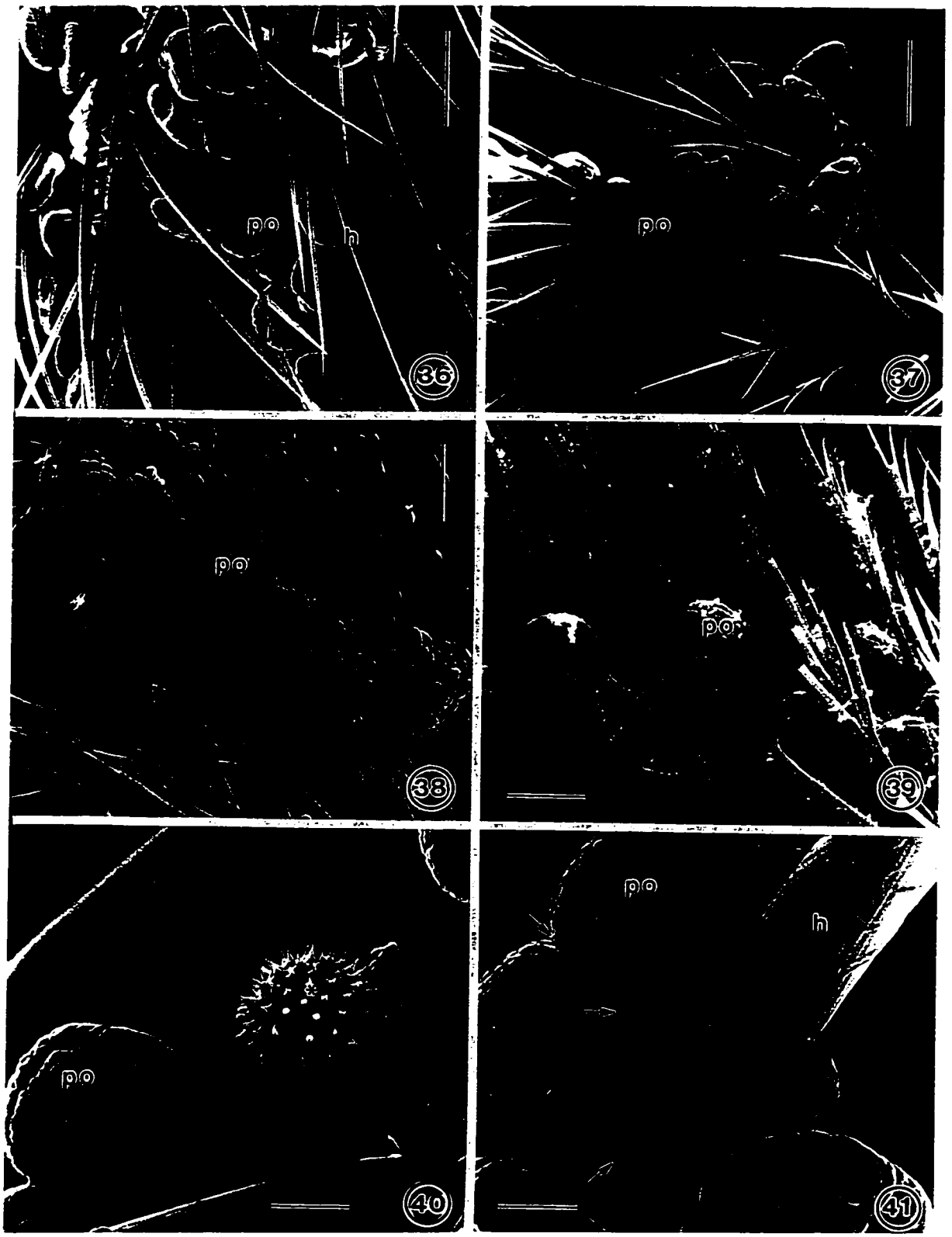
Figs. 36-37. Scanning electron micrographs (SEMs) showing *Acacia* hybrid polyads (po) on the hind legs of carpenter bee (*Ceratina* sp.). Fig. 36. Abundant polyads accumulated among scopal hairs (h) of the hind metatarsus. Bar = 50 μm. Fig. 37. A mass of polyads deposited on hairy hind tibia. Bar = 40 μm.

Figs. 38-41. SEMs showing *Acacia* hybrid polyads on the hind legs of honey bee (*Apis mellifera*). Fig. 38. A mass of polyads in the pollen basket (corbicula) on the outside of the hind tibia. Bar = 200 μm. Fig. 39. Abundant polyads were deposited on the hairy hind metatarsus. Bar = 30 μm. Fig. 40. Close-up of the pollen basket showing the Asteraceae pollen (*). Bar = 15 μm. Fig. 41. Sticky substances (arrows) (regurgitated honey produced by honey bee) among the polyads and between the polyads and a hair. Bar = 10 μm.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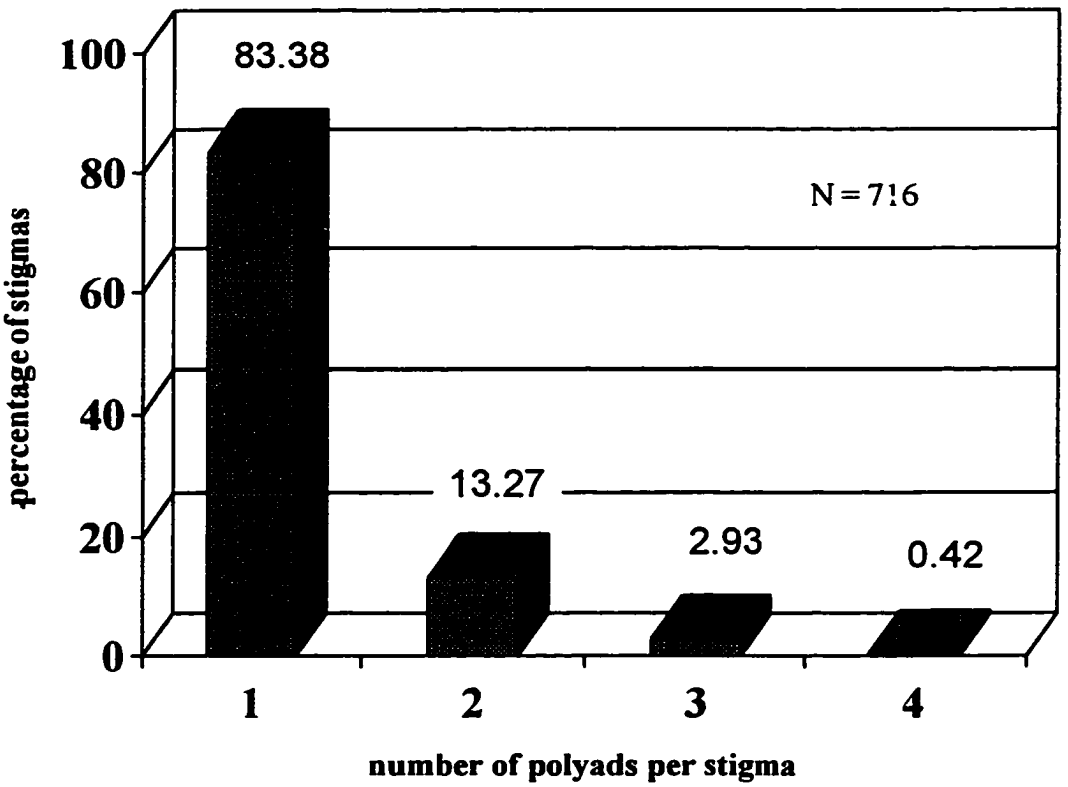

Figure 42. Frequency distribution of polyad deposition on stigmas in the *Acacia* hybrid, N = no. of flowers observed

4.4 Discussion

4.4.1 Floral biology, anthesis and female receptivity

Timing of anthesis in *Acacia* varies among species, mostly occurring during late night to noon (Bernhardt *et al.*, 1984; Ibrahim, 1991; Tybirk, 1993; Baranelli *et al.*, 1995). In the *Acacia* hybrid, anthesis is similar to that of the parental species (Ngamkhajornwiwat and Leungviriyasaeng, 1989; Ibrahim, 1991; Sornsathapornkul and Tangmitcharoen, 1992) in which most flowers begin to open about 2400 h and fully open about 0500-0600 h. The hybrid flower has a short longevity (3 days) with the peak pollen availability, floral fragrance, and stigmatic receptivity occuring during the first half day (0600-1200 h) after anthesis and decreasing thereafter. That seems to be the most suitable period for pollinator activities and effective pollination. A similar active time for pollinators (0730-1100 h) was also observed in the parental species grown in Malaysia (Ibrahim, 1991).

According to the classification system of Heslop-Harrison and Shivanna (1977), the stigma of the hybrid and other acacias is wet and non-papillate (Bernhardt *et al.*, 1984; Kenrick and Knox, 1981b, Knox *et al.*, 1989; Ibrahim, 1991). Ultrastructural studies at the earliest stage observed in this study (stage 0) show the secretion droplets already present in the intercellular space and the space between the stigmatic cells and the cuticle. Also, the peak stigmatic secretion occurs before the complete straightening of the style and anther dehiscence. Synthesis of the stigmatic secretion in the hybrid, therefore, occurs at the early floral bud stage. In *A. retinodes*, in which stigma morphology is similar to that of the hybrid, accumulation of the stigmatic secretion in the cytoplasm was observed at the

early floral bud stage when buds are still green and covered by bracteoles (Knox *et al.*, 1989).

In the hybrid and the majority of angiosperms, the stigmatic secretion is produced from the stigmatic cells (Clarke *et al.*, 1979; Sedgley, 1981; Tilton *et al.*, 1984b; Baird *et al.*, 1988; Knox *et al.*, 1989; Mckenzie *et al.*, 1990), whereas in some species it may be produced from the stylar tissues (Weber, 1994), the stigmatic cells and stylar tissues (Kandasamy and Kristen, 1987), or the ovules (Franssen-Verheijen and Willemse, 1993). In the hybrid, the stigmatic secretion accumulates in the intercellular spaces in the stigmatic zone.

Lipids are the major components in the hybrid stigmatic secretion, suggesting that the hybrid stigma is the lipophilic type reported in many Solanaceae (Konar and Linskens, 1966a, b; Herrero and Dickinson, 1979; Cresti *et al.*, 1986; Mckenzie *et al.*, 1990). This is different from *A. retinodes* stigmatic secretion which possesses complex components, including proteins, carbohydrates, and lipids (Kenrick and Knox, 1981b, Knox *et al.*, 1989). Stigmatic secretions have traditionally been considered as attractants for floral visitors and a nutrient source for pollen germination and recognition (Clarke *et al.*, 1979; Lord and Webster, 1979; Knox, 1984). Dumas *et al.* (1984) noted that a lipidic secretion is hydrophobic and functions as a liquid cuticle to prevent stigma dehydration following disruption of the cuticle (Konar and Linskens, 1966a, b) or as a food reward for insect pollinators (Lord and Webster, 1979). In the hybrid, the lipidic secretion not only functions in stigma protection, including the possibility of being rain-resistant as the flowering period usually occur during the rainy season (Chapter 3).

In this study, we have shown the pathway of secretion from intercellular spaces onto the stigmatic surface. How the droplets are secreted from the stigmatic cells into intercellular spaces is unclear. Several studies show different pathways of lipidic secretions (Herrero and Dickinson, 1979; Cresti *et al.*, 1982; Knox, 1984; Knox *et al.*, 1989). In stigmas in which the secretion is composed of lipids, proteins and carbohydrates, lipid synthesis is associated with smooth ER and lipid droplets are transported through the cell wall by membrane perfusion (eccrine mechanism) (Sedgley and Blesing, 1983; Schou, 1984). Dumas *et al.* (1984) suggest that the lipidic secretion is mainly of the holocrine type in which lipids are synthesized by ER, transported into the vacuoles, accumulated in periplasmic region, and then transported through the cell wall. In the lipophilic hybrid stigma, lipid secretion is not well understood but appears to be similar to that described by Dumas *et al.* (1984) because of the number of vacuoles and ER in the stigmatic cells, and secretion droplets in the cell walls.

Post-pollination exudates have been observed in several *Acacia* species (Kenrick and Knox, 1981a; Marginson *et al.*, 1985a,b). Their function has been suggested to ensure pollen germination of grains not in contact the stigma. However, post-pollination exudate has not been observed in the hybrid or the parental species (Ibrahim, 1991).

Protogynous dichogamy (female phase preceding male phase) is common in *Acacia* (Newman, 1934a; Philp and Sherry, 1946; Kenrick and Knox, 1981b; Sedgley *et al.*, 1992b). In *A. retinodes*, protogyny is distinct and the stigma becomes receptive one day before anther dehiscence (Bernhardt, *et al.*, 1884; Knox *et al.*, 1989). However, protogyny is weak in the hybrid with stigma receptivity occuring only a few hours before anther dehiscence. Ibrahim (1991) also noted that flowers of the parental species are

likely to be homogamous rather than protogynous. If protogynous dichogamy is one mechanism for promoting outcrossing in *Acacia* (Sedgley, 1987), it is questionable whether protogyny still exists to promote the outcrossing in the hybrid and the parental species.

Floral organ arrangement of the hybrid flower appears similar to those of many *Acacia* species as described by Newman (1934a), Kenrick and Knox (1979, 1981b, 1982), Buttrose *et al.* (1981), Bernhardt *et al.* (1984), Turnbull *et al.* (1986), Tybirk (1989, 1993), Baranelli *et al.* (1995). Hybrid flowers differ from other acacias in the numbers of ovules and anthers per flower and pollen grains per polyad. Generally, the hybrid flowers in Thailand are morphologically similar to those of the parental species as described by (Ibrahim, 1991; Ngamkhajornviwat and Luangviriyasaeng, 1991; Sornsathapornkul and Tangmitcharoen, 1992). The hybrid floral color (cream) is similar to that of *A. mangium* (creamy white) rather than *A. auriculiformis* (bright yellow). The total number of flowers and staminate flowers per spike appear intermediate between the parental species in Thailand and Malaysia, whereas the number of ovaries and anthers per flower is greater than the parental species (Chapter 3).

4.4.2 Floral morphology and insect pollinators

Several authors suggest that floral attractants in most angiosperms include primary attractants (food rewards for visitors), e.g. pollen, nectar, oil, and secondary attractants (visitor attraction from a distance), e.g. floral odor, color and temperature (Fægri and van der Pijl, 1979; Sedgley and Griffin, 1989; Proctor *et al.*, 1996). Since hybrid and other

Acacia species flowers lack nectar, pollen produced in abundant inflorescences is the main attractant (Bernhardt and Walker, 1984, 1985; Bernhardt *et al.*, 1984; Sedgley *et al.*, 1992b). Andromonoecy has been reported in several *Acacia* species (Newman, 1933, 1934a; Zapata and Arroyo, 1978; Tybirk, 1989; Ibrahim, 1991; Baranelli *et al.*, 1995) and the hybrid (the proportion of staminate flowers ranged from 0-30 % in a spike) (Chapter 3). It is likely that, in the hybrid and other acacias, the staminate flowers function for pollen donation, thus increasing pollinator visitation (Zapata and Arroyo, 1978; Stephenson, 1979; Sutherland and Delph, 1984; Bertin, 1988). Although the cream color of the hybrid flowers may not be a strong attractant, distinct floral fragrance at anthesis seems to attract most insect groups, e.g. bees, flies, wasps, beetles, and butterflies, from a distance (Fægri and van der Pijl, 1979; Barth, 1985; Proctor *et al.*, 1996). The hybrid floral color may attract only insects which respond to a wide range of the spectrum, such as butterflies (Proctor *et al.*, 1996). A number of the hybrid floral characteristics, such as small flowers grouped into a spike, closeness of male and female floral organs and exposure of anthers and style for easy pollen accessibility may facilitate pollen transfer from anthers and to stigmas. The small flowers grouped into spike may also facilitate landing of small insects such as flies or bees as shown in some *Acacia* species (Ibrahim, 1991; Sedgley *et al.*, 1992c).

Some of insects from the Hymenoptera, Diptera, Coleoptera, and Lepidoptera are recognized as the effective pollinators of tropical tree flowers (Bawa, 1990, Cruden *et al.*, 1990, Carthew, 1993; Renner and Feil, 1993). In many *Acacia* species, insects from these groups visit flowering branches, but only from hymenopterans, particularly *Apis* (honey bee) and *Trigona* (sweat bee) are effective pollinators (Bernhardt and Walker, 1984, 1985;

Bernhardt *et al.*, 1984; Ibrahim, 1991, Sedgley *et al.*, 1992c). In the hybrid, diverse insect visitors were also observed to forage on inflorescences for different times and purposes. According to the criteria of Smith (1970), most species, other than *Apis mellifera* and *Ceratina* sp., are more likely only visitors to the hybrid flowers. The small beetles which are common pollinators in Annonaceae, Araceae, Cyclanthaceae, Lauraceae, and Myristicaceae (Bawa, 1990), would be less effective pollinators in the hybrid because they were few in number and carried few polyads on their bodies. This is also true for the butterflies. Although they are common visitors to the flowers of species with brightly colored corollas, especially in the Boragiaceae, Rubiaceae, and Vochysiaceae (Bawa, 1990), only four species were attracted to the cream color of the hybrid flowers. Rather than collecting pollen, butterflies more likely forage for floral nectar which is not present in the hybrid. They are usually relatively large and lack the potential pollen collecting sites such as hairy bodies and legs, suggesting that they are not effective pollinators for the hybrid flowers. This was confirmed by the low pollen number on their bodies and legs. The five species of small flies, whose size is compatible with the hybrid flower may also not be effective pollinators. Most flies found on flowering branches are pollen-feeding, and few polyads were attached to the short hairs on their bodies. The two wasp species from the Vespidae are medium and large-sized predatory insects. They usually feed on or collect other insects or animal materials and occasionally pollen, but lack the distinct pollen collecting features (Borror *et al.*, 1981, Proctor *et al.*, 1996). The few polyads on their bodies may be obtained when they were in contact with flowers during their forage for other small insects visiting the hybrid.

Apis mellifera (honey bee) and *Ceratina* sp. (carpenter bee) are effective pollinators in many crop and tree species (Cruden *et al.*, 1990: Sedgley *et al.*, 1992c, Carthew, 1993; Ish-Am and Eisikowitch, 1993; Tangmitcharoen and Owens, 1997). They appear to be the most effective pollinators in the hybrid because they are the most common visitors and carry a heavy load of hybrid polyads. Both visited the hybrid flowers during the peak of pollen presentation and receptivity. In addition, while collecting pollen the ventral regions of their bodies contact the stigma due to the flower structure, contributing to pollination. However, this may also promote self-pollination in the hybrid as bee pollinators were observed to visit different inflorescences in the same tree prior to moving to another tree. The abundant flowers on each tree may provide an adequate source of pollen for visits by the insects, resulting in few visitations among trees. (Sedgley and Griffin, 1989). There are other floral features which may also allow self-pollination in the hybrid. These include the complete development of female and male phases prior to foraging of most flower visitors and the fact that the style is only slightly longer than the surrounding stamens. This allows pollen transfer onto the stigma of the same flower during foraging activities. van Schaik *et al.* (1993) suggested that either the plant species or floral visitors have adapted to match foraging activities or flowering time. The same bee species forages for pollen and nectar of *Tectona grandis* Linn., growing in close proximity but flowering at different time (Tangmitcharoen and Owens, 1997).

Carpenter bees are probably more efficient pollinators of the hybrid than honey bees. Although both carry large amounts of pollen and have compatible sizes for the hybrid flower, the sticky substances found among polyads on honey bees may make pollen transfer difficult from the insect to the stigma. It is unlikely that this substance is derived

from the pollen grain exine since it was not present on polyads of other insect visitors. Proctor *et al.* (1996) noted that this sticky substance is regurgitated honey produced by the honey bee and is used to moisten and compact a mass of pollen before transfer into pollen baskets. Carpenter bees, on the other hand, not only regularly visit flowers, but can also collect polyads and easily transfer them to stigmas. Although a small number of other pollen grains were carried by some honey bees, these did not appear to result in false pollination of the hybrid since only hybrid polyads were found on pollinated flowers.

There was no correlation between pollination success and exposure period of the hybrid flowers to insect visitors. Between anthesis and abscission (about 3 days), less than 20% of flowers per spike were pollinated, probably due to a few insect pollinators during the flowering period. van Schaik *et al.* (1993) noted that the abundance of pollinators may be affected by season, with the number of insects usually highest during the dry season. In the hybrid, flowering mostly occurs during rainy season (June- October) at the Muak-lek site (Chapter 3). Rain may decrease foraging activities of insect pollinators, especially bees which preferentially forage for pollen or nectar during warm and sunny days (Proctor *et al.*, 1996), resulting in low pollination success. Introduction of bee hives during the flowering period may increase pollination success since it has worked in a wide range of tree crops (Moncur and Somerville, 1989; Sedgley and Griffin, 1989).

Most pollinated flowers receive only one polyad. An increase in exposure time to pollinators may not affect the rate of pollen deposition on stigmas since the limitation of polyad number on the stigma is more likely due to the relatively small stigma size in relation to polyad (Kenrick and Knox, 1981b, 1982; Ibrahim, 1991). Increasing exposure time or pollinators, in turn, may increase the number of pollinated flowers.

The pollen to ovule ratio averaged 1.05 in the hybrid (Chapter 3), indicating that there is approximately one pollen tube to fertilize each ovule. However, several studies show that excess pollen per stigma is required to ensure seed set and pollen competition to increase seed quality (Mulcahy and Mulcahy, 1987; Snow, 1990; Spira *et al.*, 1992). Heard (1993) also suggested that approximately 150 insect visits per inflorescence may be necessary to ensure adequate pollination in *Macadamia integrifolia*. Whether or not the number of polyads per stigma after open-pollination is adequate for maximum seed set in the hybrid needs further investigation.

CHAPTER 5

In Vivo and *In Vitro* Pollen Germination and Pollen Tube Growth in a Tropical *Acacia* Hybrid (*A. mangium* Willd. x *A. auriculiformis* A. Cunn. ex Benth.)

5.1 Introduction

Acacia mangium (Leguminosae: Mimosoideae) is native to northern Queensland, western Papua New Guinea, and Irian Jaya and Maluku of Indonesia (Doran and Skelton, 1982), whereas *A. auriculiformis* is native to Queensland, western and southern Papua New Guinea, and Irian Jaya and the Kei Islands of Indonesia (Turnbull *et al.*, 1986). They have both been introduced into several Southeast Asian countries such as Indonesia, Malaysia and Thailand (Darmono and Dayanto, 1981; Chittachumnok and Sirilak, 1991; Ibrahim, 1991), where they are fast-growing, multipurpose species widely used for timber, fuelwood, tanning, agroforestry, ornamental horticulture and soil improvement (Phillips *et al.*, 1979; Abdul Razak *et al.*, 1981; Turnbull *et al.*, 1986). Recently, there has been an increased interest in their interspecific hybrid because it exhibits superior growth, form, and adaptability compared to the parental species (Tham, 1979; Korwanich, 1982; Kijkar, 1992; Pinso and Nasi, 1992). The hybrid occurs naturally where both species grow in close proximity (Darus and Rasip, 1989; Kijkar, 1992) and hybrid seeds may also be obtained by artificial cross-pollination (Sedgley *et al.*, 1992a) or biclonal seed orchards (Griffin *et al.*, 1992). Despite the interest in the hybrid performance for plantations, there have been few thorough studies of the hybrid to date. Recent studies have concentrated

on seedling morphology and identification (Rufelds, 1987, 1988; Gan and Liang, 1992), isozyme analysis (Wickneswari and Norwati, 1992), and vegetative propagation (Wongmanee *et al.*, 1989).

Detailed studies of the hybrid floral and pollination biology have been done (Chapter 4). The hybrid flower is hermaphroditic showing weak protogynous dichogamy, in which the female phase precedes the male phase by only a few hours. The hybrid floral architecture facilitates pollen transfer by pollinators onto the stigma but low pollination success is likely due to low pollinator number, and this is thought to be partly responsible for low fruit set (Chapter 4). Other reproductive processes, such as pollen quality, pollen tube growth, and pollen-pistil interaction (Knox, 1984; Kahn and Morse, 1991; Herrero, 1992; O'Brien, 1994) play key roles in successful fertilization and affect fruit and seed set (Hessing, 1989; Roa *et al.*, 1992; Smith-Huerta, 1996). There have been few studies on pollen germination and pollen tube growth in *Acacia* and no detailed studies of the hybrid. Some studies in *Acacia*, which are related to these aspects, include *in vivo* pollen germination and pollen-tube growth (Kenrick and Knox, 1985; Kenrick *et al.*, 1986; Sedgley *et al.*, 1992b; also see review by Kenrick and Knox, 1989a) and *in vitro* pollen germination and pollen storage (Sedgley *et al.*, 1992a; Sedgley and Harbard, 1993).

This study describes pollen-pistil interactions to determine if this is a cause of low fruit and seed set in the hybrid. Particular emphasis is given to pollen quality and pollen-tube growth *in vivo* and *in vitro*. The study of *in vivo* pollen-tube growth concentrates on patterns and variation in tube growth and penetration of the ovules. We also examine the ultrastructural and histochemical changes in the style before and after pollen-tube penetration to determine how the style facilitates pollen-tube growth to the ovary.

5.2 Materials and methods

5.2.1 Study site and plant materials

An experimental plot of the hybrids was established in 1990 at the ASEAN Forest Tree Seed Centre, Muak- lek, Saraburi, Thailand (latitude N 14° 40′, longitude E 101° 17′, and about 200 m elevation), using the F_1 hybrid seeds collected from *A. mangium* naturally crossed with *A. auriculiformis*. Hybrids first flower at for 1.5 years and produce abundant flowers over several months (June- October). Individual flowers are grouped into an inflorescence called a spike containing about 150 loosely arranged flowers. Grains are grouped into compound structure called a polyad, containing 16 pollen grains (Chapter 4). Four 4-year-old hybrid trees were used for this study. Several spikes containing flowers at pre-anthesis (1 to 2 hr before flower opening) were randomly tagged and periodically collected for light microscopy (LM), scanning electron microscopy (SEM), transmission electron microscopy (TEM), fluorescence microscopy (FM), and histochemistry.

5.2.2 Estimation of pollen quality

Pollen quality was estimated by *in vitro* germination using Brewbaker's solution (Brewbaker and Kwack, 1963) and viability using the fluorochromatic reaction (FCR test) (Heslop- Harrison *et al.*, 1984). For the germinability test, polyads were excised at 0700 h from freshly dehisced anthers onto microscope slides to which a few drops of

Brewbaker's solution containing 10% sucrose (optimal sucrose concentration for the hybrid pollen germination, unpublished data) were added. After 12 hr a sample of polyads was placed in a drop of decolorized aniline blue and viewed by fluorescence microscope. A minimum of 100 polyads were scored from each of four slides, representing four replications from each hybrid. The number of germinated polyads was counted when at least one pollen grain in a polyad germinated with the pollen-tube length at least twice the diameter of the pollen grain. The number of germinated polyads was also placed into classes based on proportion of pollen grains per polyad that germinated. For the pollen viability test, polyad collection was similar to that described in the *in vitro* germination test. Polyads were placed in 10% sucrose-containing Brewbaker's solution combined with a saturated fluorescein diacetate solution. Viable pollen grains in a polyad were indicated by a positive stain (bright fluorescence). Polyads were scored into four classes based on the proportion of viable pollen grains per polyad, 1-4, 5-8, 9-12, and 13-16.

Pollen longevity was determined using the FCR test as described above. Polyads were collected from freshly dehisced anthers at 0700 h and stored at room temperature for 1 to 3 days. Polyad viability was determined 3 to 4 times each day. At each time, a minimum of 100 polyads were scored from each of four replications from four hybrids. Again, polyads were scored into four classes as described above.

5.2.3 Ultrastructure and histochemistry of the style

Styles were dissected from flowers at different stages; i) pre-anthesis (a few hours before flower opening; ii) partly open; iii) fully open; and, iv) 24 hr after anthesis.

Specimens were fixed in 2.5% glutaraldehyde in 0.075 M phosphate buffer (pH 7.2 to 7.4) for TEM, LM and histochemistry. Specimens were then rinsed in the same buffer and postfixed for 1 hr in 1% osmium tetroxide in 0.075 M phosphate buffer (pH 7.2 to 7.4), dehydrated in a series of acetone solutions, embedded in Spurr's resin (Spurr, 1969) and sectioned using an ultramicrotome. For TEM, ultrathin sections (about 60 nm) were collected on single hole grids coated with 0.3 % Formvar in chloroform, then stained with 2% aqueous uranyl acetate for 15 min and 0.2% Sato's lead citrate (Sato, 1968) for 15 min and viewed using Hitachi 7000 electron microscope at 75 kV. To localize lipids, proteins and insoluble carbohydrates in the stylar secretion using the LM, semithin sections (about 1 μm) were mounted on glass slides and stained with periodic acid-Schiff's reagent (PAS) for insoluble carbohydrates (Jensen, 1962), Sudan black B for lipids (Bronner, 1975), and Amido black for proteins (Jensen, 1962).

5.2.4 *In vivo* pollen germination and pollen tube growth

In vivo pollen germination and pollen tube growth were studied using open-pollinated flowers. Several spikes containing newly-opened flowers were allowed to be open-pollinated between 0600-0700 h. Pollinated flowers were then enclosed in cellophane bags to ensure synchronous pollination. To study pollen germination and pollen tube growth *in vivo*, ten bagged spikes from each of three hybrids were collected at two-hour intervals for 1 d after pollination. Pistils were dissected from pollinated flowers and fixed in formalin-acetic acid-alcohol (FAA 50% ethanol) (Johansen, 1940) for SEM and in 2.5% glutaraldehyde in 0.075 M phosphate buffer (pH 7.2 to 7.4) for LM and

TEM. For SEM, specimens were dehydrated in a series of ethanol solutions, critical point dried, and mounted on aluminum stubs, sputter-coated with gold, and viewed using a JEOL JS M-35 SEM at 15 kV. For TEM, specimens were prepared and observed as described above. For LM, semithin sections (about 1 μm) were cut from resin-embedded specimens, stained with 0.5 % toluidine blue and viewed.

Variation in the number of polyads per stigma, the rate of *in vivo* pollen-tube growth, and the penetration of the ovules were determined using FM. About 100-150 pollinated flowers from each of three hybrids were collected every hour after pollination for 1 d and then 3 to 4 times over the next 2 d. Flowers were fixed immediately in acetic acid: 100% ethanol, 1:2 for 24 hr. Pistils were dissected from flowers and soaked in 8 N NaOH at room temperature for a few days until most tissues became nearly transparent. Pistils were rinsed in water until tissues became transparent then stained with 0.1% decolorized aniline blue in 0.1% K_3PO_4 (Martin, 1959). Pistils were then gently squashed on a slide under coverslips, and polyad germination and pollen-tube growth were observed using fluorescence microscopy.

5.2.5 Statistical analysis

Among-tree variation in pollen quality and the number of polyads, pollen tubes and penetrated ovules per pistil was assessed by analysis of variance (ANOVA) using SAS. All percent data were subjected to arc-sine transformation prior to being analyzed by ANOVA. Duncan's New Multiple Range test at $P < 0.05$ was used to compare means if there were significant differences among these variables (Zar, 1984).

5.3 Results

5.3.1 Pollen quality

Most hybrid polyads germinated *in vitro* within 1 hr, and no polyads germinated after about 6 hr. Polyads showed no significant changes in shape and size following hydration. There were significant differences among trees in fresh polyad germinability, ranging from 30 to 85% (Table 12). Polyads from tree 4 had the highest germinability, whereas trees 1,2 and 3 had moderate and low germinability. The germinated polyads also showed among-tree variation in pollen-tube number, ranging from 1 to 3 in tree 2, 1 to 5 in tree 1 and 3, and 1 to 6 in tree 6 but most polyads from each tree formed only one pollen tube (Table 12). Fluorescence microscopy showed that pollen tubes from within and among polyads stained differently with decolorized aniline blue, indicating variation in callose deposition. No callose plugs were observed in the pollen tubes.

Based on the FCR test, the number of viable fresh polyads varied among trees, ranging from 68 to 92% (Table 13). Polyads from tree 1 and 3 had high viability, whereas those from trees 2 and 4 had moderate viability. Most polyads from all trees contained 13 to 16 viable pollen grains. Polyads containing less than 13 viable grains varied among trees (Table 13). The FCR test also showed a steady decrease in the hybrid polyad longevity for polyads stored for 3 d at room temperature (Fig. 43). Fresh polyads had high viability (over 80%) with different pollen viability classes. This decreased to about 60% within one day and to about two 72 hr after anthesis and all of these polyads contained only 1 to 4 viable pollen grains.

5.3.2 Ultrastructure and histochemistry of the style before pollen tube penetration

The hybrid style is of the solid type and consists of three tissues. The outermost layer is composed of epidermal cells covered by a thick cuticle (Fig. 46). The epidermal cells are densely cytoplasmic and contain many small and large vacuoles and droplets of moderate electron density (Fig. 45). Below the epidermis are 2 to 3 layers of cylindrical, parenchymatous cortical cells compactly arranged along the style axis (Fig. 45). Vascular bundles occur in the cortical tissue. The center of the style consists of transmitting tissue whose cells are elongate similar to the parenchyma but about half their diameter.

At the floral bud stage (a few hours before anthesis), the style is irregularly folded within the bud. The cells of transmitting tissue and parenchymatous tissue are compactly arranged without any intercellular spaces (Figs. 44,45). Transmitting cells have thick cell walls and are connected with each other via plasmodesmata in longitudinal and transverse walls (Fig. 44). The cytoplasm is rich in dictyosomes, endoplasmic reticulum (ER), mitochondria, plastids containing starch, spherical homogeneous moderate electron-dense droplets, and vacuoles containing strongly electron-dense materials (Fig. 44). As the flowers open the style straightens and transmitting tissue undergoes ultrastructural changes. The cytoplasm is still dense and rich in organelles (Figs. 47,48,49) and the number of electron-dense droplets increases and some appear to fuse (Fig. 47).

In fully-opened flowers, the style becomes completely straight. Cells of the transmitting tissue are arranged loosely with small intercellular spaces in the region beneath the stigmatic zone to the ovarian chamber (Fig. 50). The intercellular spaces do not stain for lipids, proteins or insoluble carbohydrates but contain fibrillar material (Fig.

50). Transmitting cells are mostly occupied by enlarging vacuoles containing floculated and electron-dense materials (Fig. 50). Lipid droplets in the cytoplasm fuse with each other, forming larger, irregular droplets (Fig. 51). These did not stain positively for proteins with Amido black or for insoluble carbohydrate with PAS. In unpollinated flowers collected 24 hr after anthesis, transmitting cells are ultrastructurally similar to those of the flowers at anthesis and no secretion is observed in the intercellular spaces.

5.3.3 *In vivo* pollen germination

In pollinated flowers collected at 0700 (about 1 hr after anthesis began), polyads were observed on the stigmatic surface that was covered with copious exudate. When compared to polyads from newly-dehisced anthers, hydrated polyads on the stigmatic surface show no significant changes in shape and size but some pollen grains slightly separate from each other, probably due to hydration of the grains by the stigmatic exudate (Figs. 52,53). Ultrastructural studies show stigmatic exudate in intercellular spaces of the polyad (Fig. 56). Pollen grains germinate within an hour after adhering to the stigmatic surface. Pollen tubes form from the intine, emerge, penetrate the stigmatic surface, and grow between the stigmatic cells (Fig. 56). In some cases, pollen tubes formed from the pollen grains not in contact with the stigmatic surface but they failed to penetrate the stigma (Fig. 54).

For 24 hr following open-pollination, the number of polyads per stigma averaged about 1 and did not differ significantly among trees (Table 14). *In vivo* pollen germination

was high in all trees. Styles having germinated polyads ranged from 86 to 97.2% and differed significantly among trees (Table 14).

5.3.4. Pollen tube growth in the style and penetration of the ovules

Following penetration of the stigmatic surface, pollen tubes grow rapidly through the transmitting tissue and reach the ovarian chamber 6 to 7 hr after pollination. One or more pollen tubes were observed to grow through the transmitting tissue. Some were filled with small and large secretion droplets that appear homogeneous and of moderate electron density (Figs. 57,59). The small droplets were also observed in transverse and longitudinal cell walls of transmitting tissue (Fig. 59). Remnants of tube cytoplasm were observed along the tube length (Figs. 56,58). At this stage, the cells of the transmitting tissue degenerate (Fig. 57). Abundant ER remains in the collapsing cytoplasm but lipid droplets and plastids disappear (Fig. 57).

The rate of pollen-tube growth varies considerably along the style. During the first 2 hr after pollination, the pollen tubes grow at about 16 μm/min and reach about 40% of the style length. From 3 to 5 h after pollination, pollen tube growth rate is reduced to 5 to 9 μm/min and reach about 90% of the style length. When entering the ovarian chamber 6 to 7 hr after pollination (Fig. 66), pollen tubes grow at 1 to 3 μm/min. The first pollen tubes reached the ovarian chamber 6 hr after pollination, with all pollen tubes reaching ovarian chambers within about 7 hr.

Fluorescence microscopy showed variation in callose deposition in the pollen tubes growing in the transmitting tissue. Pollen tubes in the distal one-third of the style had low

callose deposition, whereas tubes in the base of the style had more callose deposition (Figs. 60,61). Callose plugs form in pollen tubes all along the style and their number and position vary among pollen tubes (Figs. 60,64). Pollen-tube abnormalities were observed in some styles. Some pollen tubes fluoresce intensely all along their length (Fig. 64), whereas others have irregular-shaped callose plugs (Fig. 65). Although nearly all pollen tubes reach ovaries, a few pollen tubes are arrested in the upper part of the style. Arrested pollen tubes are commonly characterized by swelling at the tip and callose plugs (Fig. 62) or irregular wall thickening (Fig. 63). The number of arrested pollen tubes per style averaged 0.08 to 0.09 and differ significantly among trees (Table 14). Pollen tubes produced from one polyad can be normal, abnormal, or become arrested in the style.

The mean number of pollen tubes per style 3 d after pollination differ significantly among trees, averaging 3.5, 5.0, and 5.2 in tree 1, 2, and 3, respectively. According to the classes of pollen-tube number per style, styles from trees 2 and 3 had 1 to 4 and 5 to 8 pollen tubes, respectively, whereas most styles in tree 1 had 1 to 4 pollen tubes (Table 14).

Pollen tubes penetrated the ovules 8 to 9 hr after pollination. The hybrid ovary contains about 16 ovules (Table 14) arranged in two rows (Fig. 67). In ovaries having many pollen tubes, the pollen tubes randomly penetrate the ovules. Some penetrate the ovules located near the stylar end or in the middle of the ovary. Some did not penetrate any ovules. In some cases, the ovules in the stylar end were not penetrated by pollen tubes that grew further to penetrate ovules in the peduncular end the ovary (Fig. 67). The mean number of penetrated ovules per ovary ranged from 2 to 3 and differed significantly among trees (Table 14).

Table 12. *In vitro* pollen germination of fresh polyads of the *Acacia* hybrid (mean ± s.e.).

Tree	Percent [1] germinated polyads	Percent germinated polyads with different number of pollen tubes					
		1	2	3	4	5	6
1	55.4 ± 5.32 b [2]	39.4 ± 2.43	9.6 ± 1.33	5.9 ± 2.92	0.5 ± 0.11	0.2 ± 0.04	0
2	39.7 ± 2.15 c	30.6 ± 4.42	7.3 ± 2.07	1.8 ± 0.84	0	0	0
3	30.2 ± 4.56 c	23.8 ± 2.27	5.1 ± 1.18	1.1 ± 0.52	0.1 ± 0.02	0.1 ± 0.02	0
4	84.9 ± 2.37 a	44.8 ± 1.18	17.9 ± 1.79	12.6 ± 2.65	6.4 ± 2.57	2.1 ± 0.43	1.1 ± 0.80

[1] Polyads with one or more pollen tubes.
[2] Means in the column followed by the same letter do not differ significantly at $p < 0.05$.

Table 13. Pollen viability of fresh polyads of the *Acacia* hybrid (mean ± s.e.) based on the fluorochromatic reaction (FCR test).

Tree	Percent [1] viable polyads	Percent polyads with different number of viable pollen grains			
		1-4	5-8	9-12	13-16
1	88.9 ± 4.33 a [2]	4.3 ± 0.53	15.5 ± 2.75	20.3 ± 2.92	48.8 ± 4.11
2	72.5 ± 3.15 b	15.5 ± 2.46	9.8 ± 1.72	14.3 ± 1.26	32.9 ± 1.20
3	91.9 ± 4.32 a	2.1 ± 0.31	18.1 ± 2.72	19.3 ± 2.61	52.4 ± 2.50
4	68.2 ± 2.57 b	13.7 ± 2.28	15.3 ± 3.19	9.4 ± 0.98	29.8 ± 4.23

[1] Polyads with one or more viable pollen grains.
[2] Means in the column followed by the same letter do not differ significantly at $p < 0.05$.

Table 14. Among-tree variation in *in vivo* pollen tube growth and ovule penetration of the *Acacia* hybrid 3 days after anthesis (mean ± s.e.).

Tree	Number of flowers observed	Mean [1] number of ovules per ovary	Mean [1] number of polyads per stigma	Percent styles with germinated polyads	Percent styles having different numbers of pollen tubes			Mean number of pollen tubes per style	Mean number of arrested pollen tubes per style	Mean number of penetrated ovules per ovary
					1-4	5-8	9-12 [3]			
1	115	15.8 ± 0.12	1.1 ± 0.02	86.1 ± 0.59 b [2]	66.6	19.5	0	3.5 ± 0.14 b	0.08 ± 0.02 a	1.99 ± 0.12 b
2	152	15.8 ± 0.10	1.3 ± 0.04	96.1 ± 0.81 a	42.1	48.7	5.3	5.0 ± 0.18 a	0.04 ± 0.02 b	3.09 ± 0.13 a
3	107	15.7 ± 0.12	1.1 ± 0.03	97.2 ± 0.68 a	35.5	49.5	12.2	5.2 ± 0.19 a	0.09 ± 0.04 a	2.96 ± 0.13 a

[1] There was no significant difference among trees at $p < 0.05$

[2] Means in the columns followed by the same letter do not differ significantly at $p < 0.05$.

[3] Quality class of pollen tube number was calculated based on the total number of flowers of each tree.

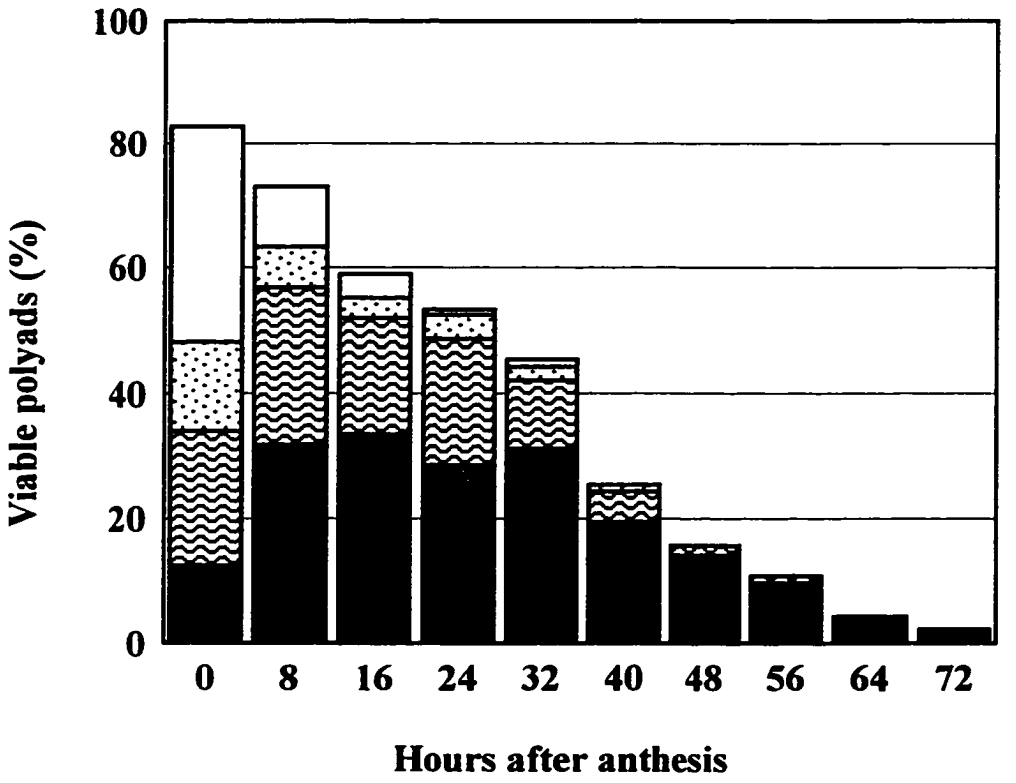

Figure 43. Longevity of the *Acacia* hybrid pollen and polyads using the fluorochromatic reaction (FCR test); (□) polyad with 13-16 viable grains, (⊡) 9-12, (▨) 5-8, (■) 1-4.

Figs. 44-46. Transmission electron micrographs (TEMs) of longitudinal sections of the *Acacia* hybrid styles from a floral bud 1 to 2 hr before anthesis. Fig. 44. The cells of the transmitting tissue showing abundant homogeneous, electron dense droplets (dl) and starch -containing plastids (p), dictyosomes (d), mitochondria (m), and endoplasmic reticulum (er). Electron dense materials (arrows) are observed in the vacuole; cw, cell wall; pm, plasmodesmata. Bar = 3 μm. Fig. 45. The epidermal cells (ep) of the style showing dense cytoplasm containing a number of small and large vacuoles (v) and some moderately electron-dense droplets (*), pa, parenchyma cell. Bar = 5 μm. Fig. 46. The thick cuticle (*) of the style. Bar = 0.5 μm.

Figs. 47-49. TEMs of longitudinal sections of the *Acacia* hybrid style from a partly-opened flower. Fig. 47. Cells of transmitting tissue showing abundant droplets and cytoplasmic organelles including dictyosomes with electron-lucent vesicles (arrowhead). Fusion of the droplets is observed (*). Bar = 0.5 μm. Fig. 48. The cells of transmitting tissue showing mitochondria, smooth (ser) and rough (rer) endoplasmic reticulum lining the cell wall; pl, plasmalemma. Bar = 0.5 μm. Fig. 49. The cells of transmitting tissue showing droplets and starch-containing plastids with several plastoglobules (arrow). Bar = 1 μm.

Fig. 50-51. TEMs of longitudinal sections of the *Acacia* hybrid style from a fully-opened flower. Fig. 50. The transmitting tissue showing the loosely-arranged cells and intercellular spaces (*) containing fibrillar materials. The cells consist of abundant cytoplasmic organelles and large vacuoles (v) containing electron dense materials (arrow). Bar = 3 μm. Fig. 51. The cells of the transmitting tissue showing starch-containing plastids, mitochondria, and fused droplets near the cell wall. Bar = 1 μm.

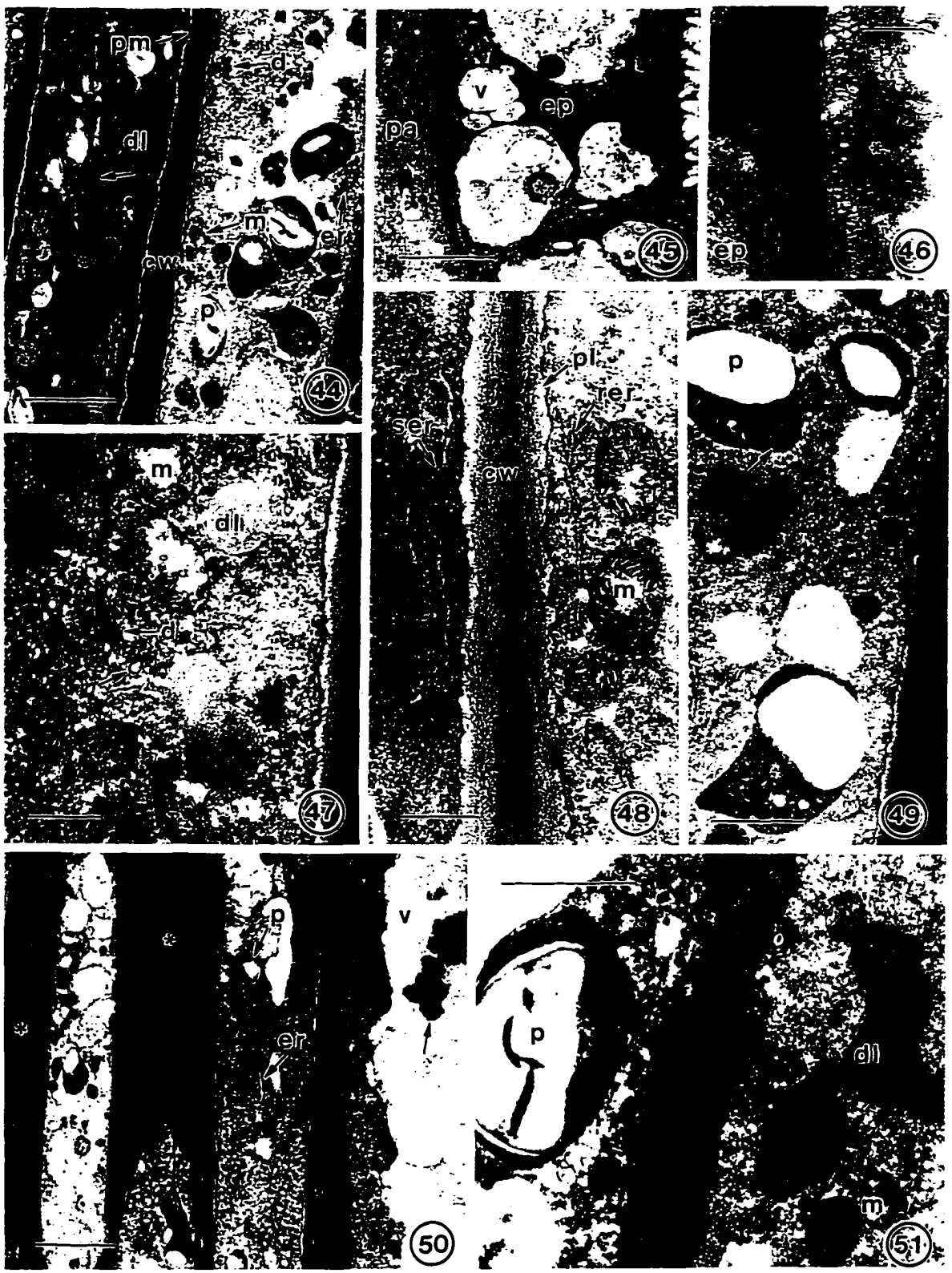
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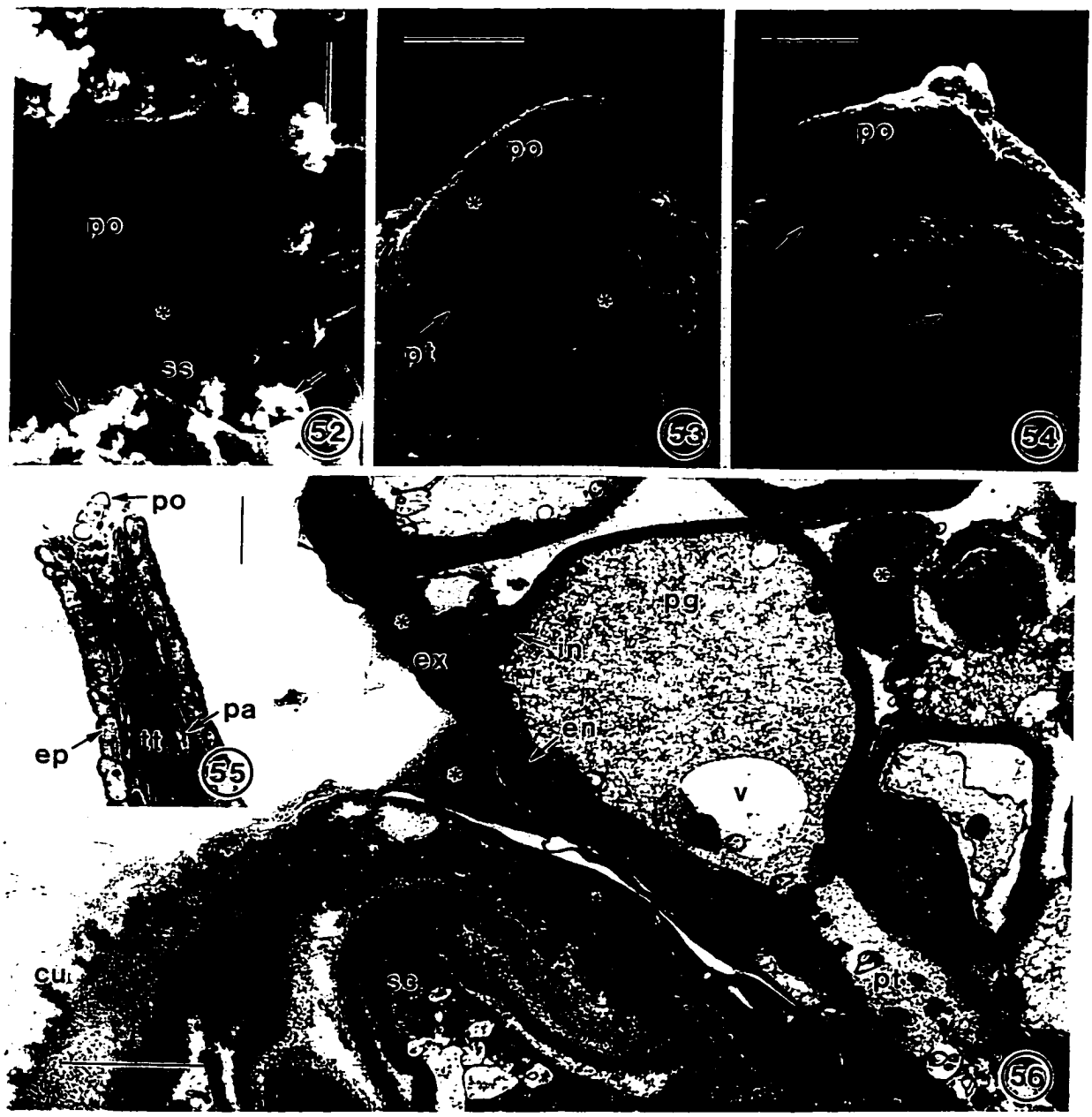
Fig. 52. Scanning electron micrograph (SEM) of a portion of a polyad (po) adhering to the *Acacia* hybrid stigmatic surface (ss) after pollen hydration showing separation of the polyad pollen grains, ruptured cuticle (*) and stigmatic exudate (arrows). Bar = 2 μm.

Fig. 53. SEM of germinated polyads on the *Acacia* hybrid stigmatic surface a few hours following open-pollination showing a pollen tube (pt) penetrating the stigmatic surface and copious exudate (*). Bar = 20 μm.

Fig. 54. SEM of germinated polyads on the *Acacia* hybrid stigmatic surface 24 hr following open-pollination showing some aborted pollen tubes (arrows) that fail to penetrate the stigma surface. Bar = 20 μm.

Fig. 55. Light micrograph of a longitudinal section of the *Acacia* hybrid stigma and style 24 hr following open-pollination showing a germinated polyad; tt, transmitting tissue; pa, parenchyma cells; ep, epidermal cells. Bar = 40 μm.

Fig. 56. Transmission electron micrograph showing a germinated pollen grain (pg) of a polyad on the *Acacia* hybrid stigmatic surface 24 hr following open-pollination. The pollen tube is growing between the stigmatic cells (sc). The stigmatic exudate (*) is observed among pollen grains of the polyad; ex, exine; en, endexine; in, intine; v, vacuole; cu, cuticle. Bar = 3 μm.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Figs. 57-59. Transmission electron micrographs of the transmitting tissue of the *Acacia* hybrid style 24 hr following open-pollination. Fig. 57. Pollen tube growing between the cells of the transmitting tissue (tt), dc, degenerating cytoplasm. Secretion droplets (sd) are observed in the intercellular spaces; cw, cell wall; n, nucleus; pm, plasmodesmata. Bar = 3 μm. Fig. 58. More than one pollen tube occurs in the intercellular spaces and remnants of tube cytoplasm (*). Bar = 1 μm. Fig. 59. Large secretion droplets in the intercellular spaces and small droplets (arrows) in the cell wall. Bar = 1 μm.

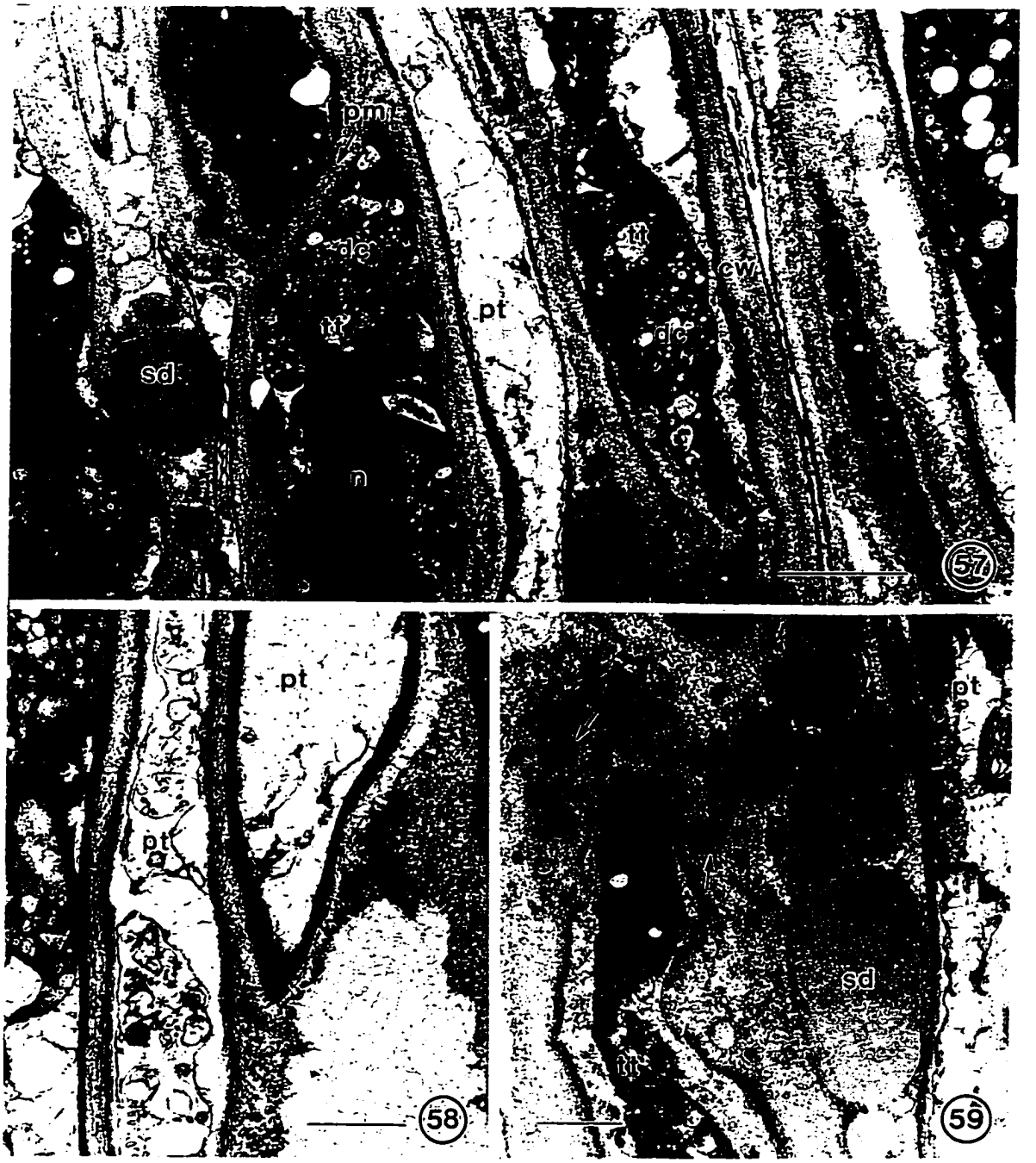
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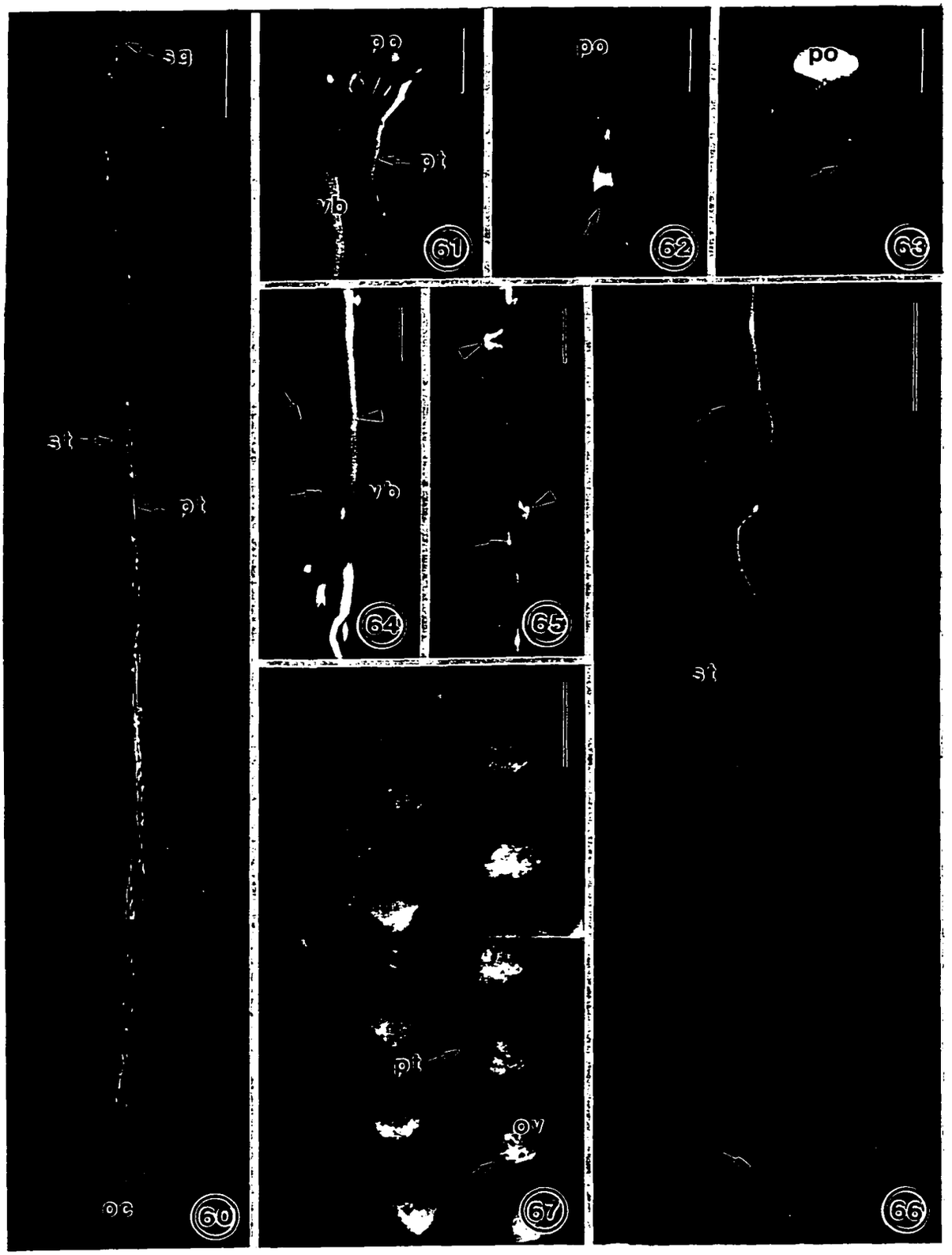
Fig. 60. Fluorescence micrograph (FM) of the *Acacia* hybrid pistil 24 hr following open-pollination, showing pollen-tube (pt) pathway from the stigma (sg) through the style (st) to the ovules (oc). Bar = 200 μm.

Figs. 61-63. FMs showing pollen-tube growth in the upper region of the style of the *Acacia* hybrid 24 hr following open-pollination. Fig. 61. Normal pollen tubes; po, polyad; vb, vascular bundle. Fig. 62. Arrested pollen tube with swollen tip and intensely-stained callose plug (arrow). Fig. 63. Arrested pollen tube with irregular wall thickening (arrow). Bar = 30 μm in Figs. 61-63.

Fig. 64-65. FMs showing pollen tubes in the mid-region of the style of the *Acacia* hybrid 24 hr following open-pollination. Fig. 64. Normal pollen tubes (arrows) and pollen tube with intense fluorescence (arrowhead). Fig. 65. Pollen tubes showing normal (arrow) and irregular callose plugs (arrowheads). Bar = 15 μm in Figs. 64-65.

Fig. 66. FM showing pollen-tube tips (arrows) in the stylar base of the *Acacia* hybrid 6 to 7 hr following open-pollination. Bar = 40 μm.

Fig. 67. FM showing arrangement of the ovules (ov) and pollen tubes contacting the ovules (arrow) in the ovarian chamber of the *Acacia* hybrid 24 hr following open-pollination. Bar = 75 μm.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5.4 Discussion

5.4.1 Pollen quality

Protocols for *in vitro* pollen germination based on Brewbaker and Kwack' s solution have been employed in many species but optimum conditions vary among species (Towill, 1987; Cheng and McComb, 1992; Voyiatzi, 1995; Tangmitcharoen and Owens, 1997). In the hybrid, the results from *in vitro* pollen germination using Brewbaker and Kwack' solution with 10% sucrose were less reliable as they underestimated germination in all trees compared to *in vivo* pollen germination or the FCR test but Sedgley *et al.*(1992a) also reported that germination of fresh *A. anceps* polyads using Brewbaker and Kwack' solution was highly variable among replications. Pollen viability of the parental species and other acacias have been estimated using different methods but the FCR test (Heslop-Harrison *et al.*, 1984) gave the best indication of viability (Sedgley and Harbard, 1993). My results are in agreement with their results.

Viability of *Acacia* polyads vary considerably among species (Newman, 1934a; Kenrick and Knox, 1985; Sedgley and Harbard, 1993). In the hybrid, fresh polyad viability based on FCR test is high, with overall mean of 80%, and is close to the parental species (85% in *A. mangium* and 82% in *A. auriculiformis*) (Sedgley and Harbard, 1993). However, viability is of short duration. Polyads collected at the start of anthesis (0700 h) had the highest viability, with a high number of viable grains, when compared to those estimated from 8 to 72 hr following anthesis. This indicates that failure of pollen germination on the stigma may be due partly to lack of pollen vigor or viability if flowers

are pollinated several hours after anthesis. The study also indicates that only fresh polyads should be used for controlled pollination. Sedgley and Harbard (1993) reported that polyads of some acacias can be stored up to 1 year and retain the ability to germinate but the storage methods vary among species. This has not been determined for the hybrid polyads.

5.4.2 Structure and secretion of the style

The style of the hybrid is of the solid type and has structure and morphology similar to that of many acacias, including the parental species (Kenrick and Knox, 1981b; Knox *et al.*, 1989; Ibrahim, 1991; Baranelli *et al.*, 1995). At anthesis, the style is straight, long, and narrow. The central transmitting tissue is surrounded by a few layers of cortical parenchyma cells and the outer layer consists of epidermal cells covered by a thick cuticle. The hybrid transmitting tissue undergoes structural changes during flower opening. The cells of the transmitting tissue are compactly arranged during the floral bud stage but become loosely arranged, forming conspicuous intercellular spaces in the style of the fully-opened flower. This may facilitate pollen-tube penetration (Knox, 1984) and has been observed in solid styles of many species, including acacias (Kenrick and Knox, 1981b; Ghosh and Shivanna, 1984; Tilton *et al.*, 1984b; Ciampolini *et al.*, 1996)

Stylar secretions have been observed in many species having either solid or hollow styles (Kenrick and Knox, 1981b; Kuruvilla and Shah, 1988; Janson *et al.*, 1994; Weber, 1994, Tangmitcharoen and Owens, 1997). In solid styles the exudate is usually observed in the intercellular spaces, whereas in hollow styles the exudate is present in the stylar

canal (Knox, 1984). Several studies also show that stylar secretions mostly occur at or before anthesis and play an important role in supporting pollen-tube growth in species such as avocado (Sedgley, 1979), petunia (Herrero and Dickinson, 1979), acacias (Kenrick and Knox, 1981b), lily (Janson *et al.*, 1994) and kiwifruit (Gonzalez *et al.*, 1996). Timing of hybrid style secretion is different from the species described above. Histochemical studies show that, in mature styles of unpollinated and pollinated hybrid flowers, intercellular spaces do not stain for proteins but prominent secretion droplets are observed in some intercellular spaces after pollen-tube growth. In *Persea*, *Prunus*, and other acacias, degeneration of stigmatic and transmitting cells, that are rich in cytoplasmic products such as starch or lipids, occurs after pollination but before pollen tube passage (Vasil and Johri, 1964; Sedgley, 1979b; Uwate and Lin, 1981; Jobson *et al.*, 1983). It is suggested that, during their degeneration, the cytoplasmic products are secreted into intercellular spaces, providing nourishment for pollen-tube growth (Sedgley, 1979b; Kenrick and Knox, 1989a). The hybrid style has a secretion process similar to these species - degeneration of the transmitting cells and appearance of an intercellular matrix only after pollination. Also, the disappearance of the secretion in some intercellular spaces after pollen-tube growth may be due to the fact that it has been used for pollen-tube nutrition but some may remain, as observed in *Actinidia* (Gonzalez *et al.*, 1996). Stylar secretion, triggered by pollination, is also reported in species such as *Citrullus* (Sedgley and Scholefield, 1980; Sedgley, 1982), *Prunus* (Herrero and Gascon, 1987) and *Pyrus* (Uwate *et al.*, 1982).

The major components of the stigmatic and stylar secretion are proteins, carbohydrates, lipids or pectins but this varies among species (Kenrick and Knox, 1981b;

Knox, 1984; Weber, 1994; Ciampolini *et al.*, 1995, 1996). Histochemical and ultrastructural studies of the hybrid style suggest that lipid droplets and plastids containing starch grains are abundant in the cytoplasm of the transmitting cells at all developmental stages. However, these disappear as transmitting cells degenerate after pollen-tube growth, suggesting that lipids and carbohydrates are likely the major components of the stylar secretion. Ultrastructural studies also show evidence of lipid secretion into the intercellular spaces. Before pollination, abundant lipid droplets accumulate in periplasmic regions. Vacuoles enlarge and contents are secreted into intercellular spaces by penetrating through the cell walls, as indicated by the presence of small lipids droplets in the cell walls of the transmitting cells. Synthesis of lipid droplets in the cytoplasm may be associated with ER and dictyosomes, as these organelles are abundant and involved in these secretory activities (Mauseth, 1991). At the ultrastructural level, the intercellular spaces contain electron dense fibrillar materials which do not stain for carbohydrates, proteins or lipids. TEM studies of the stylar secretion in *Smyrnium* and *Vitis* revealed that similar fibrillar material consists of pectins (Weber, 1994; Ciampolini *et al.*, 1996). These pectins are thought to be disintegrated middle lamellae which form during cell separation and intercellular space formation (Ciampolini *et al.*, 1996).

In some species, such as *Vitis* (Considine and Knox, 1979; Ciampolini, 1996) and *Trifolium* (Heslop-Harrison and Heslop-Harrison, 1982), the style and stigma possess similar histochemical secretions and cytoplasmic organelles. In other species, such as *Lilium*, *Lycopersicum* or *Tibouchina*, stylar secretion is different from that of the stigma (Dickinson *et al.*, 1982; Kadej *et al.*, 1985; Ciampolini, *et al.*, 1995). In the hybrid, the stigmatic secretion in the hybrid is lipophilic type and peak receptivity occurs in a partly-

opened flower before style straightening and pollination (Chapter 4). The present studies suggest that the stigmatic and stylar secretions in the hybrid are different not only in components but also in the time that they occur. The stigmatic secretion appears to be independent of pollination, whereas the stylar secretion requires pollination.

5.4.3 *In vivo* pollen germination and pollen-tube growth

In angiosperms, pollen germination on the stigma involves complex pollen-stigma interactions (Dumas *et al.*, 1984). In the hybrid and other acacias, the number of pollen grains which contact the stigmatic surface depends on how the polyad lands on the surface (Kenrick and Knox, 1982). The post-pollination exudation on the stigma, therefore, may be a compensating mechanism to ensure the germination of all polyad grains, as observed in *A. subulata* or *A. iteaphylla* (Kenrick and Knox, 1981a; Marginson *et al.*, 1985). Post-pollination exudation was not observed in the hybrid. TEM and SEM studies show that the hybrid stigma produces copious exudate before anthesis (Chapter 4). After polyads land on the stigmatic surface, the quantity of this exudate is large enough to envelope the entire polyad and some is absorbed into the polyad through slits between polyad pollen grains during hydration, as was also observed in *A. baileyana* (Kenrick and Knox, 1989a). This may ensure the germination of more polyad grains, as shown by the germination of some hybrid polyad grains that did not contact the stigmatic surface.

In acacias, *in vivo* pollen germination occurs rapidly, within a few hours after pollination (Kenrick and Knox, 1989a). Degenerated stigmatic cells observed before pollen-tube passage are common in the hybrid and other acacias (Sedgley, 1979; Kenrick

and Knox, 1989a). Kenrick and Knox (1989a) suggested that degeneration of stigmatic cells occurs before pollen-tube penetration and that cytoplasmic products are secreted into intercellular spaces, nourishing early pollen tubes in the stigmatic zone. In the hybrid, pollen tubes produced from grains not in contact with the stigmatic surface aborted when they fail to penetrate the stigmatic surface. This may be due to the fact that the stigmatic exudate at anthesis assists in pollen capture and the stigmatic secretion triggered by pollination provides essential nutrients required for pollen germination and further tube growth (Sedgley and Griffin, 1989).

In angiosperms, pollen tubes grow through the style to the ovules in hours or days (Kuruvilla and Shah 1988; Sedgley and Smith, 1989; Fuss and Sedgley, 1991; O'Brien, 1994; Tangmitcharoen and Owens, 1997). Mulcahy and Mulcahy (1982, 1983), and Janson *et al.* (1994) suggest that, in bicellular pollen, such as *Petunia* and *Lilium*, pollen tubes undergo a two-phase growth in the style, as determined by speed and the appearance of callose plugs. In phase I, pollen-tube growth is slow and no callose plugs are observed (autotrophic), whereas in phase II, pollen tubes grow fast and contain callose plugs (heterotrophic). Biphasic growth of the pollen tubes is also observed in other acacias and the hybrid but tube growth rate and callose plug formation are different from *Petunia* or *Lilium*. In *A. retinodes*, pollen tubes from self- and cross-pollination reached the ovules within 11 hr and tube growth rate was 3.3 to 4.5 μm/min for first 6 hr and less than half that rate for the next 5 hr (Kenrick and Knox, 1985; Kenrick *et al.*, 1986). In the hybrid, although the style length (2.7 mm) is about twice the length of the *A. retinodes* style (1.5 mm), the pollen tubes may reach the ovules within 8 hr because growth rate during both phases is much higher than *A. retinodes*. In the hybrid, phase I of pollen-tube growth

takes about 2 hr and differs from phase II in which tube growth is about twice as fast and tube walls fluoresce faintly.

The hybrid shows timing and major components of the stigmatic and style secretion different from those of *A. retinodes* (Kenrick and Knox, 1981b; Knox *et al.*, 1989). This may affect speed of the pollen-tube growth between *A. retinodes* and the hybrid. The stylar secretion gradient, in which the upper transmitting tissue contains larger amounts of exudate than the lower parts is observed in some species such as *Glycine* (Tilton *et al.*, 1984b). This phenomenon also may exist in the hybrid style. The large amount, as well as optimal component and/or concentration in the upper style, may account for rapid pollen-tube growth in phase I. This may also be true for *in vitro* pollen germination. Pollen tube lengths from *in vitro* pollen germination are much less than *in vivo* because Brewbaker's solution may not provide some substances essential for further growth of the pollen tubes (Read *et al.*, 1992).

Gametophytic self-incompatibility (SI) is evident in many acacias (Bernhardt *et al.*, 1984; Kenrick and Knox, 1985; Kenrick *et al.*, 1986; also see review by Kenrick and Knox, 1989a) including the parental species (Ibrahim, 1991; Sedgley *et al.*, 1992b). Cytological studies of *in vivo* pollen-tube growth in *A. retinodes* revealed that pollen tubes from self- and cross-pollination have similar growth in the style but tubes from self-pollination are arrested in the nucellus (Kenrick and Knox, 1985; Kenrick *et al.*, 1986). In other gametophytic SI patterns, self pollen tubes are arrested in the style and are commonly characterized by abortion or irregular growth such as swelling or twisting of the tube tip or wall thickening (Kahn and DeMason, 1986; Hessing, 1989; Ellis *et al.*, 1991; Scribailo and Barrett; 1991). Knox and Kenrick (1983) pointed out that in a 16-

grain polyad system such as acacias, a polyad originates from a single sporogeneous cell which undergoes mitotic division to form four pollen mother cells. These then undergo meiosis. A polyad, therefore, can be incompatible, half-compatible or fully compatible based on S-allele constitution (Knox and Kenrick, 1983). Hybrid polyads possess characteristics similar to those of other acacias. This suggests that pollen tubes arrested in the upper style following open-pollination are likely from the incompatible pollen grains of a polyad.

Several studies show that excess pollen per stigma is required to ensure good seed set and pollen and pollen-tube competition increases offspring quality (Mulcahy and Mulcahy, 1987; Stephenson and Bertin , 1983; Snow, 1990; Spira *et. al.*, 1992; Holm 1994). The pollen number on a stigma shows a positive correlation with pollen germinability and pollen-tube growth rate (Lee, 1980; Mulcahy and Mulcahy, 1987). Pollen-tube competition occurs by fast-growing pollen tubes, probably having superior genotypes, having better opportunities for the limited nutrients available in the maternal tissue (transmitting tissue). Thus, they are more likely to fertilize ovules (Tilton *et al.*, 1984b; Mulcahy and Mulcahy, 1987). In the hybrid, following open-pollination, the pollen to ovule ratio averaged 1 (Chapter 3) and the pollen tube number per style (range from 3.5 to 5.2 with maximum range of 9 to 12) appears less than the ovule number (16). In some cases when the pollinated flower receives more than one polyad, the pollen tube number is still less than the ovule number. A similar phenomenon is also observed in the parental species (Sedgley *et al.*, 1992b). This suggests that pollen and pollen-tube competition may not be evident in the hybrid or in other acacias.

Pollen-tube penetration of the ovule in the hybrid occurs within a few hours after pollen tubes enter the ovarian chamber, whereas, in some species, such as avocado, almond or pear, it may be delayed hours to days due to immaturity of the ovary or delay in the ovular secretion required for pollen-tube growth (Sedgley, 1979a; Pimienta *et al.*, 1983; Arbeloa and Herrero, 1987). Therefore, the rapid pollen-tube penetration of ovules in the hybrid may be due partly to the fact that the ovules are already mature at anthesis (Chapter 4). In each pollinated flower, nearly all pollen tubes except arrested tubes entered the ovarian chamber but only about half randomly penetrated the ovules. Low percentages of penetrated ovules per flower are also observed in the parental species (Sedgley *et al.*, 1992b). The causes for failure in ovule penetration and variation in position of fertilized ovules in the hybrid and other acacias are unclear. Some studies show that pollen tubes preferentially penetrate ovules that contain micropylar exudate as reported in *Beta* or *Gasteria* (Oleson and Bruun, 1990; Franssen- Verheijen and Willemse, 1993). My study, however, shows that patterns of pollen germination and behavior during pollen-tube growth can affect seed set in the hybrid.

CHAPTER 6

Ultrastructure and Histochemistry of the Ovule, Fertilization and Formation of the Zygote in a Tropical *Acacia* hybrid (*A. mangium* Willd. x *A. auriculiformis* A. Cunn. ex Benth.)

6.1 Introduction

The hybrid between *Acacia mangium* and *A. auriculiformis* (Leguminosae: Mimosoideae) is a fast growing tree. *Acacia mangium* is native to northern Queensland, western Papua New Guinea, and Irian Jaya and Maluku of Indonesia (Doran and Skelton, 1982), whereas *A. auriculiformis* is native to Queensland, western and southern Papua New Guinea, and Irian Jaya and the Kei Islands of Indonesia (Turnbull *et al.*, 1986). They have been widely introduced to many Southeast Asian countries for reforestation programs (Darmono and Dayanto, 1981; Chittachumnok and Sirilak, 1991; Pinyopusarerk *et al.*, 1993). In Thailand, the hybrid shows great potential in adaptability and growth (Kijkar, 1992). Preliminary studies show that fruit and seed set in the hybrid are partially limited during pre-fertilization due to insufficient pollination and low numbers of penetrated ovules (Chapter 4, 5). At post-pollination, many pollen tubes are observed in the ovary but only about half appear to penetrate the ovules. Causes for non-penetration of ovules are not well understood. No detailed studies of fertilization have been carried out in the hybrid.

Acacia consists of approximately 1,200 species, widely distributed in the tropical and subtropical regions. Many aspects of reproductive biology have been extensively studied in *Acacia*, such as floral biology (Kenrick and Knox, 1981b, 1982; Knox *et al.*, 1989, Baranelli *et al.*, 1995), pollination (Bernhardt *et al.*, 1984, Sedgley *et al.*, 1992c, Tybirk, 1993), and post-pollination events (Kenrick and Knox, 1981a, 1985; Sedgley *et al.*, 1992b). Little work has concentrated on fertilization. In some acacias, such as *A. retinodes*, the embryo sac before and after pollen tube penetration has been investigated using light microscopy (Kenrick and Knox, 1986) but there were no ultrastructural observations. Fertilization in angiosperms including patterns of synergid degeneration, gametic delivery and fusion or cytoplasmic inheritance, has been most extensively studied in herbaceous plants (Yan *et al.*, 1991; Janson and Willemse, 1995; also see review by Huang and Russell, 1992; Russell, 1992; Mogensen, 1996) with few studies of tree species (see review by Sedgley and Griffin, 1989).

Because flowers of most acacias and the hybrid are very small and grouped into inflorescences, detailed studies of fertilization events are difficult to obtain. The present study addresses many aspects of fertilization in the hybrid using light microscopy, histochemistry and electron microscopy. I describe the: 1) ultrastructure and histochemistry of the ovule before pollen tube penetration; 2) ultrastructural changes of the cells of the embryo sac after pollen tube penetration; 3) patterns of gametic delivery and fusion, and cytoplasmic inheritance; and, 4) an ultrastructural comparison between the egg and zygote.

6.2 Materials and methods

6.2.1 Study site and plant materials

Hybrids were grown in an experimental plot at the ASEAN Forest Tree Seed Centre, Muak-Lek, Saraburi, Thailand (latitude N 14° 40′, longitude E 101° 17′, and about 200 m elevation). They produce abundant flowers over several months (June-October). Flowers usually open at 0500-0600 h and last 3-4 days. Floral buds were tagged one day before anthesis and those that opened the following day were allowed to be open-pollinated between 0600-0700 h. Pollinated flowers were enclosed in cellophane bags to ensure a synchronous pollination period. Collections of flowers were made at anthesis and every 6 to 8 h for 1 w after open-pollination. Pistils were dissected and fixed in 2.5% glutaraldehyde in 0.075 M phosphate buffer (pH 7.2 to 7.4) for light microscopy (LM), transmission electron microscopy (TEM) and histochemistry.

6.2.2 Electron microscopy

Specimens were rinsed in 0.075 M phosphate buffer (pH 7.2 to 7.4) r and postfixed for 1 h in 1% osmium tetroxide in 0.075 M phosphate buffer (pH 7.2 to 7.4). Specimens were then dehydrated in a series of acetone solutions, embedded in Spurr's resin (Spurr, 1969) and sectioned at 60 nm using an ultramicrotome. Ultrathin sections were collected on single hole grids coated with 0.3 % Formvar in chloroform, then stained

with 2% aqueous uranyl acetate for 15 min and 0.2% Sato's lead citrate (Sato, 1968) for 15 min and viewed using a Hitachi 7000 electron microscope at 75 kV.

6.2.3 Light microscopy and histochemistry

Pistils from flowers were used for LM and histochemistry. Specimens were dehydrated and embedded in Spurr's resin as described above. Semithin sections (about 1 μm) were cut and stained with 0.5 % toluidine blue in 0.1% sodium carbonate (pH 11.1) for general observation (Trump *et al.*, 1961), periodic acid-Schiff's reagent (PAS) for insoluble carbohydrates (Jensen, 1962), Amido black for proteins (Jensen, 1962) and Sudan black B for lipids (Bronner, 1975).

6.3 Results

6.3.1 Ultrastructure and histochemistry of the ovule at anthesis

The mature pistil has a long, narrow style and a hairy ovary. The ovary has one locule, containing about 15 ovules. Many trichomes are observed in the ovarian chamber (Fig. 68). They are long, slender and attached to the inner ovary wall opposite the placenta. At anthesis, the ovarian chamber is filled with secretion but during specimen preparation for SEM this appears to be washed away. It usually spreads over the region where trichomes are present and covers all the ovules. This secretion contains a granular

material (Fig. 69) and stains intensely with toluidine blue (Fig. 71) and faintly with PAS and Amido black for insoluble carbohydrates and proteins, respectively (Figs. 72,73).

The mature ovule lacks a distinct micropyle due to the partial development of both integuments, exposing the nucellus. The nucellus is composed of a few cell layers that contain many plastids with starch grains and lipid bodies that stain positively by PAS and Sudan black B, respectively (Figs. 72,74). These stored products are not observed in the integuments and the chalazal regions of the ovules. The cells of the micropylar nucellus form a straight pathway between cells toward the egg apparatus (Fig. 69). Nucellar cells contain large vacuoles and a few cytoplasmic organelles. At anthesis, no channel or degeneration of the micropylar nucellar cells is observed. No ovular exudate is observed but a portion of the ovarian secretion comes in contact with the micropylar nucellus (Fig. 69). At anthesis, a few layers of crushed nucellar cells are observed adjacent to the embryo sac in the region of the central cell (Fig. 70). Moderately electron-dense materials observed in the crushed nucellar cells stain positively with Sudan black B for lipids (Fig. 74). The strongly electron-dense material is likely the collapsing nucellus and stains positively with Amido black for proteins (Fig. 73).

The egg apparatus consists of the egg and two synergids. All three cells are polarized, rich in cytoplasmic organelles (Figs. 75,76) and separated from each other by thin cell walls lacking plasmodesmata (Fig. 77). The egg cell is large and attaches to the embryo sac wall at the micropylar region. It contains a large micropylar vacuole that pushes most cytoplasm into the chalazal region (Fig. 76). Its dense cytoplasm contains abundant mitochondria without well-developed cristae, plastids containing large starch grains and ribosomes but no ER or dictyosomes are observed (Fig. 78). A few lipid

bodies are observed (Fig. 76). The egg nucleus is located chalazally and there is a large micropylar vacuole (Fig. 76). The nucleus contains a single prominent nucleolus and the chromatin is evenly distributed (Fig. 78).

Both synergids appear identical and their walls are attached to the micropylar embryo sac and egg-cell wall (Figs. 75, 76). In contrast to the egg cell, the synergid contains a large chalazal vacuole and a few small plastids, some with small starch grains (Fig. 76). A few dictyosomes are observed in the cytoplasm but many mitochondria are present, particularly in the micropylar region of the cell near the filiform apparatus (FA) (Fig. 79). A nucleus with a single nucleolus is usually located centrally at the micropylar end of the large vacuole (Figs. 75,76). A prominent, irregular FA occupies the micropylar region of each synergid (Fig. 79). Ultrastructurally, the FA is continuous with the micropylar embryo sac wall and shows finger-like projections into the cytoplasm and contains a microfibrillar matrix (Fig. 79). The FA stains intensely with toluidine blue (Fig. 71) and slightly with PAS and Amido black (Figs. 72,73) but does not react with Sudan black B.

The central cell is the largest cell in the embryo sac. It is separated from the egg apparatus by a prominent gap which contains electron-lucent bodies (Figs. 76, 81). Wall ingrowths occur along the embryo sac wall (Fig. 80). The cell is mostly occupied by a large vacuole, pushing the cytoplasm peripherally. Compared to the egg cell, the central cell appears to be more active metabolically as evidenced by abundant cytoplasmic organelles such as mitochondria, dictyosomes and ER (Figs. 80, 81). Abundant plastids with large starch grains and lipid bodies are present throughout the cell (Figs. 72,74). Two prominent polar nuclei are located in the micropylar region adjacent to the egg cell

(Fig. 80). At anthesis, the polar nuclei are closely paired but do not fuse. Each polar nucleus has a double nuclear membrane and a single prominent nucleolus containing a micronucleolus (Figs. 80,81).

Three irregular-shaped antipodals are located in the chalazal region of the embryo sac (Fig. 82). They are arranged in one plane perpendicular to the embryo sac axis. Each cell contains large and small vacuoles, and dense cytoplasm rich in organelles such as mitochondria, ER, and plastids with starch grains. The nucleus with a single nucleolus is located adjacent to the large vacuole (Fig. 82).

6.3.2 The ovule during and after pollen tube penetration

Pollen tubes penetrate the micropylar nucellus about 12 h after pollination. Only one pollen tube grows between the nucellar cells and enters the embryo sac (Fig. 84). No degeneration of the micropylar nucellar cells is observed after pollen tube penetration. In some ovules, the pollen tube is arrested when it reaches the embryo sac and is characterized by wall thickening at tip (Fig. 86). Degeneration of one of the two synergids does not occur until penetration of the pollen tube into the nucellus (Fig. 83). During degeneration, the synergid cell wall increases in thickness and becomes more electron transparent, the large micropylar vacuole present at anthesis disappears and the cytoplasm and nucleus collapse, resulting in an increase in electron density. Cytoplasmic organelles become disorganized but are still noticeable. After complete degeneration, the cytoplasm is highly electron dense, and cell walls and cytoplasmic organelles can not be identified (Figs. 83, 87). The osmiophilic material observed adjacent to the chalazal

region of the persistent synergid (Fig. 84) may be the degenerate cytoplasm extruded from the degenerate synergid. No ultrastructural changes occur in the persistent synergid as the other synergid degenerates. In all ovaries containing pollen tubes, the degenerate synergid is observed only in fertilized ovules, whereas, in the unfertilized ovules, both synergids remain intact. In about 20 fertilized ovules observed, either synergid degenerates with about equal frequency.

The pollen tube penetrates and discharges its contents into the degenerating synergid via the FA (Fig. 84). The portion of the pollen tube within the degenerate synergid remains intact and contains remnants of the tube cytoplasm with ER and many mitochondria lining the tube wall (Fig. 85). In a few ovules, the pollen-tube tip branched and penetrated both the degenerate and persistent synergid (Fig. 87). In fertilized embryo sacs, no cytoplasmic bodies from sperm cells were observed in the degenerate synergid except two strongly electron-dense bodies (Fig. 87). From serial sections, the body in the micropylar region appears larger than the other one. Following pollen-tube penetration of the embryo sac, integuments, nucellus, egg cell, and antipodals show no ultrastructural changes. The polar nuclei remain unfused (Fig. 84).

6.3.3 Gametic fusion

By 3 days after open-pollination (DAP), one sperm nucleus migrates toward the egg cell nucleus and the other lies between the polar nuclei (Figs. 88, 89). Sperm nuclei appear identical. They are disc-shaped and have condensed electron dense chromatin when compared to the large nuclei of the egg and central cell (Figs. 88, 89). No nucleoli

are observed in the sperm nuclei. Following plasmogamy, the fertilized egg cell shows some ultrastructural changes, including slight shrinkage of the cell, formation of wall invagination in the chalazal region, and an increase in the number of plastids with starch grains. The central cell remains unchanged and the polar nuclei are unfused.

Fusion of polar nuclei with a sperm nucleus occurs shortly before fusion of the egg and the sperm nucleus. The sperm nucleus migrates toward the micropylar end of one of the polar nuclei (Fig. 90), then aligns between the two polar nuclei, where their concave surfaces come together (Figs. 91,92). Polar nuclear fusion starts with the linkage of the nuclear envelopes followed by fusion of nucleoplasm through these nuclear bridges (Figs. 93, 95). Abundant ER is observed in the fusion site (Figs. 92, 95). The sperm nucleus contacts both polar nuclei and complete fusion among polar nuclei and sperm nucleus occurs, resulting in the primary endosperm nucleus (Fig. 96). The fusion between the egg and sperm nucleus was not observed in this study. However, when the sperm nucleus aligns adjacent to the egg nucleus, there is no ER observed in the fusion site (Fig. 89).

In some fertilized ovules 4 DAP, more than two electron dense bodies were observed in the same degenerate synergid (Fig. 98) after pollen-tube penetration (Fig. 99). In serial sections, the two bodies near the FA are the same size and smaller than the other electron dense bodies. Also, in the same sections no sperm nucleus was observed in the egg cell that remains intact. Thus, these two bodies are interpreted as aborted sperm nuclei, whereas the large body is likely a degenerate synergid nucleus. A similar phenomenon also occurs in the central cell in which the polar nuclei may abort before the arrival of the sperm nucleus (Fig. 100). The aborted polar nuclei collapse and their nucleoplasms appear very electron dense.

6.3.4 Formation of the endosperm and zygote

The newly formed primary endosperm nucleus is observed about 4 DAP and moves toward the micropylar region of the embryo sac (Fig. 96). When compared to the polar nuclei, the primary endosperm nucleus is large, double-membrane bound, irregular-shaped and contains two prominent nucleoli, probably from each of the polar nuclei. At later stages, the primary endosperm nucleus becomes more spherical with a single nucleolus and are surrounded by abundant starch grains (Fig. 97).

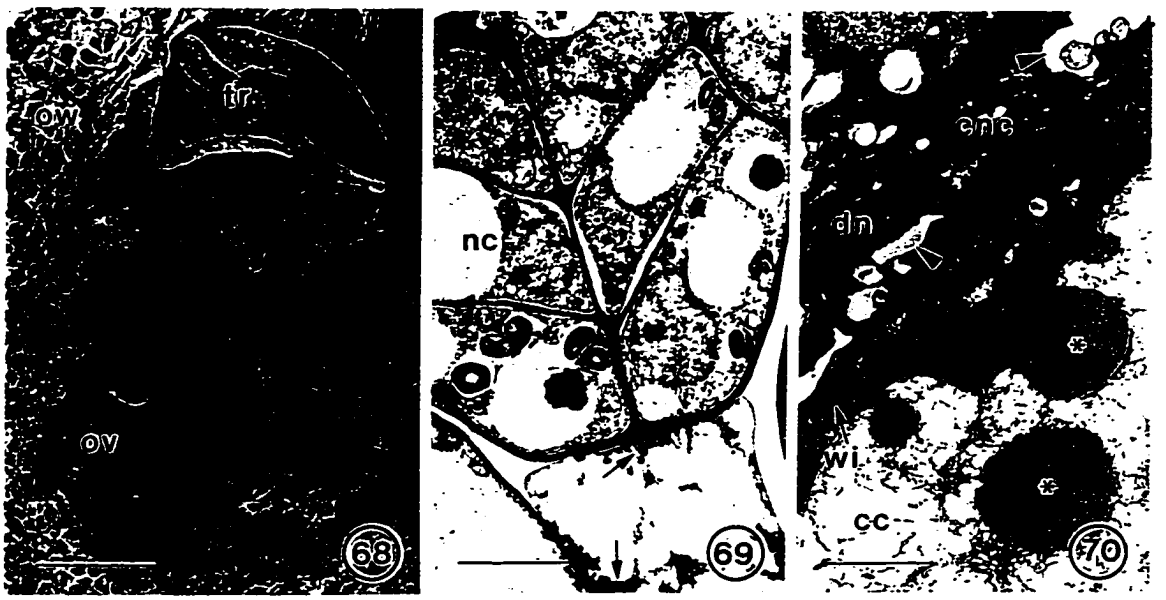
The zygote is observed about 5 DAP (Fig. 103) and degeneration of the antipodals and the persistent synergid has begun (Fig. 101). The zygote is still attached to the micropylar wall of the embryo sac and is separated from the degenerate synergid by a thin wall (Fig. 102). The zygote remains in direct contact with the central cell cytoplasm as the micropylar wall of the central cell disappears after fertilization (Fig. 103). The zygote is reduced in size with thin cell walls (Fig. 103). Wall invaginations observed in the egg cell after plasmogamy become more pronounced in the chalazal part of the zygote (Fig. 103) The zygote contains a large micropylar vacuole and many small vacuoles which are distributed throughout the cell. Throughout the dense cytoplasm are many plastids with large starch grains and mitochondria without well-defined cristae. ER and dictyosomes, not observed in the egg cell, are present in small numbers in the micropylar region of the zygote (Fig. 102).

Fig. 68. Scanning electron micrograph of the *Acacia* hybrid ovary at anthesis showing abundant trichomes (tr) in the ovarian chamber, ov, ovule; ow, ovary wall. Bar = 75 μm.

Fig. 69. Transmission electron micrograph (TEM) of the *Acacia* hybrid ovule at anthesis showing arrangement of the micropylar nucellar cells (nc). A portion of the secretion containing granular material (arrows) is observed adjacent to the nucellus. Bar = 3 μm.

Fig. 70. TEMs of the *Acacia* hybrid ovule at anthesis showing crushed nucellar cells (cnc) adjacent to the embryo sac wall. Degenerating nuclei (dn) appear electron-dense, whereas spherical (*) and irregular (arrowheads) bodies contain moderately electron-dense materials, cc, central cell; wi, wall ingrowths. Bar = 4 μm.

Figs. 71-74. Histochemical stains of the *Acacia* hybrid ovary at anthesis. Fig. 71. Section stained with toluidine blue showing reserve accumulations (arrow) in the central cell. Note the ovarian chamber is filled with secretion (*) which stains intensely, sy, synergid; pn, polar nuclei; fa, filiform apparatus. Fig. 72. Section stained with PAS. Plastids containing starch grains (arrows) in the central cell and nucellus stained intensely. Fig. 73. Degenerate nuclei (arrows) of the crushed nucellar cells, the filiform apparatus (arrowhead) and the ovarian secretion (*) stained with Amido Black for proteins. Fig. 74. Section stained with Sudan Black B showing lipid bodies (arrows) in the central cell and nucellus. Bar = 30 μm in Figs. 71-74.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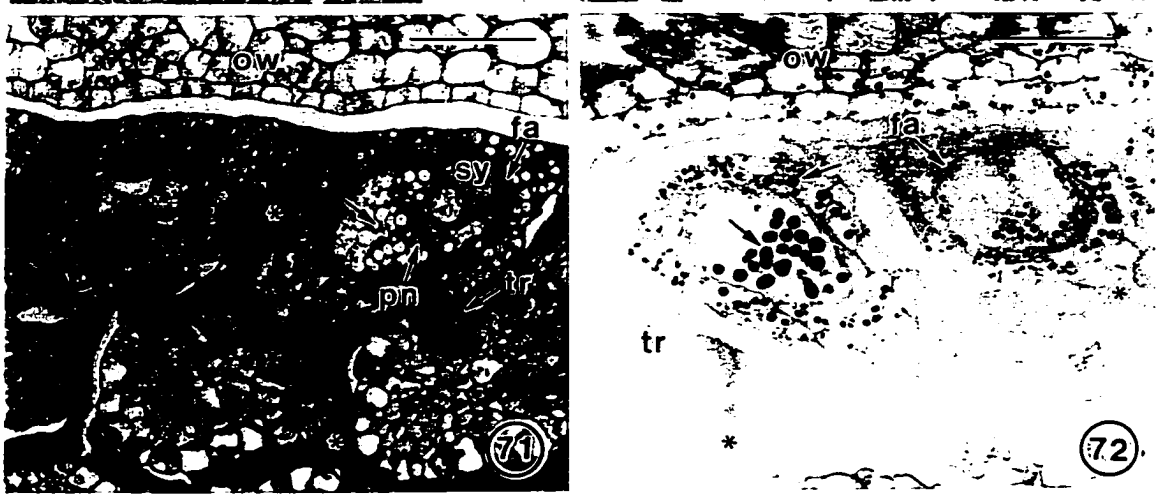
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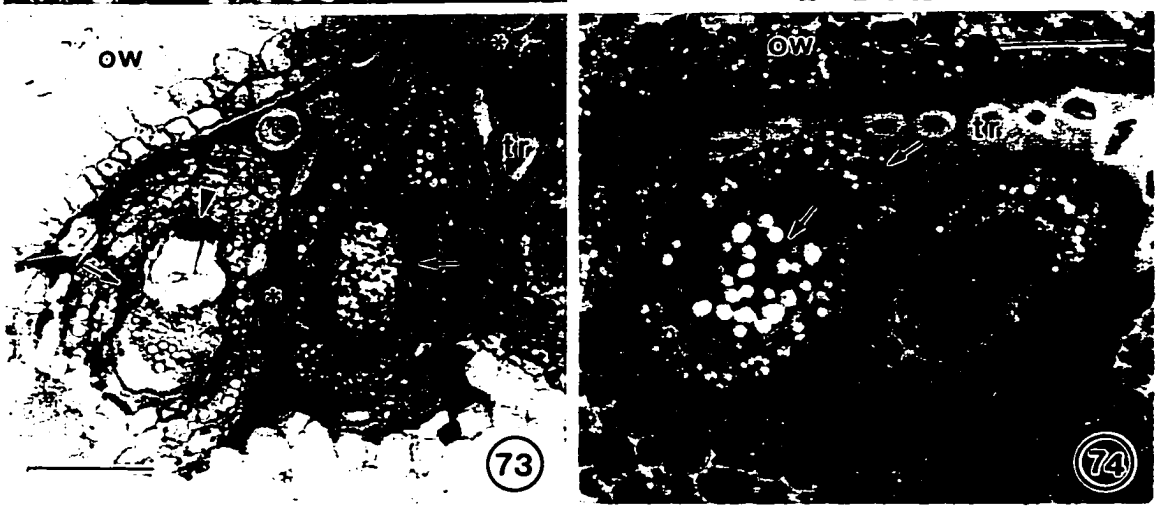
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Figs. 75-79. Transmission electron micrographs (TEMs) of the egg apparatus of the *Acacia* hybrid embryo sac at anthesis. Fig. 75. The egg apparatus showing the egg (eg) and two synergids (sy); n, nucleus; v, vacuole; fa, filiform apparatus; cc, the central cell. Bar = 5 μm. Fig. 76. Side view of the egg apparatus showing the egg, one synergid, and gap (arrows) between the egg apparatus and central cell, pn, polar nuclei, p, plastids; lb, lipid bodies. Bar = 5 μm. Fig. 77. Thin cell walls (arrows) between two synergids and the egg, d, dictyosomes. Note no plasmodesmata are visible. Bar = 1 μm. Fig. 78. Dense cytoplasm of the egg cell showing abundant mitochondria (m), plastids containing starch and ribosomes (r), Nu, nucleolus. Bar = 1 μm. Fig. 79. Finger-like projections of the filiform apparatus. Bar = 1 μm.

Figs. 80-81. TEMs of the central cell of the *Acacia* hybrid embryo sac at anthesis. Fig. 80. Polar nuclei (pn) surrounded by dense cytoplasm rich in plastids containing starch. Bar = 3 μm. Fig. 81. Close up of portion of Fig. 80 showing abundant dictyosomes near the polar nuclei and abundant small electron-lucent bodies (arrows) between the egg cell and central cell. Bar = 1 μm.

Fig. 82. TEM of the *Acacia* hybrid embryo sac at anthesis showing the antipodals in the chalazal end of the embryo sac. Dense cytoplasm contains abundant organelles, i.e. mitochondria, plastids containing starch, endoplasmic reticulum (er). Bar = 3 μm.

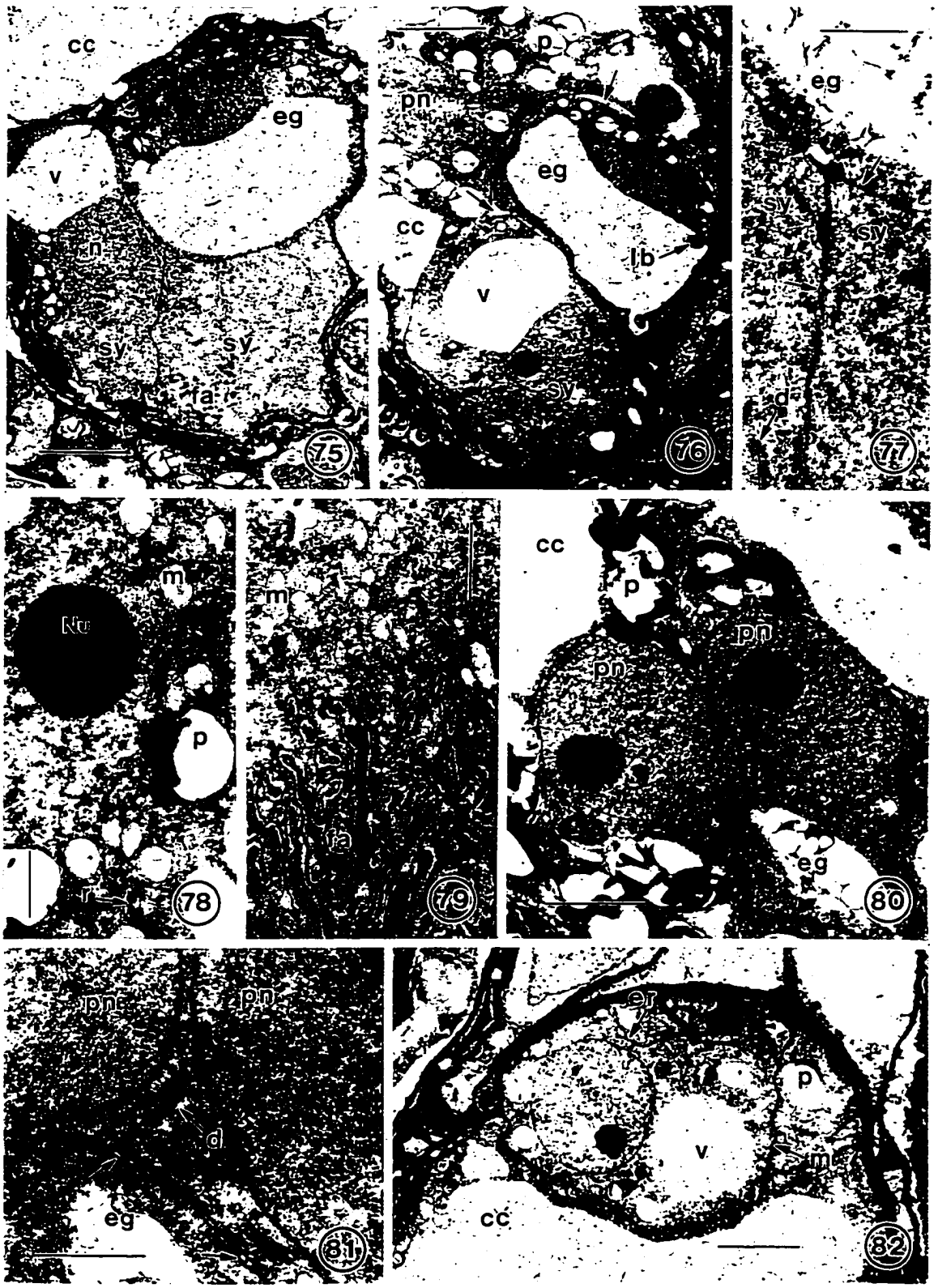
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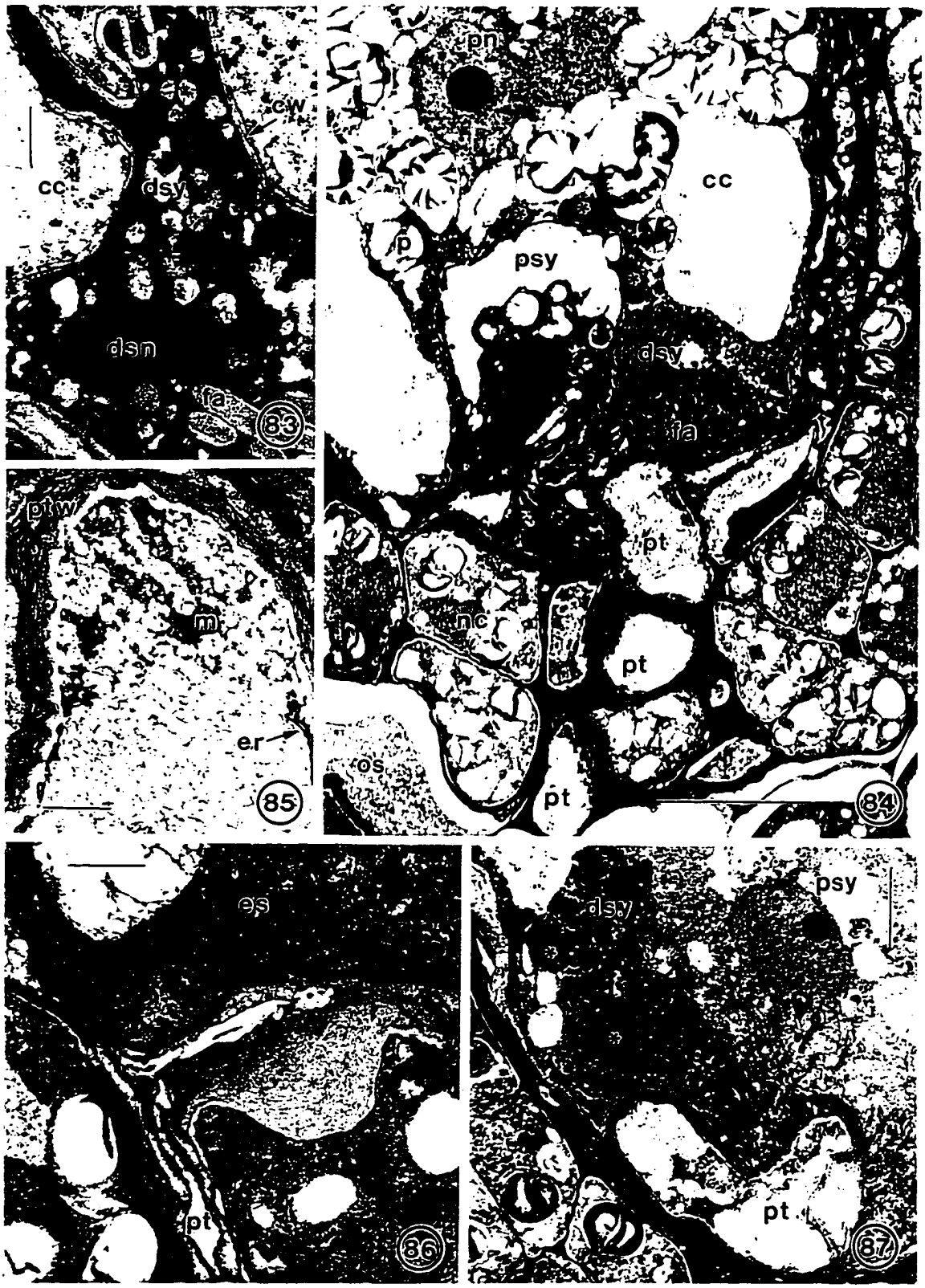
Fig. 83. Transmission electron micrograph (TEM) of the *Acacia* hybrid embryo sac showing a degenerating synergid (dsy) during pollen tube penetration of the nucellus, dsn, degenerating synergid nucleus; cw, cell wall; fa, filiform apparatus; cc, the central cell. Bar = 0.5 μm.

Fig. 84. TEM of the *Acacia* hybrid ovule about 2 d after open-pollination (DAP) showing pollen tube (pt) penetrating the micropylar nucellus (nc) and entered the degenerate synergid via the filiform apparatus. Electron-dense materials (*) accumulate at the micropylar region of the persistent synergid (psy), pn, polar nuclei; p, plastids containing starch; v, vacuole; os, ovarian secretion. Bar = 8 μm.

Fig. 85. TEM of portion of a pollen tube in the degenerate synergid of the *Acacia* hybrid embryo sac, showing the remaining cytoplasm and organelles including mitochondria (m) and endoplasmic reticulum (er), ptw, pollen tube wall. Bar = 1 μm.

Figs. 86-87. TEMs of the *Acacia* hybrid ovule about 3 DAP. Fig. 86. The arrested pollen tube in the micropylar nucellus adjacent to the embryo sac (es) is characterized by wall thickening of the tube tip (*). Bar = 1 μm. Fig. 87. Branching pollen tube following entering the embryo sac. One tube penetrates the degenerated synergid, whereas the other penetrates the persistent synergid. Note two electron dense bodies (*) in the degenerate synergid. Bar 3 μm.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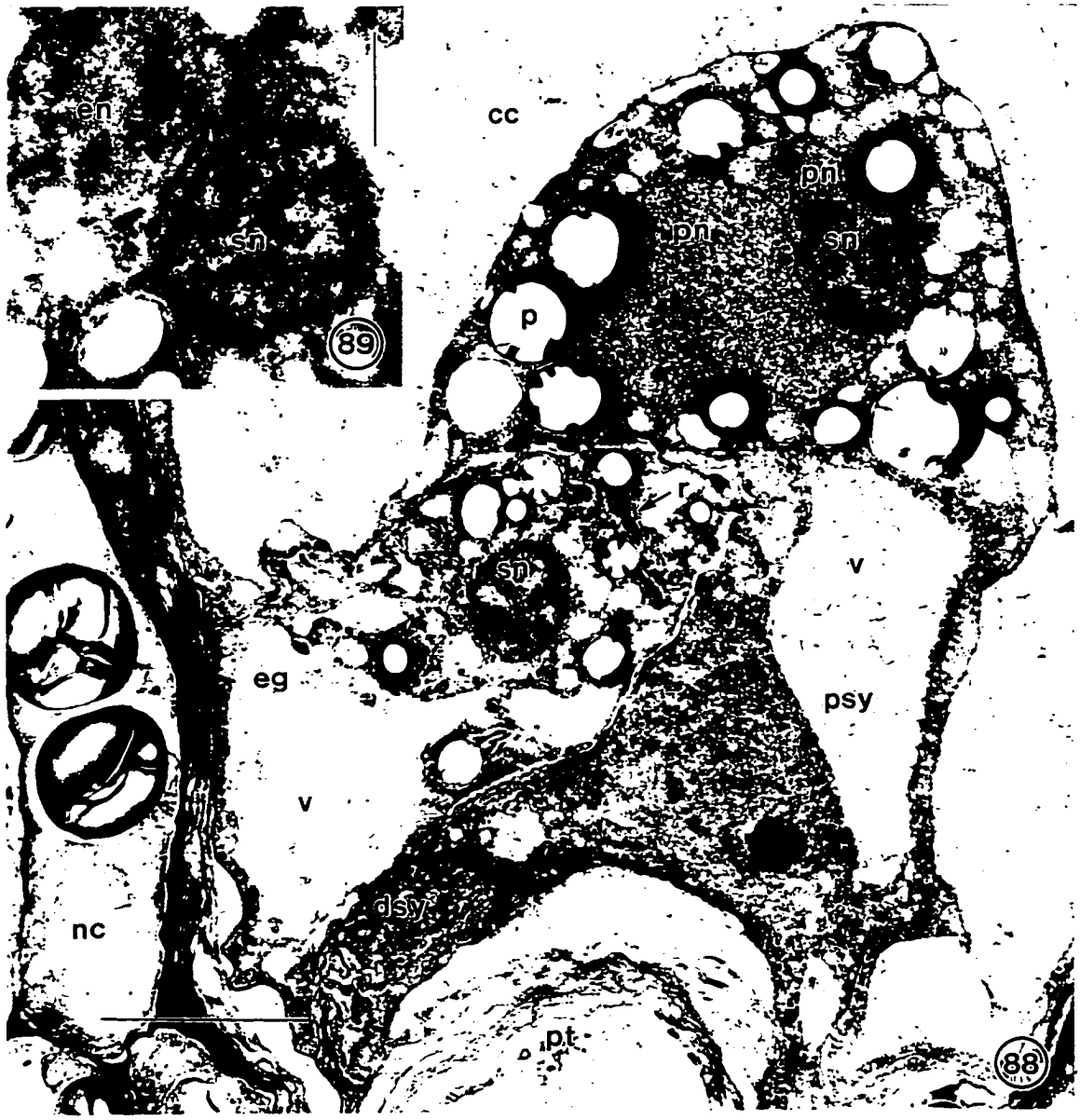
Figs. 88-89. Transmission electron micrographs of the *Acacia* hybrid pollen tube penetrating the embryo sac about 3 DAP. Fig. 88. Sperm nuclei (sn) are in the egg cell (eg) and central cell (cc), r, ribosomes; pn, polar nuclei; dsy, degenerate synergid; psy, persistent synergid; nc, nucellus; pt, pollen tube; v, vacuole; p, plastids containing starch. Bar = 4 μm. Fig. 89. A portion of the same embryo sac as in Fig. 88 showing the alignment of the sperm nucleus to the egg nucleus (en) before karyogamy. Bar = 1 μm.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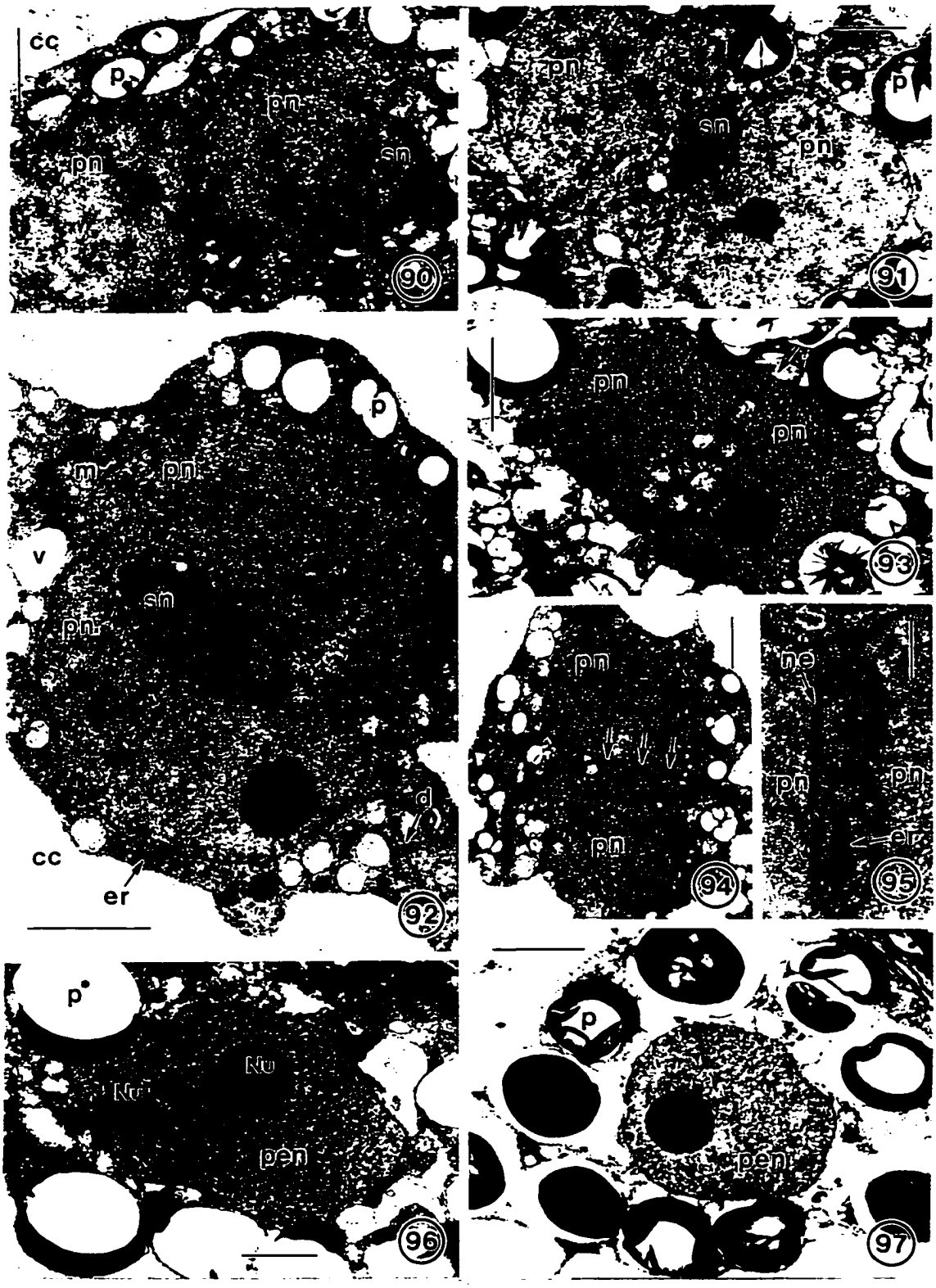
Figs. 90-95. Transmission electron micrographs (TEMs) of the central cell (cc) of the *Acacia* hybrid embryo sac showing the fusion of the sperm nucleus (sn) with the polar nuclei (pn). Fig. 90. The sperm nucleus approaches the micropylar end of one of the polar nuclei, p, starch-containing plastids. Bar = 2 μm. Fig. 91. The sperm nucleus aligns in the concavities between the two polar nuclei. No fusion is observed at this stage. Bar = 2 μm. Fig. 92. The sperm nucleus being engulfed by the polar nuclei, v, vacuole; m, mitochondria; d, dictyosome; er, endoplasmic reticulum. Bar = 2 μm. Fig. 93. Nucleoplasmic fusion between the two polar nuclei via nuclear bridges (arrows). Bar = 3 μm. Fig. 94. Later stage of fusion showing formation of many nuclear bridges (arrows) between the two polar nuclei. Bar = 2 μm. Fig. 95. Close up of part of Fig. 94 showing endoplasmic reticulum in the fusion site, ne, nuclear envelops. Bar = 0.5 μm.

Figs. 96-97. TEMs of the central cell of the *Acacia* hybrid embryo sac after karyogamy. Fig. 96. Newly-formed primary endosperm nucleus (pen). Note two nucleoli (Nu) from polar nuclei. Bar = 2 μm. Fig. 97. Later stage of the primary endosperm nucleus with a single nucleolus. Bar = 3 μm.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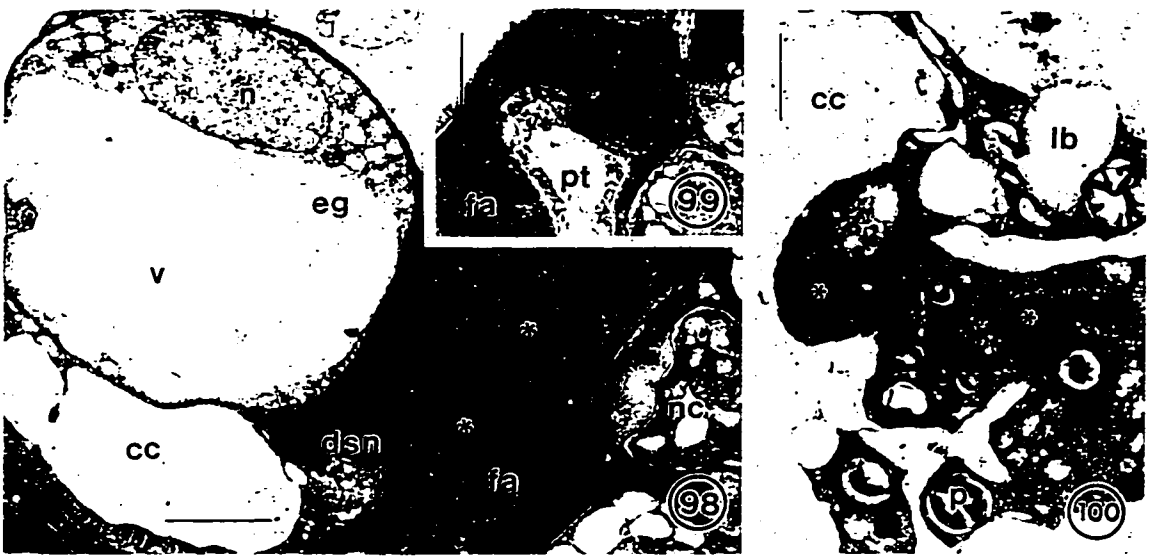
Figs. 98-99. Transmission electron micrographs (TEMs) of the egg apparatus of the *Acacia* hybrid embryo sac about 4 DAP. Fig. 98. Both aborted sperm nuclei (*) in the degenerate synergid (dsy). Note the egg cell (eg) remains unchanged, n, nucleus; m, mitochondria; dsn, a portion of the degenerate synergid nucleus; nu, nucellus; fa, filiform apparatus; v, vacuole; cc, central cell. Fig. 99. A portion of the same embryo sac as in Fig. 98. showing the pollen tube (pt) penetrating the degenerate synergid. Bar = 3 μm in Figs. 98-99.

Fig. 100. TEM of the central cell of the *Acacia* hybrid embryo sac about 4 DAP showing aborted polar nuclei (*), p, starch-containing plastids; lb, lipid bodies. Bar = 2 μm.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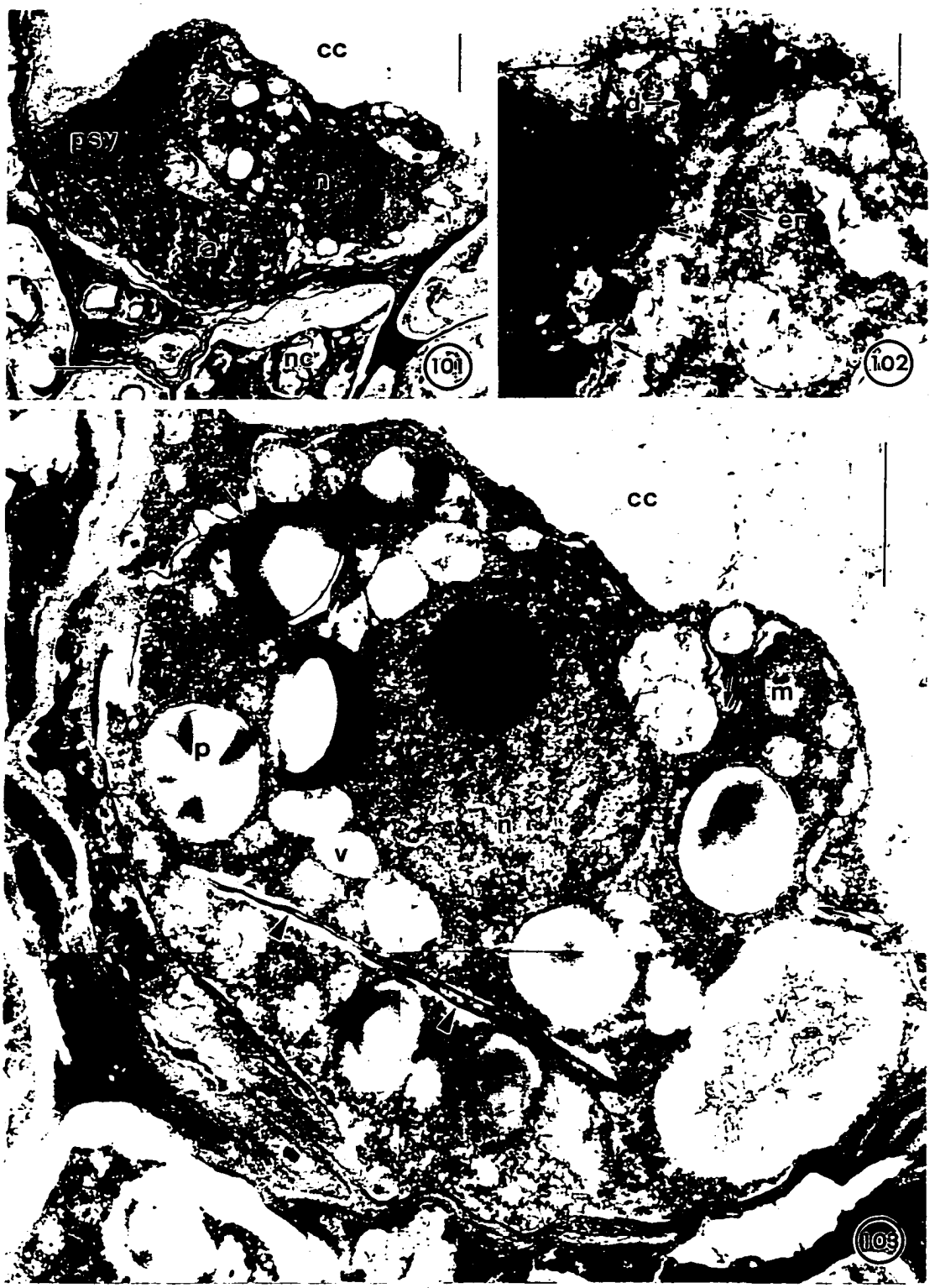
Figs. 101-103. Transmission electron micrographs of the *Acacia* hybrid zygote about 5 DAP. Fig. 101. Zygote (z) and degeneration of the persistent synergid (psy), n, nucleus; fa, filiform apparatus; cc, central cell; nc, nucellus. Bar = 3 μm. Fig. 102. Close up of a portion of Fig. 101 showing dictyosomes (d) and endoplasmic reticulum (er) in the chalazal region of the zygote cytoplasm. Note the zygote is separated from the degenerate synergid by a thin cell wall (arrows). Bar = 0.5 μm. Fig. 103. Dense cytoplasm of the zygote at the same stage as Fig. 101, containing abundant starch-filled plastids (p), mitochondria (m) with poorly developed cristae, vacuoles (v). Note wall invaginations in the chalazal region of the zygote (arrows) and a part of the degenerating-synergid cell wall (arrowheads). Bar = 2 μm.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6.4 Discussion

6.4.1 The ovule before pollen tube penetration

Morphology of the hybrid ovule was reported in Chapter 4. Prior to fertilization the nucellar cells are rich in starch and lipids and a few layers of the crushed nucellar cells are observed adjacent to the embryo sac wall. In *Vicia* (Johansson and Walles, 1993a), disintegration of the starch-rich nucellus occurs during megasprogenesis and continues until after fertilization, providing nutrients for growth of the megaspore mother cell, embryo, and endosperm. Nucellar degeneration in the hybrid may have a function similar to that in *Vicia*. The digested nucellar cells may release nutrients, particularly carbohydrates and lipids into the central cell through the wall ingrowths for the future development of the endosperm and the embryo.

The hybrid embryo sac consists of four cell types (egg, synergids, central cell, and antipodals), as in many angiosperms (Mogensen and Suthar, 1979; Folsom and Peterson, 1984; Sumner, 1992; also see review by Johri *et al.*, 1992). In the hybrid these cells are ultrastructurally and histochemically different from those of many other species and from each other. No plasmodesmata are observed in the wall between the egg cell and synergids in the hybrid and some species such as *Proboscidea* (Mogensen, 1978), indicating that the egg cell may be somewhat physiologically separated from the synergids, especially regarding nutrient transfer. The egg cell possesses a thin, uniform wall, whereas in *Glycine*, the wall of the egg cell is variable in thickness (Folsom and Peterson, 1984). The egg cell of the hybrid and most angiosperms (Cass *et al.*, 1985; Sumner and van

Caeseele, 1989) is polarized due to the presence of a large micropylar vacuole restricting most cytoplasm and the nucleus to the chalazal region. It contains mitochondria with poorly developed cristae and ribosomes suggesting that the hybrid egg cell is inactive as observed in some species such as *Capsella* (Schulz and Jensen, 1968) or *Petunia* (van Went, 1970b). Starch and lipids are the main stored products in the hybrid egg cell as observed in sunflowers (Newcomb, 1973) and cotton (Jensen *et al.*, 1977)

In the hybrid, the synergids are distinguished ultrastructurally from the egg cell, whereas, in *Lilium longiflorum*, all three cells are similar in ultrastructure (Janson and Willemse, 1995). In contrast to most angiosperms (Huang and Russell, 1992), no plasmodesmata are observed in the thin wall between the hybrid synergids, suggesting that no metabolites transfer occurs between these two cells. The synergids contain a large chalazal vacuole similar to that of *Capsella* (Schulz and Jensen, 1968) and *Quercus* (Mogensen, 1972), whereas, in *Petunia* and *Brassica* the synergid contains many small vacuoles (van Went, 1970a; Sumner and van Caeseele, 1989). When compared to the egg cell, the synergid appears metabolically more active because of the presence of many mitochondria, ribosomes, ER, and dictyosomes, as reported in many other species (Schulz and Jensen, 1968; Folsom and Peterson, 1984, also see review by Huang and Russell, 1992).

The presence of the FA in the synergids is common (Huang and Russell, 1992) with the exception of some species such as *Nicotiana rustica* (Sehgal and Gifford, 1979) and *Lilium longiflorum* (Janson and Willemse, 1995). The hybrid FA possesses features similar to that of *Glycine* (Folsom and Cass, 1989) in which it proliferates from the base as wall ingrowths and appears as finger-like projections into cytoplasm of the synergid. The

presence of the FA suggests that the synergids act as transfer cells according to Pate and Gunning (1972). The FA varies histochemically among angiosperms and may consist of insoluble carbohydrates (Sumner and van Caeseele, 1989), proteins (Huang and Russell, 1992) or lipids (Mogensen and Suthar, 1979). In the hybrid, the FA contains both insoluble carbohydrates and proteins but not lipids. The presence of abundant mitochondria adjacent to FA suggests high metabolic activities. Several studies suggest that the synergid is a source of chemotropic substances, particularly calcium (Chaubul and Reger 1990, 1992), and the FA may function in absorbing these substances and in releasing them to guide the pollen tube into the embryo sac (Jensen *et al.*, 1983).

The characteristics of the hybrid central cell are similar to those of most angiosperm as described by Huang and Russell (1992). The cell occupies most of the embryo sac volume and contains a large central vacuole, two polar nuclei, and a peripheral cytoplasm rich in organelles such as plastids, mitochondria, dictyosomes and ER, suggesting that the cell is metabolically active. Starch and lipids are the main nutrient reserves. These features are distinct from those of the egg apparatus and antipodals. The cell is bordered by a cell wall which is totally separated from the egg apparatus and no plasmodesmata are observed in the wall between the central cell and egg apparatus, as in *Arabidopsis* (Mansfield *et al.*, 1991)

The formation of wall ingrowths along the embryo sac wall is common in the central cells of the hybrid and most angiosperms (Huang and Russell, 1992). In many species, wall ingrowths are formed only in the micropylar region of the cell (Newcomb, 1973; Folsom and Peterson, 1984; Sumner and van Caeseele, 1990) after fertilization (Schulz and Jensen, 1968; Mansfield *et al.*, 1991). In the hybrid, wall growths are usually

observed at anthesis in the chalazal region and extend toward the micropylar region, as in *Euphorbia* (Gori, 1977). Tilton *et al.*(1984a) suggest that the wall ingrowths in the central cell have functions similar to those in other transfer cells, as described by Pate and Gunning (1972). In the hybrid, wall ingrowths of the central cell occur along the embryo sac wall at anthesis. This may be related to the nucellar degeneration, in that ingrowths may increase metabolite transfer between the nucellus and the central cell.

6.4.2 The ovule during and after pollen tube penetration

In the hybrid and many other angiosperms, gynoecial secretions play an important role in supporting pollen-tube growth and guidance from the stigma to the ovary (Tilton *et al.*, 1984b; Franssen-Verheijen and Willemse, 1993; Weber, 1994; Gonzalez *et al.*, 1996; see review by Kenrick and Knox, 1984; also see Chapter 4, 5). In some species, after entering the ovarian chamber, pollen tubes penetrate the ovules that secrete a micropylar exudate, suggesting non-random fertilization (Oleson and Bruun, 1990; Yan *et al.*, 1991; Franssen- Verheijen and Willemse, 1993). In those species, the cells of the micropylar nucellus or integuments contain abundant dictyosomes and ER which are involved in synthesis and secretion of the ovular exudate. In the hybrid, both stigmatic and stylar secretions are present (Chapter 4, 5) but no ovular exudate is observed before or after pollen penetration of the ovules. The hybrid ovule lacks a micropyle (Chapter 4) and there is no evidence of abundant secretory organelles in the micropylar nucellus. However, at anthesis, the ovarian chamber is filled with a secretion containing insoluble carbohydrates and proteins. A large portion of the secretion is observed in the region of the trichomes

and enclosing the ovules and a small portion is observed adjacent to the micropylar nucellus. Judged by its abundance and position, the secretion is likely derived from the many trichomes, that may be glandular, rather than the ovules. Secretion by glandular trichomes has been observed in many plants and has different functions, depending on where the trichomes are located (Esau, 1977; Wagner, 1991). The ovarian secretion in the hybrid may be involved in supporting pollen tube growth and guidance to the ovules. A similar phenomenon was observed in *Lilium* (Janson *et al.*, 1994) in which a placental fluid is produced and is involved in directing pollen tubes to the ovules. The ultrastructure of the trichomes and the composition of the secretion need further investigation. All of the ovules in a hybrid ovary are covered by this secretion. This suggests that each ovule has the opportunity to be fertilized by a pollen tube. Random fertilization may occur in the hybrid. Failure of pollen tube penetration of an ovule (Chapter 5) may be due partly to inviability of the ovule.

Degeneration of one of the two synergids during fertilization is common in the hybrid and most angiosperms but the time of degeneration varies among species. In most species observed, synergid degeneration occurs soon after pollination (Mogensen and Suthar, 1979; Jensen *et al.*, 1983; Folsom and Cass, 1992) but in some cases, it occurs before pollination (Engell, 1988). In the hybrid, synergid degeneration occurs only in ovules penetrated by pollen tubes and begins before the pollen tube enters the embryo sac, suggesting that degeneration may be triggered by the pollen-tube penetration of the nucellus. Synergid degeneration after pollen tube penetration of the ovule has been reported in other species such as *Petunia hybrida* (van Went, 1970c), *Helianthus annuus* (Newcomb, 1973) and *Quercus gambelii* (Mogensen, 1972). Random synergid

degeneration has been reported in *N. tabacum* (Huang and Russell, 1992), whereas preferential degeneration may occur in *Helianthus* (Yan *et al.*, 1991) or *Hordeum* (Mogensen, 1984) in which the synergid located closest to the ovular vascular trace tends to degenerate. In the hybrid, both synergids are almost identical in cell size and ultrastructure and appears that random synergid degeneration occurs. However, a larger number of fertilized ovules need to be observed to confirm this.

Penetration of one pollen tube into the degenerate synergid via the FA has been observed in the hybrid and many other angiosperms i.e. *Glycine* (Tilton *et al.*, 1983, Dute *et al.*, 1989), *Helianthus* (Yan *et al.*, 1991), *Nicotiana* (Mogensen and Suthar, 1979) and *Brassica* (Sumner, 1992). With some exceptions, in other species the ovule may be penetrated by more than one pollen tube (Palser *et al.*, 1989) and entry of the pollen tube into the embryo sac may occur between one of the synergids and the egg cell or the embryo sac wall (Johri *et al.*, 1992). The hybrid, the cytoplasm of the degenerate synergid appears electron dense. The presence of two electron-dense oval bodies, called X-bodies, in the degenerate synergid after pollen tube discharge is common in the hybrid and some other species (Janson and Willemse, 1995). Observations of early degenerating synergids in the hybrid appear similar to observations of Jensen (1974) and Huang *et al.* (1993) in which the large body is thought to be the remnant of the degenerate synergid nucleus. The small body appears to be the remnant of the vegetative nucleus based on its shape and the time when it is present. Russell (1992) suggests that synergid degeneration provides conditions favorable for gamete release and gamete fusion. In the hybrid, the deterioration of the degenerate synergid wall may allow the male nuclei to directly contact and be transmitted into the egg and central cells.

In a few fertilized ovules, a pollen tube enters the embryo sac and branches, each branch appears intact and both degenerate and persistent synergids are penetrated. However, the male gametes seem to be released only into the degenerate synergid as evidenced by the presence of the remnants of the vegetative nucleus. This phenomenon may be considered an abnormality of pollen tube growth. In other cases, the pollen tube becomes arrested in the nucellus or both male gametes abort in the degenerate synergid. In *A. retinodes* pollen tubes from self-pollination are arrested in the nucellus, which has been interpreted as a primitive type of gametophytic self-incompatibility (Kenrick *et al.*, 1986). Seavey and Bawa (1986) also proposed models of late-acting self-incompatibility as pollen tubes enter the ovary at later pre-fertilization stages or during the post-zygotic period. In the hybrid, pollinated flowers were collected after open-pollination and pollen tubes produced may be from either compatible or incompatible pollen grains. It is possible that pollen tubes arrested in the nucellus are incompatible grains but this needs to be tested using control crosses. Similarly, male gametes that abort prior to plasmogamy may be released from an incompatible pollen tube.

6.4.3 Patterns of gametic fusion and cytoplasmic inheritance

In angiosperms, the time between pollination and fertilization may take minutes in *Taraxacum* to months in *Corylus* (Marshall and Grace, 1992). In the hybrid *Acacia*, both male nuclei were observed in the egg and central cell about 3 DAP and karyogamy occurs in the following few days as in *A. retinodes* (Kenrick and Knox, 1989a). The time of fertilization in the hybrid appears to coincide with flower abscission which usually occurs

by the end of the day 3 (Chapter 4), suggesting that abundant flower abscission observed results from a lack of fertilization. In some species, the fertilized egg shows ultrastructural changes prior to karyogamy, such as an increase in the number of starch grains and polysomes as in *L. longiflorum* (Janson and Willemse, 1995) and cotton (Jensen, 1968). This is also true in the hybrid in which before karyogamy there is an increase in starch grains and formation of wall invaginations in the micropylar wall.

The times and patterns of double fertilization vary among angiosperms. In avocado, the fusion of the polar nuclei of the central cell with a sperm usually occurs before fusion between the egg cell and a sperm (Sedgley, 1979a). Also, the polar nuclei may fuse before or during fertilization and in the latter case polar nuclear fusion may occur simultaneously with the sperm (Palser *et al.*, 1989; Janson and Willemse, 1995) or before the arrival of the sperm (Folsom and Cass, 1992). In the hybrid, the polar nuclei remain unfused until migration and alignment of the sperm nucleus between the polar nuclei and triple fusion occurs shortly before the fusion of the egg and the other sperm nucleus. The pattern of sperm alignment to the polar nuclei in the hybrid has not been reported in other angiosperms. Polar nuclear fusion starts with the linkage of the nuclear envelopes along the margins of the bowl-shaped nuclei. The polar nuclei start to fuse, then appear to engulf the sperm nucleus. The ER observed in the fusion site may be involved in the fusion of the polar nuclei (Jensen 1964, van Went 1970c; Janson and Willemse, 1995). Jensen (1964) showed that polar nuclei are linked by fused ER and shortening of ER enables them to come into contact and merge. It is assumed that this form of polar nuclear fusion enables the sperm nucleus to contact both polar nuclei simultaneously then fusion among polar nuclei and sperm nucleus occurs. Janson and Willemse (1995) suggest

that, in *Lilium*, fusion of the egg nucleus with the sperm nucleus is less associated with the ER. Fusion, therefore, may occur by alignment and contact of the egg and sperm nucleus. This also appears to be the case in the hybrid in which no ER has been observed in the fertilized egg.

Most angiosperms exhibit maternal and rarely biparental inheritance of plastids and mitochondria (Russell, 1992; Mogensen, 1996). In uniparental inheritance, the exclusion of the sperm cytoplasmic organelles can occur during pollen development (Schröder and Hagemann, 1986), pollen tube growth (Yu *et al.*, 1992) or gametic fusion (Mogensen, 1988). In the hybrid, no cytoplasmic structures from the sperm were observed in the degenerate synergid, egg or central cell. Whether the sperm cytoplasm is extruded during pollen development or pollen tube growth is not known. This suggests that the hybrid possesses maternal cytoplasmic inheritance.

6.4.4 Formation of the zygote and endosperm

Striking ultrastructural differences between the egg and zygote have been observed in many species. Reduction in the size of the egg cell following fertilization occurs in *N. tabacum* (Mogensen and Suthar, 1979), cotton (Jensen, 1968), and sunflower (Sumner, 1992). An increase in the number of the cytoplasmic organelles such as ER, dictyosomes, mitochondria, polysomes, and starch grains in the zygote has been reported in cotton (Jensen, 1968), *Capsella* (Schulz and Jensen, 1968) and sunflower (Sumner, 1992). Also, complete formation of the egg cell wall after fertilization is observed in *Quercus* (Singh and Mogensen, 1975), tobacco (Mogensen and Suthar, 1979), and sunflower (Sumner,

1992). In the hybrid, ultrastructural changes between the egg and zygote stages are noticeable. These include reduction in size of the egg cell and an increase in the number of starch grains and small vacuoles. ER and dictyosomes are absent in the egg cell but a few are observed in the zygote.

The hybrid zygote is surrounded by a complete cell wall and its chalazal wall increases in wall invaginations. The cytoplasm contains mitochondria with poorly-developed cristae, and a few dictyosomes and ER, suggesting that shortly after fertilization the hybrid zygote is inactive. The zygote of some species such as *Q. gambelli* (Mogensen, 1972) and *N. tabacum* (Mogensen and Suthar, 1979) appears similar to that of the hybrid and usually remains undivided for several days. This suggests that the zygote of these species and probably the hybrid require a long period for cellular organization and physiological changes before the first division (van Went and Willemse, 1984).

CHAPTER 7

Zygotic Embryo Development and Embryo Sac Nutrition in a Tropical *Acacia* Hybrid (*A. mangium* Willd. x *A. auriculiformis* A. Cunn. ex. Benth.)

7.1 Introduction

Acacia mangium (Leguminosae: Mimosoideae) is naturally distributed in northern Queensland, western Papua New Guinea, and Irian Jaya and Maluku of Indonesia (Doran and Skelton, 1982), whereas *A. auriculiformis* is native to Queensland, western and southern Papua New Guinea, and Irian Jaya and the Kei Islands of Indonesia (Turnbull *et al.*, 1986). Both species have been introduced to many Southeast Asian countries for reforestation programs (Darmono and Dayanto, 1981; Chittachumnok and Sirilak, 1991; Pinyopusarerk *et al.*, 1993). Their natural hybrids exhibit heterosis (hybrid vigor) in growth performance, form, and adaptability when compared to the parental species (Tham, 1979; Kijkar, 1992; Pinso and Nasi, 1992). This has resulted in considerable interest in using them for plantations. In Thailand, studies have shown that hybrid seed production is very low (Chapter 3) and partly limited by low pollination and fertilization success (Chapter 4, 5). Low seed production is also caused by seed and fruit abortion which may be attributed to resource limitations (Briggs *et al.*, 1987; O'Donnel & Bawa, 1993; Tybirk, 1993; Guitian, 1994; also see review by Stephenson, 1981) or abnormal seed development

(Palupi, 1996). To determine the causes of seed and fruit abortion, it is essential to understand the normal patterns and timing of seed and fruit development.

Some studies have emphasized on phenology and pollination biology of the parental species (Ibrahim and Awang, 1992; Josue, 1992; Sedgley *et al.*, 1992b,d) and pre- and post-pollination in the hybrid (Chapter 4, 5, 6). However, no studies have been done on the hybrid seed and fruit development. General seed and fruit development of some acacias have been studied using light microscopy (Newman, 1934b; Dnyansagar, 1958; Souza, 1993; also see Johri *et al.*, 1992) but there have been no detailed ultrastructure investigation. Anatomical and ultrastructural studies have been done on many aspects of post-fertilization of many other angiosperms, including embryo development (Jones and Rost, 1989; Johansen and Walles, 1993b; Chamberlin *et al.*, 1994; Taylor and Vasil, 1995), endosperm development (Singh and Mogensen, 1976; Dute and Peterson, 1992; Briggs, 1993a, 1993b) and embryo sac nutrition (Chamberlin *et al.*, 1993; Johansen and Walles, 1994) However, little work has been done on mimosoid plants.

The purpose of this study is to describe the developmental stages of the embryo, endosperm and ovular tissues in relation to seed and fruit morphology and abortion using light and electron microscopy. Structural adaptations of the ovule were determined with respect to embryo sac nutrition during embryo development. Since most legume seeds accumulate various storage products, particularly proteins, the hybrid seed storage products at maturity were investigated histochemically and ultrastructurally.

7.2 Materials and methods

7.2.1 Study site and plant materials

Acacia hybrid pods and seeds at various developmental stages were collected from two trees growing in the experimental plot at the ASEAN Forest Tree Seed Centre, Muak-Lek, Saraburi, Thailand (latitude N 14° 40′, longitude E 101° 17′, and about 200 m elevation). Floral spikes were marked at the stage of initial pod set, as indicated by color and size changes of the pistil (about 1 w after open-pollination), and developing pods were collected every 7-10 d until maturation. Observations and measurements from five spikes of each tree were taken to determine the number of pods per spike, pod size and color, and water content (WC). Pod WC was measured from initial pod set until maturity, whereas the first measurement of seed WC was made when developing seeds were large enough to be excised. Developing pods were also collected from an additional three spikes from each tree for light and electron microscopy, and histochemical study.

7.2.2 Light and electron microscopy

Developing seeds were dissected and fixed in FAA (50% ethanol) for light (LM) and scanning electron microscopy (SEM) and in 2.5% glutaraldehyde in 0.075 M phosphate buffer (pH 7.2 to 7.4) for LM and transmission electron microscopy (TEM). Small portions of pods during early development, up to 65 days after

pollination (DAP) were used, whereas during later development entire seeds were bilaterally trimmed before fixation.

To determine stages of embryo and seed development, FAA-fixed specimens for LM were dehydrated through a tertiary butyl alcohol series (Johansen, 1940), embedded in TissuePrep and sectioned at 6-8 μm. Sections were stained with 1% safranin and 1% aniline blue. For SEM, specimens were dehydrated in a alcohol series, critical-point dried, coated with gold, and viewed using a JOEL 35 at 15 kV. Specimens fixed in 2.5% glutaraldehyde were rinsed in the same buffer and postfixed for 1 h in 1% osmium tetroxide in 0.075 M phosphate buffer (pH 7.2 to 7.4). Specimens were then dehydrated in a series of acetone solutions, embedded in Spurr's resin (Spurr, 1969) and sectioned at 0.5 to 1 μm for LM and 60 nm for TEM using an ultramicrotome. Semithin sections were stained with 0.5 % toluidine blue in 0.1% sodium carbonate (pH 11.1) (Trump *et al.*, 1961). Ultrathin sections were collected on 200-mesh grids, stained with 2% aqueous uranyl acetate for 15 min and 0.2% Sato's lead citrate for 15 min (Sato, 1968) and viewed using an Hitachi 7000 electron microscope at 75 kV.

7.2.3 Histochemical study

Histochemical analyses of the mature embryos were carried out to localize lipids, proteins and insoluble carbohydrates. Mature embryos were excised from seedcoats and fixed in 2.5% glutaraldehyde in 0.075 M phosphate buffer (pH 7.2-7.4). Specimens were then prepared for LM as described above. Semithin sections (about

1μm) were stained with 0.5 % toluidine blue in 0.1% sodium carbonate (pH 11.1) (Trump *et al.*, 1961) for general observations, periodic acid-Schiff's reagent (PAS) for insoluble carbohydrates (Jensen, 1962), Sudan black B for lipids (Bronner, 1975), and PAS-Amido black for proteins (Jensen, 1962).

7.3 Results

7.3.1 Pod morphology and abortion

Development of the *Acacia* hybrid pods are classified into six morphological stages. During 7 to 13 DAP pods containing fertilized ovules undergo slight elongation of the ovary and a withering of the stamens, corolla and calyx (stage 1). During stage 2, the pods continue to elongate slowly but their shape remains unchanged. During stage 3 (55 to 75 DAP) substantial changes in pod size and shape are observed. Pods become more flattened and their length ranges from 3 to 10 cm. During stage 4, further elongation and broadening result in spiral-shaped pods. By this stage, some pods have attained their maximum length of up to 20 cm. Mature pods at stage 5 are broad and spiral, and vary in size from 15 to 20 cm long and 0.6 to 0.9 cm wide. Pod desiccation occurs during stage 5 and 6 and is characterized by a color change from green to brown. Complete development of the hybrid pods from pollination to dehiscence (stage 6) occurs in 140 to 145 d (Table 15).

During the course of pod development the number of pods per spike declines significantly, particularly during early development (stages 1 and 2). About 7 DAP,

pod number per spike is about 15 and decreases to about two within 55 DAP (Fig. 104) then remains relatively unchanged.

Pods also show changes in WC during development. About 7 DAP, pod WC is about 40% then increases reaching a maximum of about 70% by 44 DAP (stage 2). This high WC remains until pod maturity. During the desiccation period, pod WC decreases abruptly and averages 13% at the dehiscence (stage 6) (Fig. 105A). Seed WC could not be measured until 106 DAP at which time it was over 70% and remained constant until seed maturity. However, seed desiccation began earlier and required more time than pod desiccation. Seed WC decreased dramatically from stage 4 through stage 5. At the pod dehiscent stage, seed WC reached its minimal level of about 6% (Fig. 105B).

7.3.2 Development of the ovule after fertilization

7.3.2.1 Embryo

Fertilization in the hybrid occurs about 3 DAP and the zygote is observed about 5 DAP (Chapter 6). About 7 DAP, the zygote is still attached to the micropylar wall of the embryo sac and is separated from the central cell by a thin wall (Fig. 106). Ultrastructurally, the cell is densely cytoplasmic and contains large and many small vacuoles and starch grains throughout the cell. No lipid bodies are observed. The cell contains a large nucleus with a prominent single nucleolus. During development of the outer and inner integuments (Fig. 110), the zygote remains quiescent and degenerate

synergids are still observed adjacent to the zygote (Figs. 111,113,114). About 55 DAP, when the integuments completely enclose the embryo sac, the zygote shows some changes, including an increase in cell size and the number of nucleoli (Fig. 115). The first zygotic division occurs about 65 DAP. The transverse division of the zygote results in almost equal-size terminal and basal cells. The basal cell remains attached to the embryo sac wall. Both cells appear to have similar ultrastructures. Both have irregular shapes, thin cell walls and wall evaginations in some regions (Figs. 116,117). They have many large and small vacuoles, and dense cytoplasm containing abundant organelles, particularly mitochondria with well-developed cristae and ribosomes which aggregate to form polysomes (Fig. 119). At this stage, nearly all starch grains disappear and no dictyosomes and vesicles are present. The spherical nuclei are centrally located within each cell and contain a few prominent nucleoli (Fig. 116).

At this stage, the degenerate synergid still remains in contact with the two-cell embryo. This synergid is likely the one that the pollen tube penetrated as shown by the remnants of the pollen tube. From serial sections the chalazal region of the synergid contains fibrillar material (Fig. 118) and forms a thick layer which appears to enclose the embryo (Fig. 117). Some regions of this layer are irregularly thickened and fold (Fig. 117). The embryo cell walls are separated from this layer by a pericytoplasmic space (Fig. 117). These features are not observed in the newly-formed zygote.

The terminal cell of the two-cell embryo divides vertically, followed by vertical division of the basal cell, resulting in a four-cell embryo. Cells of the four-cell embryo then undergo several oblique, transverse and vertical divisions which give rise to a globular embryo (Fig. 120). Following this stage, cell divisions in the proembryo

become somewhat irregular but are mostly tangential and oblique. During the late globular stage (about 76 DAP), the embryo substantially increases in size and a conspicuous, densely cytoplasmic protoderm is formed, whereas the cells in the central region are vacuolate (Fig. 123). There is no differentiation of the suspensor during the globular stage.

About 87 DAP (Table 16), two cotyledonary primodia are initiated from the globular embryo (Fig. 124). As they elongate, the embryo begins to become heart-shaped (Fig. 125). The cotyledons continue to elongate into the middle of embryo sac resulting in a linear cotyledon stage (Fig. 126). At this stage, a shoot apical meristem and provascular tissues of the embryo axis become noticeable. At later development (about 114 DAP) the cotyledons enlarge dramatically and their provascular tissues are initiated below the adaxial surface (Fig. 127). A radicle apical meristem is formed in the micropylar embryo axis (Fig. 127) and the shoot apical meristem elongates to become narrow cone-shaped (Fig. 128) and later differentiates into a plumule (epicotyl) (Figs. 129,130). Complete embryo development occurs about 121 DAP (Table 16).

At maturity, the embryo axis is oval, small and enclosed by two laterally attached cotyledons (Figs. 129,130). Provascular tissues are well-defined in the hypocotyl (Fig. 130). The plumule consists of compound leaves. No root cap is observed at the tip of the well-developed radicle (Fig. 130). The cotyledons are large, broad, fleshy and have flat adaxial and concave abaxial surfaces (Fig. 131). Vascular bundles are well-developed along the cotyledon axis near the adaxial surface (Fig. 131). The cotyledons consist of a one-cell epidermal layer, a few-cell subepidermal

layer and large ground tissues containing large, vacuolated, spherical parenchymatous cells (Fig. 130).

Ultrastructurally, the radial and inner tangential walls of the oblong epidermal cells of the cotyledons are thick with many plasmodesmata and the outer tangential walls are much thicker and covered by a thin cuticle. Epidermal cells have many vacuoles and dense cytoplasm containing abundant organelles, including plastids lacking starch but with well-developed thylakoids, mitochondria with well-defined cristae, dictyosomes, and rough endoplasmic reticulum (ER). In some cases, a stack of long rough ER is arranged parallel to the nucleus (Fig. 132). Subepidermal cells are small and isodiametric. The parenchymatous cells of the ground tissues have thick cell walls with plasmodesmata and large vacuoles which push the cytoplasm to the periphery. Their dense cytoplasm contains many organelles such as plastids with starch and well-developed thylakoids, mitochondria with well-defined cristae and rough ER with short-cisternae (Figs. 134, 135).

Histochemical tests show that the mature hybrid embryo contains storage carbohydrates, lipids and proteins localized in different regions of the cotyledons. Starch is abundant in the periphery of the cotyledonary subepidermal and ground tissue cells but absent from the cotyledonary epidermal cells and embryo axis (Fig. 136). Protein bodies are localized only in the vacuoles of the cotyledonary epidermal, subepidermal and embryo-axis cells (Figs. 137). Ultrastructurally, protein bodies are large, variable in size and shape, strongly electron dense and mostly attached to the inner surface of the tonoplast (Fig. 133). Abundant lipid bodies are observed in all

cotyledonary cells but are absent from the embryo axis (Fig. 138). They were small, spherical and moderately electron dense (Figs. 132,135).

7.3.2.2 Endosperm

The primary endosperm nucleus (PEN) is observed in the middle of central cell about 7 DAP. The PEN is spherical with a single nucleolus and surrounded by many plastids containing large starch grains (Fig. 106). Wall ingrowths containing fibrillar materials (Fig. 107) are observed along the central cell walls. The first division of the PEN occurs shortly after its formation, forming two free daughter nuclei (Fig. 108). This is accompanied by a decrease in starch. About 14 DAP, the two free nuclei separate and relocate in the periphery of the chalazal region of the embryo sac (Fig. 111). At that time, starch grains are absent from the central cell. The two-free nuclear endosperm stage is observed until 34 DAP, when the outer integument completely encloses the embryo sac and the funiculus has elongated.

Free nuclear divisions follows and about 55 DAP, some free endosperm nuclei appear to connect with others via cytoplasmic strands (Fig. 114). Ultrastructurally, each free nucleus has a thin nuclear envelope, a few prominent nucleoli, several small inclusions and is surrounded by a thin parietal layer of cytoplasm rich in organelles (Fig. 139). The number of free-nuclei and first cellularization were not observed, however, they occurred after 65 DAP.

Cellularization of the free nuclear endosperm is complete about 76 DAP, during the early globular embryo stage (Table 16) at which time they fill the embryo

sac and enclose the developing embryo (Fig. 120). At this stage, the nucellus already degenerated and the cellular endosperm comes in contact with the embryo sac wall (Fig. 121). During embryo development, the endosperm cells increase in size and have large vacuoles and little cytoplasm (Fig. 121). Endosperm degeneration begins in the chalazal embryo sac and progresses toward the micropylar region during the heart-shaped embryo stage. During the linear cotyledon stage, most cellular endosperm disappears leaving a small amount enclosing the developing embryo (Fig. 126). As the embryo enlarges, this endosperm is displaced toward the periphery of the embryo sac (Fig. 127.) and its remnants remain along the embryo sac wall at the mature embryo stage (Fig. 146).

7.3.2.3 Nucellus

The nucellus of the fertilized embryo sac about 7 DAP is a few cells thick and cells have large nuclei and vacuoles and dense cytoplasm containing abundant starch (Fig. 106). Crushed nucellar cells occur adjacent to the embryo sac. The nucellar cells undergo divisions and accumulate along the embryo sac wall (Fig. 113). Newly-formed nucellar cells are large, irregular, densely cytoplasmic with no distinct vacuoles, and thus are distinguishable from the nucellar cells (Fig. 113). Remnants of the pollen tube still remain in the micropylar nucellus (Fig. 113).

About 55 DAP the nucellar cells are most abundant in the periphery at the chalazal and micropylar ends of the embryo sac (Fig. 114). Nucellar cells have irregular shapes, electron-dense cell walls and many large and small vacuoles (Fig.

140). The dense cytoplasm contains many plastids with starch grains, mitochondria with well-developed cristae, dictyosomes with derived vesicles, rough ER with dilated cisternae, and ribosomes (Figs. 140,141). Abundant spherical, electron-dense lipid bodies are observed in the cytoplasm (Fig. 142), whereas large, electron-dense protein bodies are localized in large vacuoles (Fig. 141). Nucellar-cell nuclei become irregular in shape and contain prominent nucleoli (Fig. 140). Some nucellar cells in the chalazal channel are large with very little cytoplasm and large vacuoles containing fibrillar material (Figs. 114,144).

After 65 DAP, nucellar cells begin to degenerate and disappear concurrent with completion of the endosperm cellularization (Fig. 120). Most nucellar cells degenerate except in the micropylar region where the zygote is attached (Fig. 120) . Cells in the cup-like micropylar nucellus at the two-cell embryo stage have large, irregular-shaped nuclei, dense cytoplasm and many small and large vacuoles (Fig. 116). Plastids with starch grains are common. Irregular-shaped lipid bodies are observed in the dense cytoplasm and strongly electron-dense materials accumulate in some intercellular spaces (Fig. 116). The micropylar nucellus remains until the late cotyledon stage, then degenerates when the embryo is almost mature.

7.3.2.4 Integuments

After formation of the zygote and endosperm, the integuments begin to enclose the embryo sac. Both integuments are two to three cells thick and contain no stored products (Fig. 109). Integumentary cells have large, irregular-shaped nuclei, many

large and small vacuoles and dense cytoplasm containing abundant organelles (Fig. 109). Development of the outer integument is more rapid than that of the inner integument. By 24 DAP, the outer integument completely encloses the embryo sac (Fig. 112). About 44 DAP, the integumentary epidermis is distinct and provascular tissues are initiated in the outer integumentary tissues adjacent to the funiculus (Fig. 113). By 55 DAP, the cells of the outer integument continue anticlinal and periclinal divisions, whereas the inner integumentary cells divide primarily anticlinally and reach a maximum length (Fig. 114) when both ends adjoin the micropylar nucellus (Fig. 115). The inner integument remains as a two-cell layer and encloses about three-quarters of the embryo sac (Fig. 113). At this stage, the integuments in the chalazal region develop projections into the embryo sac and these connect to the large chalazal nucellar cells (Fig. 114). At the early globular embryo stage, about 76 DAP, the inner integument can be seen as two distinct one-cell layers (Fig. 121). Cells of the layer abutting the outer integument have little cytoplasm and a large vacuole containing abundant granular materials (Fig. 122). Cells of the inner layer abutting the embryo sac form the integumentary tapetum or endothelium and have large nuclei, dense organelle-rich cytoplasm and many small vacuoles containing electron-dense materials (Fig. 122). The endothelium disappears during the late globular embryo stage, whereas the outer layer remains attached to the outer integument until seed maturity and becomes a portion of the testa (seedcoat) (Figs. 125,126,127). During late embryo development, the outer integument undergoes no further cell divisions and begins to develop into the seedcoat.

The mature seedcoat has different layers which are formed from the inner and outer integuments. The outer one-cell layer differentiates from the integumentary epidermis and consists of palisade-shaped, thick-walled macrosclereids (Figs. 145,146). During seed desiccation, a thick cuticle with pleurogram and a fracture line forms on the surface of this layer (Figs. 145,146). Two layers of hourglass-shaped osteosclereids are present. One differentiates from subepidermal layers of the outer integument and is subjacent to the palisade layer and the other differentiates from the inner layer of the outer integument and is adjacent to the embryo sac (Figs. 145,146). The middle multi-cellular layer develops from the middle layers of the outer integument and consists of thick-walled parenchymatous cells (Figs. 145,146). A vascular bundle extends from the funiculus through the hilar region and ends in the chalaza. The inner one-cell layer, containing thin-walled parenchyamatous cells, is derived from the inner integument (Figs. 145,146).

Table 15. Stages of pod development in the *Acacia* hybrid.

Stage	DAP [1]	Pod size		Ovary or pod morphology
		length (cm)	width (cm)	
1	7-13	0.1- 0.2	0.05-0.09	Ovary elongates slightly and becomes green; stamen, corolla, and calyx wither
2	14-54	0.2-2.5	0.1-0.2	Green round pod
3	55-75	3.0-10.0	0.2-0.5	Green flat pod
4	76-130	10.0-20.0	0.5-0.9	Green spiral pod
5	131-139	15.0-20.0	0.6-0.9	Browning pod
6	140-145	15.0-20.0	0.6-0.9	Dehiscent pod

[1] DAP = days after pollination.

Table 16. Stages of embryo and endosperm development in the *Acacia* hybrid

Stage	DAP [1]	Embryo stage	Endosperm development
1	7	Early zygote	Free nuclear endosperm
2	55	Late zygote	Free nuclear endosperm
3	65	Two to eight-cell embryo	Free nuclear endosperm
4	76	Early to late globular embryo	Complete endosperm cellularization
5	87	Heart-shaped embryo	Beginning of cellular endosperm degeneration
6	97	Early linear cotyledon stage	Degenerating cellular endosperm
7	114	Late linear cotyledon stage	Degenerating cellular endosperm
8	121	Mature embryo	Remnants of cellular endosperm

[1] DAP = days after pollination.

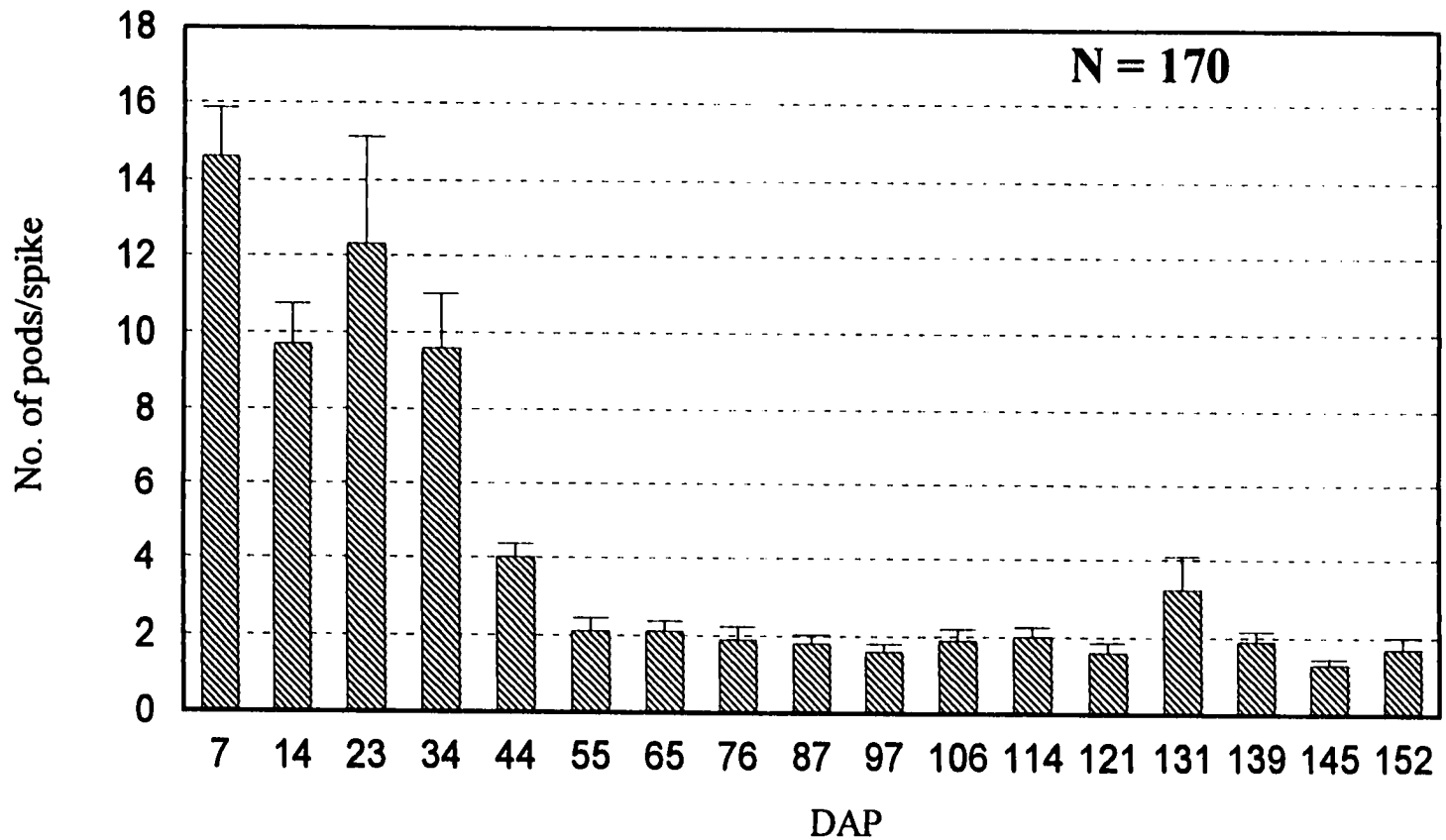

Figure 104. Reduction in pod number during development in the *Acacia* hybrid; DAP=days after pollination.

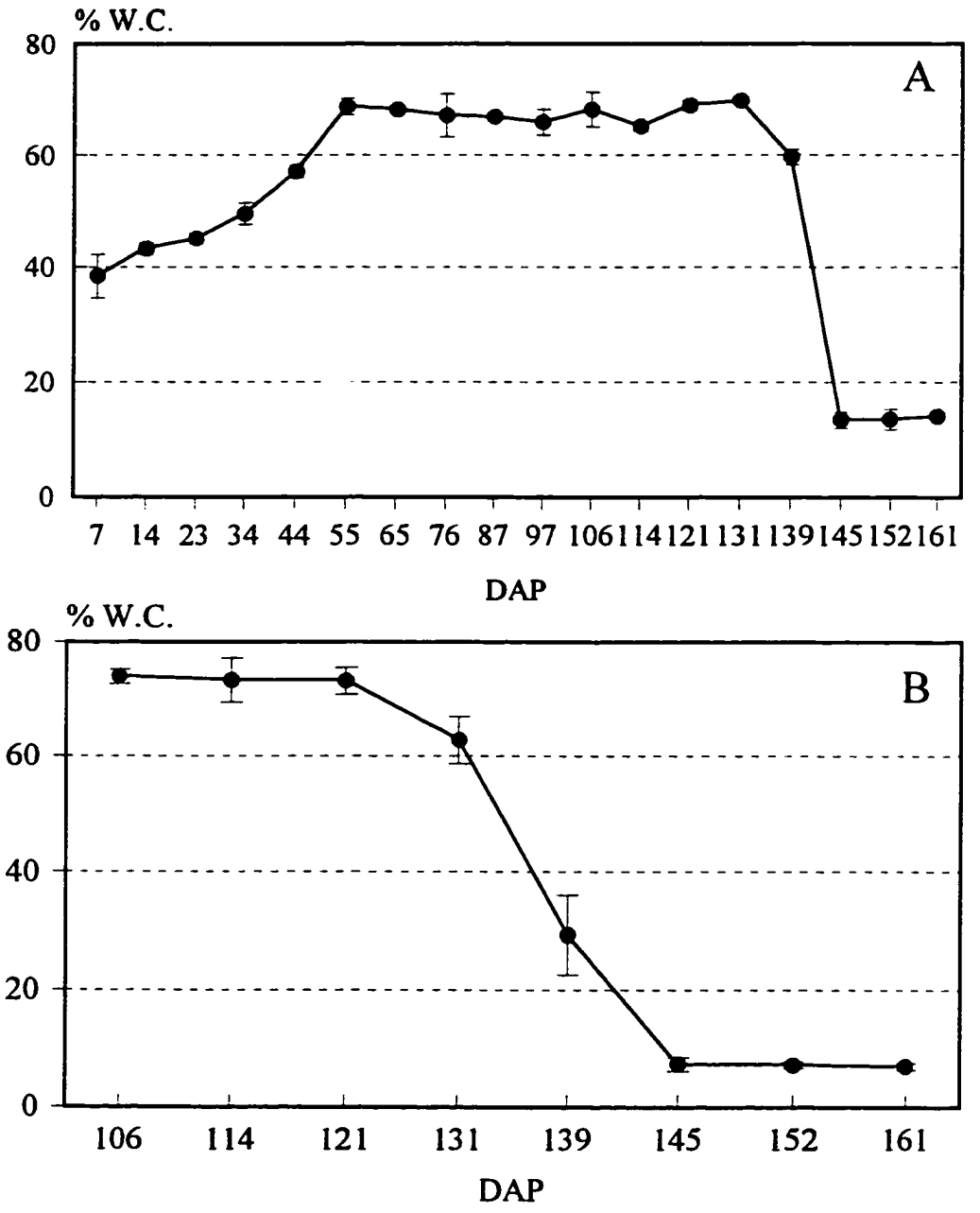

Figure 105. Changes in fruit (A) and seed (B) water content (WC) percentage during fruit development in the *Acacia* hybrid; n=10, DAP=days after pollination, vertical bars epresenting standard errors.

Fig. 106. Transmission electron micrograph (TEM) of the *Acacia* hybrid ovule 7 DAP showing the primary endosperm nucleus (pen) and zygote (z), nu, nucellus; dnu, degenerate nucellus; p, starch-filled plastids; Nu, nucleolus; wi, wall ingrowths; cc, central cell. Bar = 6 μm.

Fig. 107. TEM of the *Acacia* hybrid embryo sac 7 DAP showing fibrillar-containing wall ingrowths adjacent to the embryo sac wall. Bar = 0.5 μm.

Fig. 108. TEM of the *Acacia* hybrid central cell 7 DAP showing two daughter endosperm nuclei (en). Note reduction of the number of plastids containing starch grains. Bar = 3μm.

Fig. 109. TEM of a portion of the *Acacia* hybrid integuments 7 DAP. The inner integument (ii) is separated from the outer integument (oi) by a small gap (arrow), n, nucleus; v, vacuole. Bar = 3 μm.

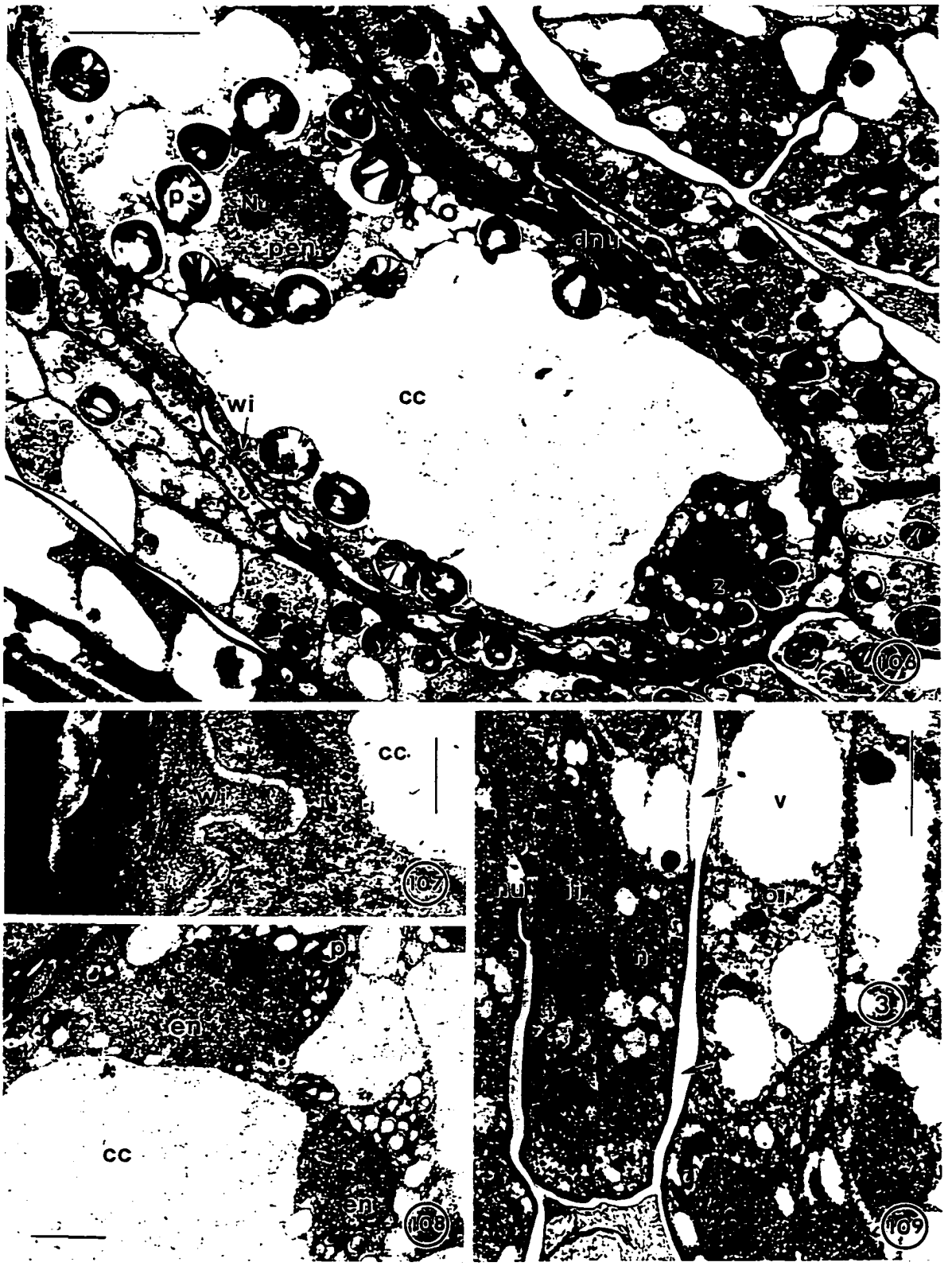
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Fig. 110. Scanning electron micrograph (SEM) showing fertilized (fo) and unfertilized ovules (uo) in the *Acacia* hybrid ovary 14 DAP. The fertilized ovule is half enclosed by the outer integument (oi), fn, funiculus; nu, nucellus. Bar = 100 μm.

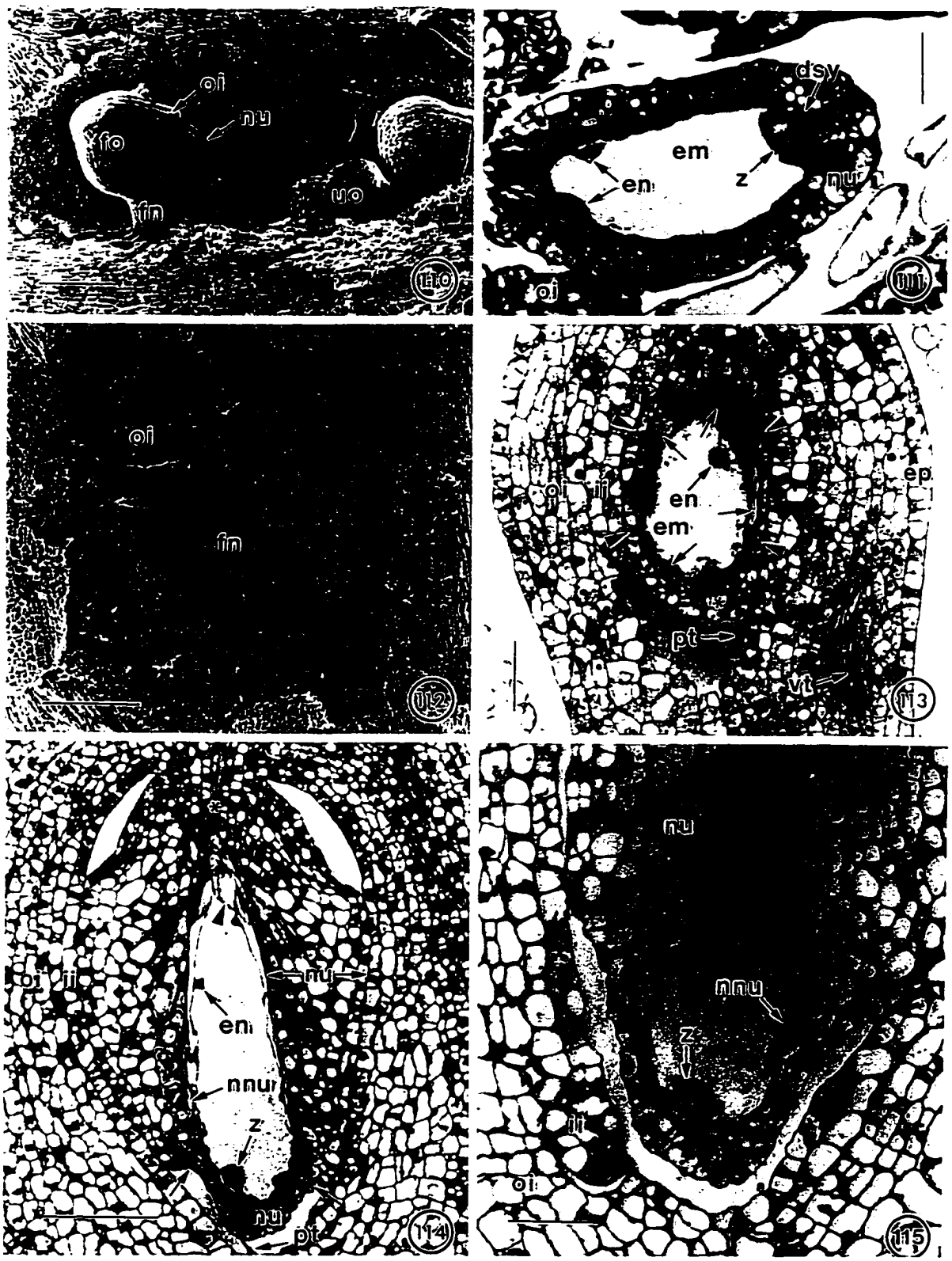
Fig. 111. Light micrograph (LM) of the fertilized *Acacia* hybrid ovule 14 DAP showing the zygote (z) attached to the micropylar embryo sac wall and the free endosperm nuclei (en) in the chalazal region, dsy, degenerate synergid; em, embryo sac. Bar = 30 μm.

Fig. 112. SEM of the fertilized *Acacia* hybrid ovule 24 DAP showing elongation of the funiculus and the outer integument completely enclosing the embryo sac. Bar = 150 μm.

Fig. 113. LM of the fertilized *Acacia* hybrid ovule 44 DAP showing a few layers of the nucellar cells (arrows) in the periphery of the embryo sac. Note that newly-formed nucellar cells are distinguished from the nucellar at anthesis (arrowheads), pt, pollen tube; vt, provascular tissue; ii, inner integument; ep, epidermis. Bar = 30 μm.

Fig. 114. LM of the fertilized *Acacia* hybrid ovule 55 DAP showing the dormant zygote, further development of the nucellar cells (nu) in the periphery of the embryo sac and the haustorial-like nucellar cells (arrowhead) in the chalazal channel connected to the integumentary projections (*), nnu, newly-formed nucellar cells. Bar = 40 μm.

Fig. 115. LM of the fertilized *Acacia* hybrid ovule 55 DAP showing the zygote which has increased in size and contains a large nucleus with a few prominent nucleoli. Bar = 20 μm.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Figs. 116 and 116a. Transmission electron micrograph (TEM) of the *Acacia* hybrid embryo sac about 65 DAP. Fig. 116. Showing the terminal (tc) and basal cells (bc) of the two-celled embryo (em). Note beginning of the cell plate (arrowheads) between the two embryonic cells and accumulation of strongly electron-dense material (arrows) in the intercellular spaces of the micropylar nucellus (nu), n, nucleus; v, vacuole; dsy, degenerate synergid; fa, filiform apparatus; pt, pollen tube; en, free endosperm nucleus; lb, lipid bodies; cc, central cell. Bar = 5 μm. Fig. 116a. Showing arrangement of the microtubules (phragmoplast) (arrows) of the cell plate of Fig. 116. Bar = 0.2 μm.

Fig. 117. TEM showing thin walls (arrows) of the two-cell *Acacia* hybrid embryo and thick, fibrillar-containing layer (la) derived from the degenerate synergid. Note irregular thickening (*) and folding (arrowhead) of the layer. Bar = 1 μm.

Fig. 118. TEM of the micropylar degenerate synergid showing the region (arrow) where the thick, fibrillar-containing layer originates. Bar = 1 μm.

Fig. 119. TEM of a dense cytoplasm of the two-cell *Acacia* hybrid embryo showing abundant mitochondria (m) and ribosomes (r), n, nucleus; ne, nuclear envelope. Bar = 0.5 μm.

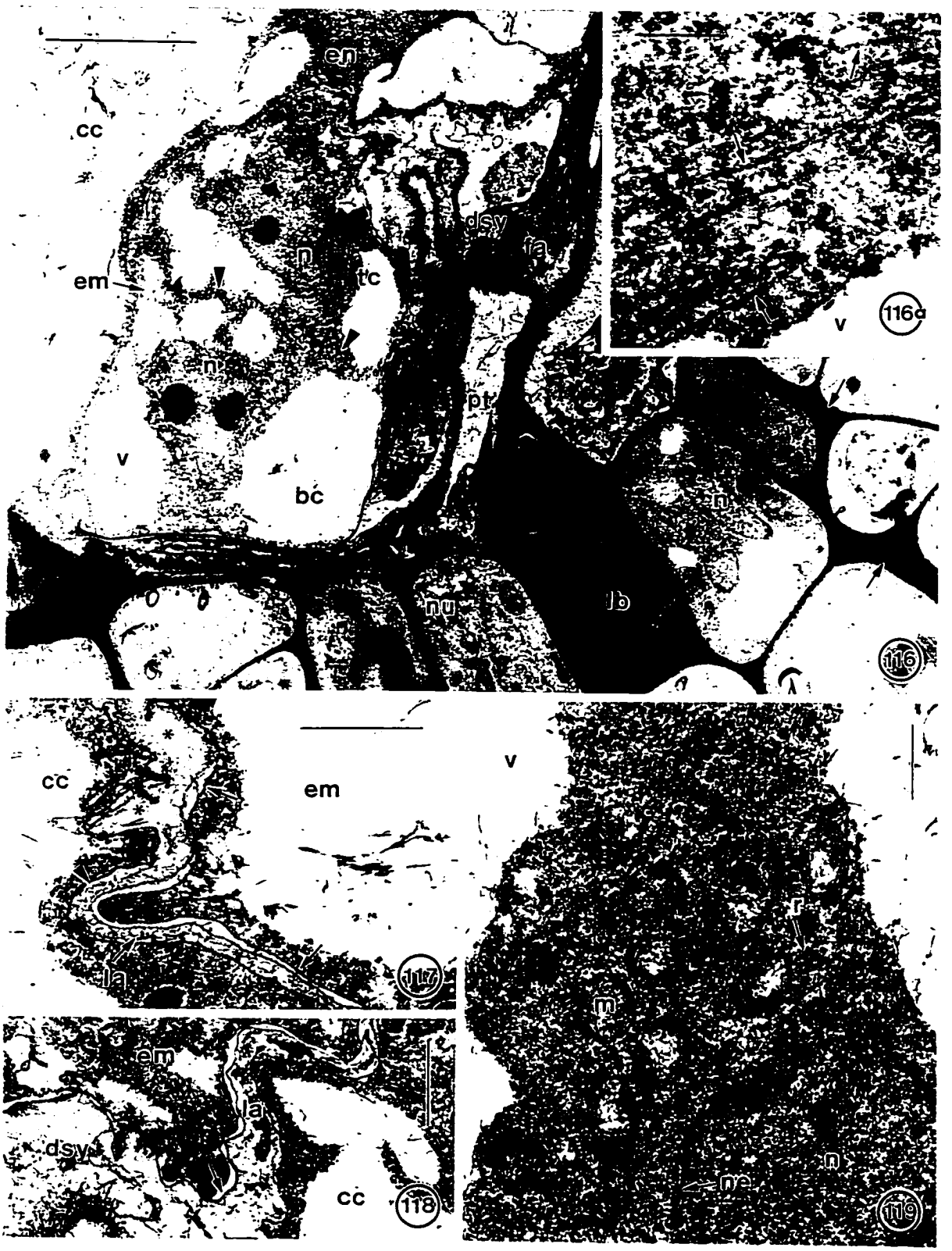
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Fig. 120. Light micrograph (LM) showing the early globular *Acacia* hybrid embryo (em), ce, cellular endosperm; nu, micropylar nucellus. Bar = 20 μm.

Fig. 121. LM of a portion of the *Acacia* hybrid ovule at the early globular stage showing the cellular endosperm, endothelium (et) and inner (ii) and outer integuments (oi). Bar = 50 μm.

Fig. 122. Transmission electron micrograph of the two-cell layer of the *Acacia* hybrid inner integument at the early globular stage showing the endothelial cells containing many vacuoles (v) with strongly electron-dense material (arrows) and dense cytoplasm with abundant organelles. Note cells of the other layer containing peripheral little cytoplasm and large vacuoles with granular material (*). Bar = 3 μm.

Fig. 123. LM showing the late globular *Acacia* hybrid embryo (em) with distinct protoderm (arrows). Bar = 50 μm.

Fig. 124. LM showing cotyledonary primodia (arrows) of the *Acacia* hybrid embryo. Bar = 30 μm.

Fig. 125. LM showing the heart-shaped *Acacia* hybrid embryo surrounded by cellular endosperm (ce), co, cotyledons. Bar = 60 μm.

Fig. 126. LM of the *Acacia* hybrid embryo at linear cotyledon stage showing elongated cotyledons, initiation of the shoot apical meristem (arrow) and provascular tissue (arrowhead) of the embryo axis. Bar = 200 μm.

Fig. 127. LM showing later developmental stage of the *Acacia* hybrid embryo. Note thickened cotyledons, root apical meristem (*), provascular tissues (pv). Bar = 150 μm.

Fig. 128. LM of the *Acacia* hybrid embryo at late development showing the cone-shaped shoot apical meristem (*). Bar = 50 μm.

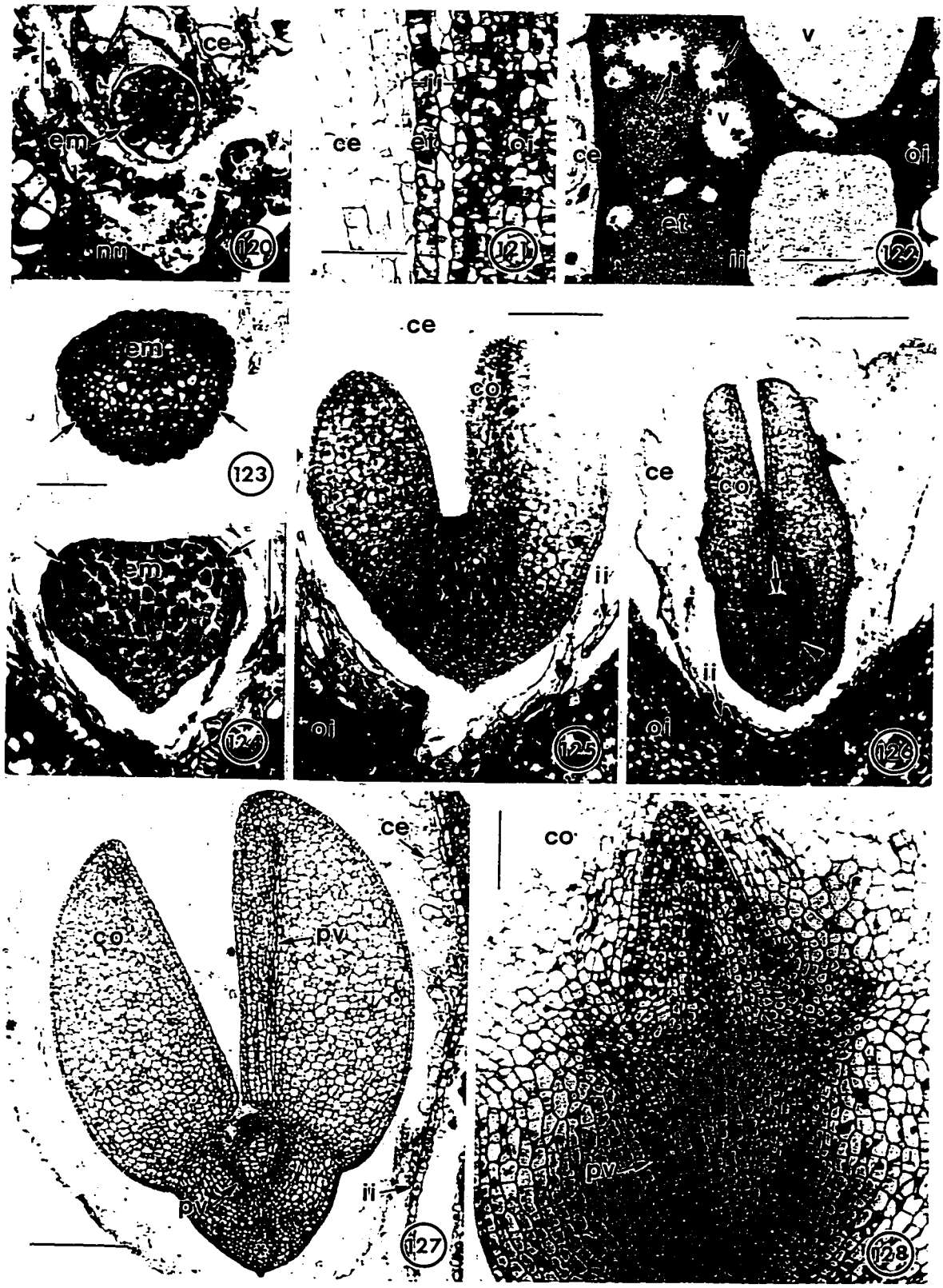
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Fig. 129. Scanning electron micrograph showing a portion of a mature *Acacia* hybrid embryo, pl, plumule; hy, hypocotyl; co, cotyledons. Note the region (*) where a cotyledon is attached to. Bar = 500 μm.

Fig. 130. Longitudinal section of the *Acacia* hybrid embryo axis showing plumule, radicle (ra) without root cap and hypocotyl containing provascular tissue (pv). Bar = 400 μm.

Fig. 131. Cross section of a portion of a mature *Acacia* hybrid cotyledon showing epidermis (ep), subepidermal layer (sep), vascular tissues (arrowhead) and ground tissue (gt). Bar = 8 μm.

Figs. 132-133. Transmission electron micrographs (TEMs) of the epidermal cells of a mature *Acacia* hybrid cotyledon. Fig. 132. Dense cytoplasm containing abundant plastids (p) without starch, mitochondria (m), rough ER (er), dictyosomes (d) and lipid bodies (lb). Note thickening of the adaxial cell wall (*) covered by thin cuticle (arrow), pd, plasmodesmata; n, nucleus. Bar = 2 μm. Fig. 133. Accumulation of the protein bodies (pb) in the vacuoles (v). Bar = 4 μm.

Figs. 134-135. TEMs of the parenchymatous cells of a mature *Acacia* hybrid cotyledonary ground tissue. Fig. 134. Cytoplasm contains abundant mitochondria, starch-filled plastids and lipid bodies. Bar = 1 μm. Fig. 135. Abundant starch-filled plastids and lipid bodies in the periphery of the cell, cw, cell wall. Bar = 2 μm.

Figs. 136-138. Histochemical stains of the mature *Acacia* hybrid embryo. Fig. 136. Section stained with PAS showing starch-filled plastids (arrows) in the cells of the cotyledonary subepidermal layer and ground tissue. Bar = 25 μm. Fig. 137. Section stained with PAS-Amido Black showing proteins bodies (arrows) in the cells of the plumule, and the cotyledonary epidermis and subepidermal layer. Bar = 20 μm. Fig. 138. Section stained with Sudan Black B showing lipid bodies (arrows) in the cotyledonary cells. Bar = 20 μm.

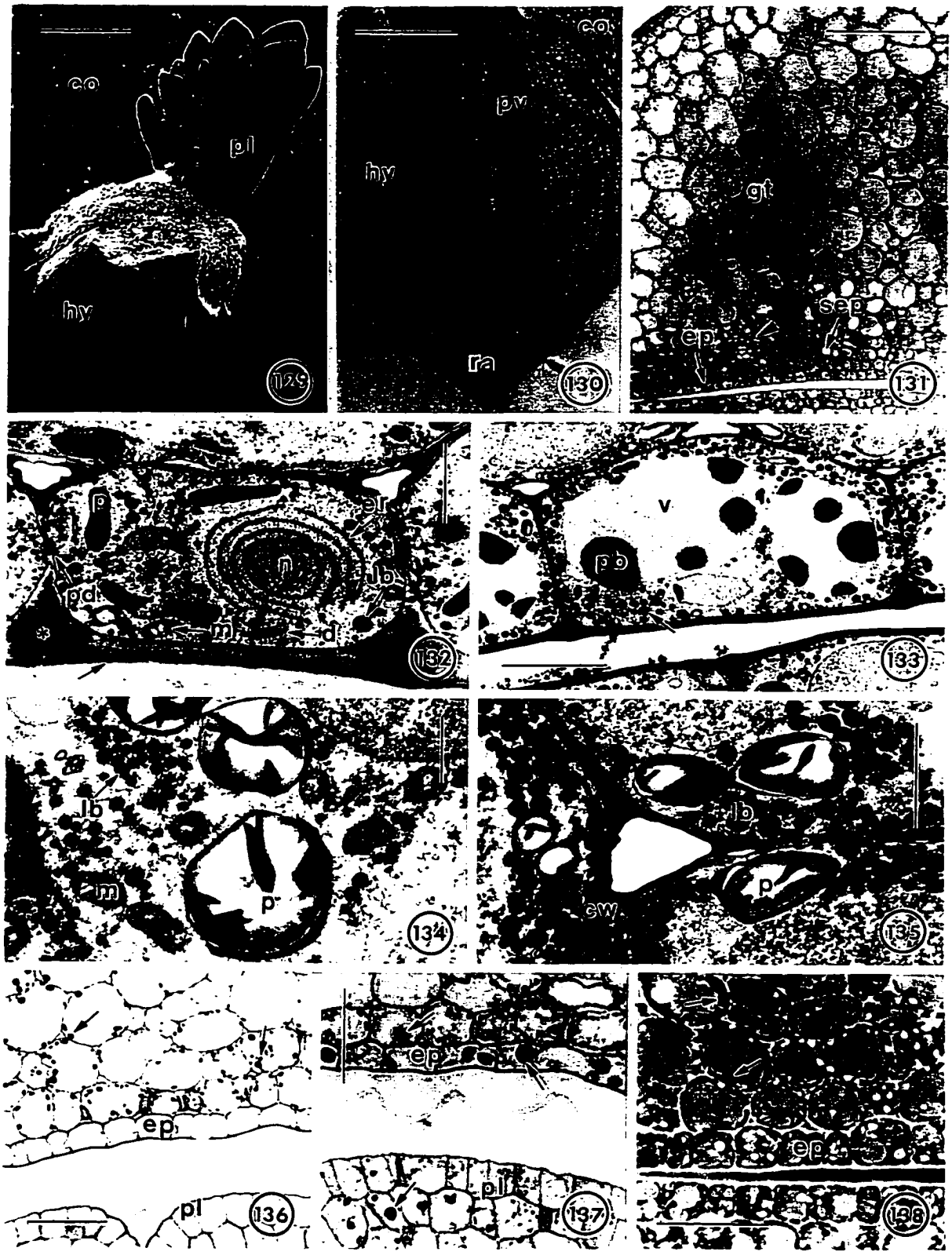
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Fig. 139. Transmission electron micrograph (TEM) showing the *Acacia* hybrid free endosperm nucleus (nc) at the late zygotic stage (about 55 DAP), Nu, nucleolus; cc, central cell. Bar = 2 μm.

Fig. 140. TEM of the *Acacia* hybrid nucellar cells (nu) at the late zygotic stage (about 55 DAP) showing electron-dense cell walls (cw) and dense cytoplasm containing abundant mitochondria (m), plastids (p) with starch grains, dictyosomes (d) and rough ER (er) with dilated cisternae, n, nucleus; v, vacuole. Bar = 3 μm.

Figs. 141-142. TEMs of a portion of an *Acacia* hybrid nucellar cell at the late zygotic stage (about 55DAP). Fig. 141. Accumulation of a protein body (pb) in the vacuole, lb, lipid body; ve, vesicle; pm, plasmalemma. Bar = 1 μm. Fig. 142. Abundant lipid bodies in the cytoplasm and a dictyosome. Bar = 0.25 μm.

Fig. 143. TEM of the micropylar region of the *Acacia* hybrid embryo sac at the late zygotic stage (55 DAP) showing the boundary between the micropylar (mnu) and chalazal nucellus (cnu), dnu, degenerate nucellus; ii, inner integument. Bar = 3 μm.

Fig. 144. TEM of the chalazal region of the *Acacia* hybrid embryo sac at the late zygotic stage (about 55 DAP) showing haustorial-like nucellar cells (hnu) containing fibrillar materials (*). Bar = 5 μm.

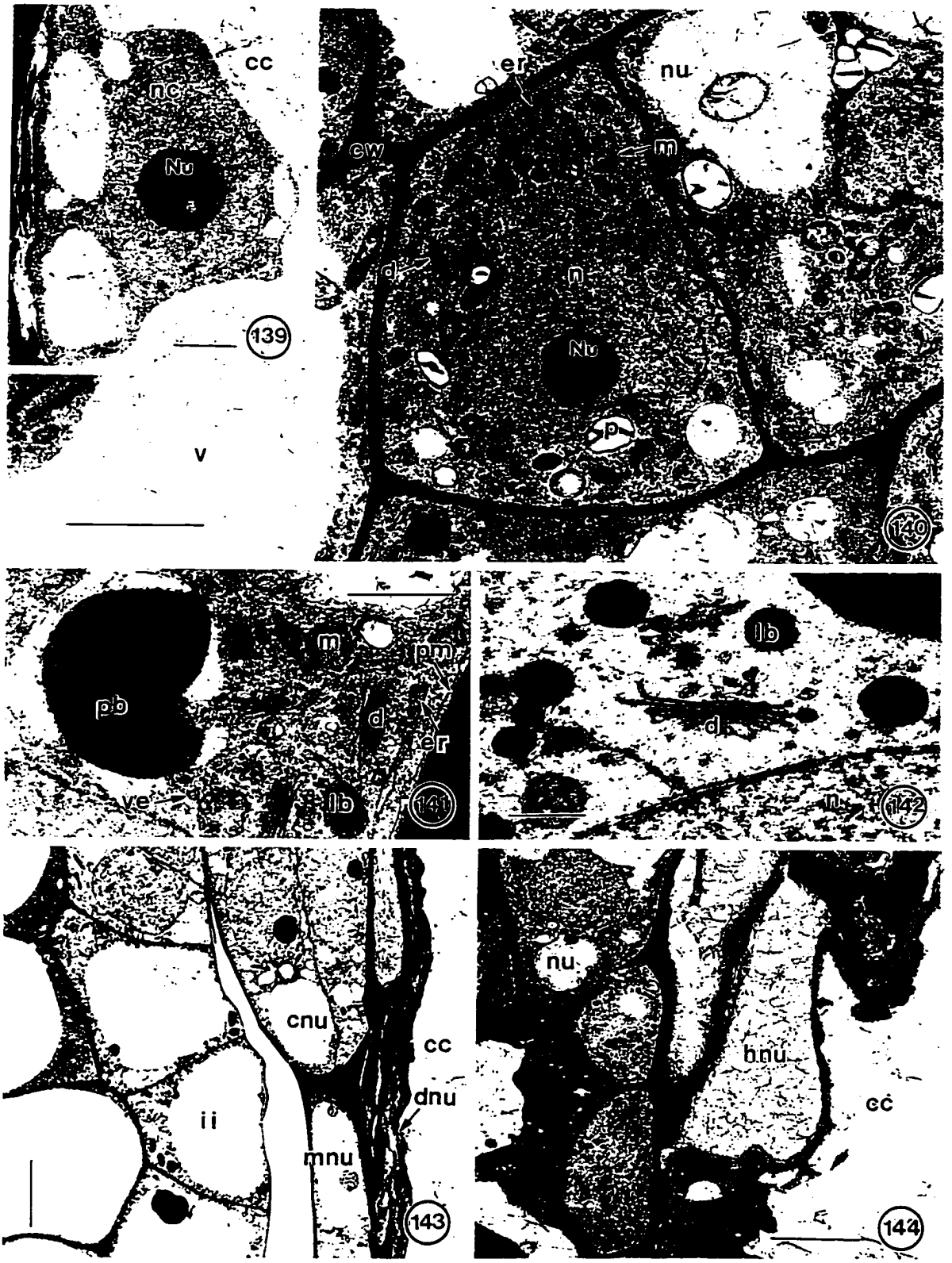
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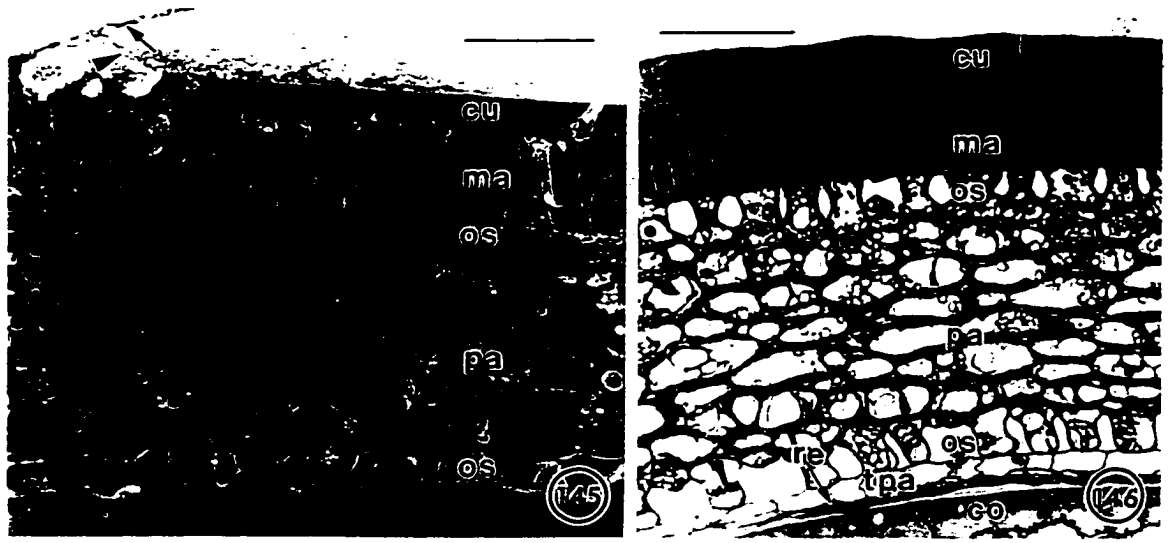
Fig. 145. Scanning electron micrograph showing the structure of a mature *Acacia* hybrid seedcoat consisting of cuticle (cu) with pleurogram (arrow) and fracture line (arrowhead), macrosclereids (ma), osteosclereids (os) and multi-cell layer of parenchymatous cells (pa). Bar = 40 μm.

Fig. 146. Light micrograph of a mature *Acacia* hybrid seedcoat showing cuticle, palisade-shaped, thick-walled macrosclereids, hourglass-shaped osteosclereids, thick-walled parenchymatous cells and thin-walled parenchymatous cell (tpa) derived from the inner integument, re, remnants of the endosperm; co, cotyledon. Bar = 40 μm.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7.4 Discussion

7.4.1 Development of the embryo, endosperm and seed coat

A quiescent period of the zygote is reported in many angiosperms and varies from hours to months (Raghavan, 1986; Sedgley and Griffin, 1989). In the *Acacia* hybrid the zygote has a two-month dormant period. Causes of zygotic dormancy are not well understood but may be related to a delay in the embryo nutrition (Sedgley and Griffin, 1989). In the hybrid, there are at least three possible causes of zygotic dormancy. Firstly, the newly-formed zygote appears metabolically inactive as indicated by the low number and poor development of cytoplasmic organelles (Chapter 6) involved in cell metabolism such as mitochondria, ER and golgi (Mauseth, 1991). This was also observed in other species such as *Quercus gambelli* (Mogensen, 1972) and *Nicotiana tabacum* (Mogensen and Suthar, 1979). These zygotes usually remain undivided for several days. This also appears to be the case in the hybrid zygote, suggesting that the zygote requires a long period for cellular organization and physiological changes before the first cell division (van Went and Willemse, 1984). Secondly, the zygotic dormant period coincides with a period of pod abortion which relates to final fruit set and may be partially determined by nutrient resource allocation by the mother plant (Stephenson, 1981). This resource allocation may also delay zygote development. Thirdly, the hybrid ovule has immature integuments at anthesis (Chapter 4) and cellularization of the free-nuclear endosperm does not occur until the integuments completely enclose the embryo sac. This is followed by degeneration of

the nucellus. Because the cellular endosperm functions as a nutrient source for the embryo (Johri *et al.*, 1992), a delay in zygotic development may be caused by a delay in endosperm development.

The hybrid zygote undergoes a transverse division to form a terminal and a basal cell. Each cell then divides vertically, forming a four-cell embryo, similar to that observed in other *Acacia* species, such as *A. baileyana* (Newman, 1934b), *A. auriculiformis* (Dnyansagar, 1958) and *A. paniculata* (Souza, 1993), and some other members of Mimosoideae, such as *Mimosa hamata* (Dnyansagar, 1951) and *Adenanthera pavonina* (Dnyansagar, 1958). Based on systematic division of angiosperm proembryos (Johansen, 1950), the terminal cell may divide vertically or transversely, whereas the basal cell usually undergoes only transverse divisions. Patterns of the hybrid proembryo cell divisions are different from those of most angiosperms but correspond to the *Trifolium* variation of the Onagrad type except, for formation of the suspensor (Johansen, 1950). This pattern has also been described in other acacias (Newman, 1934b; Narasimhachar, 1948; Dnyansagar, 1958) and some mimosoid species (Dnyansagar, 1952, 1957, 1958).

In many angiosperms, the terminal cell differs ultrastructurally from the basal cell. The terminal cell has dense, organelle-rich cytoplasm, a few vacuoles and some stored products and gives rise to an embryo proper, whereas the basal cell has little cytoplasm and many small and large vacuoles and develops into a suspensor, as in *Vicia* (Folsom and Cass, 1992), *Pennisetum* (Taylor and Vasil, 1995) and *Solanum* (Briggs, 1996). The terminal and basal cells of the hybrid proembryo have similar ultrastructures in which both cells are densely cytoplasmic and contain many large and

small vacuoles. Also, no suspensor is observed throughout the course of embryo development. This indicates that both terminal and basal cells develop into the embryo proper as observed in some mimosoid plants (Dnyansagar, 1958). Embryos lacking suspensors have been reported in all acacias studied thus far (Guignard, 1881; Newman, 1934b; Narasimhachar, 1948; Dnyansagar, 1958; Souza, 1993, also see review by Lersten, 1983) and many other members of the Mimosoideae, such as *Mimosa* (Dnyansagar, 1951, 1954 also see review by Johansen, 1950) and *Calliandra* (Dnyansagar, 1958).

During the late globular to mature embryo stages, the embryo undergoes cell divisions and differentiation to form cotyledons, plumule, hypocotyl, radicle, and a vascular system as observed in other acacias (Narasimhachar, 1948; Dnyansagar, 1958; Souza, 1993). The mature hybrid embryo possesses features similar to those of most members of Mimosoideae (Gunn, 1981; Smith, 1981; van Staden *et al.*, 1989). The embryo axis is small, straight and consists of a well-developed plumule with compound leaves and a radicle with tapered tip and lacking a root cap. Provascular bundles are well developed in the oval-shaped hypocotyl. Cotyledons are large, fleshy, lack palisade mesophyll and enclose nearly all of the embryo axis except the radicle tip. The cotyledonary vascular system is well developed along the adaxial surface.

Seed storage products are common in legumes but their components and localizations vary among species (Smith, 1981). In albuminous seeds, such as in *Cercis siliquastrum*, only lipids and proteins are usually stored in the embryo and endosperm (Baldan *et al*, 1995), whereas in *Trifolium repens*, proteins and starch accumulate in the embryo and endosperm (Jakobsen, *et al.*, 1994). In exalbuminous

seeds such as *Vicia*, starch and proteins are predominant products in the embryo (Johansson and Walles, 1994; Borisjuk *et al.*, 1995). The hybrid seeds are exalbuminous. Lipids are mainly stored in the cotyledons and starch accumulates in nearly all cotyledonary cells except the epidermis. Proteins occur only in the cotyledonary epidermis and subepidermal layer, and embryo axis. The time and pattern of reserve accumulations in hybrid seeds were not observed. In other legumes such as *Glycine* (Zheng *et al.*, 1992) and *Vicia* (Borisjuk *et al.*, 1995), accumulation of storage products occurs during late embryo development. Two mechanisms have been proposed for the formation of the embryonic protein bodies in many legumes. Protein bodies may be derived from vacuoles as in *Lupinus albus* (Davey and van Staden, 1978) and *Cercis siliquastrum* (Baldan *et al*, 1995), or develop from rough ER (Bain and Mercer, 1966) or smooth ER (Craig *et al.*, 1979) as in *Vicia faba* (Adler and Muntz, 1983). In the hybrid, protein bodies are localized only in the large vacuoles, suggesting that the mechanism of their formation may be similar to that of *Vigna unguiculata* and *Lupinus sp*. Lipid body formation in the hybrid embryo may be associated with secretory organelles such as dictyosomes and vesicles which are abundant in the embryonic cells, as suggested in *Cercis siliquastrum* (Baldan *et al*, 1995).

Formation of the hybrid endosperm is of the nuclear type, as in nearly all legumes (Dnyansagar, 1951; 1957, 1958; Souza, 1993; Chamberlin *et al.*, 1994; also see review by Johri *et al.*, 1992). However, the pattern and time of cellularization of the free nuclei vary among species. In many legumes such as *A. auriculiformis* (Dnyansagar, 1958), *Prosopis spicigera* and *Desmanthus virgatusis* (Dnyansagar,

1957) and *Glycine max* (Dute and Peterson, 1992; Chamberlin *et al*, 1994), wall formation begins at the early globular embryo stage in the micropylar region of the embryo sac and extends chalazally. Wall formation in the hybrid appears similar to that of *A. apiculata* (Souza, 1993), in which it occurs during the zygote stage first in the chalazal region and proceeds toward the micropyle and centripetally. This is different from cell wall formation in *A. auriculiformis*. In the hybrid and many other legumes (Rau, 1953; Bhojwani and Bhatnagar, 1975; Souza, 1993), the free endosperm nuclei completely cellularize. However, in some cases, cellularization may occur only around the embryo, as in *Phaseolus* or only in the chalazal region of the embryo sac, as in *Crotalaria* (Bhojwani and Bhatnagar, 1975). Patterns of hybrid endosperm degeneration appear similar to that of *A. paniculata* (Souza, 1993) in which it begins in the chalazal embryo sac and proceeds toward the micropylar region.

In many mimosoids, the seedcoat forms from the outer integument and the inner integument degenerates during embryo development (van Staden *et al.*, 1989). However, there are variations in seedcoat formation and structure among *Acacia* species. In *A. auriculiformis*, the seedcoat differentiates only from the outer integument and consists of macrosclereids, multi-layered parenchyma, and hypodermal and inner osteosclereids (Pukittayacamee and Hellum, 1988). The seedcoat forming from the outer integument is also reported in *A. grandicornuta* but inner osteosclereids are absent (Robbertse, 1974). In *A. paniculata*, the seedcoat develops from the outer integument which differentiates into macrosclereids and multi-layered parenchyma, and the inner integument which differentiates into the inner layer consisting of thin-walled parenchymatous cells. Structure of the hybrid seedcoat is different from these species

and *A. auriculiformis* but may be similar to that of *A. mangium* which has not been reported. The hybrid seedcoat is formed from both the inner and outer integuments. The outer integument gives rise to layers of macrosclereids, multi-layers of parenchyma, hypodermal and inner osteosclereids, whereas the inner integument differentiates into a single layer of thin-walled parenchymatous cells.

7.4.2 Nutrition of the embryo sac

The hybrid ovule shows a number of modifications which appear to allow it to provide nourishment and to facilitate nutrient movement into the developing endosperm and embryo. Immediately after fertilization, the ovule consists of the nucellus and integuments, and the embryo sac contains the zygote, degenerate synergids, and the central cell containing the primary endosperm nucleus with abundant starch and wall ingrowths. Compared to the zygote, the primary endosperm is active and undergoes the first division shortly after fertilization as in sunflower (Yan *et al.*, 1991). This may be due to the fact that, before and after fertilization the central cell contains abundant nutrient reserves and cytoplasmic organelles involved in synthesizing metabolites required for division of the primary endosperm nucleus (Chapter 4). In *Glycine*, nutritional reserves of the central cell break down before fertilization, nourishing the egg, zygote, embryo and endosperm (Folsom and Cass, 1992). However, in the hybrid, the central cell nutrient reserves, such as starch and lipids, decrease then disappear during the two-nucleate endosperm stage. This indicates that the large amounts of metabolites stored in the hybrid central cell are used only by the

early developing endosperm since the zygote remains dormant for about two months and its first division does not occur until the cellular endosperm occupies about half the embryo sac.

Degeneration of the nucellus after fertilization providing nutrients for the embryo sac is reported in some legumes such as *Vicia* (Johanssan and Walles, 1993a, b) and *Glycine* (Chamberlin *et al.*, 1994). This may also be the case in the hybrid. The nucellar cells substantially increase in number after fertilization and the cells contain abundant nutrient reserves, particularly starch and lipids. Post-fertilization development of the nucellus is likely supported by the nutrients translocated from the maternal tissues via the integumentary vascular bundles which extend through the funiculus. The endosperm cellularization is completed when most nucellar cells, except in the micropylar region, disappear. This suggests that nucellar degeneration provides the nutrition necessary for endosperm development and the central cell wall ingrowths may increase metabolite transfer from the degenerating nucellus into the embryo sac, as suggested by Vijayaraghavan *et al.* (1988). It is also evident that these nutrients may also be consumed by the developing embryo because the nucellar degeneration occurs during development of the embryo from the two-cell stage into the globular stage.

Haustorium is well known in many Families and can occur in many ovular cell types such as endosperm, synergid or antipodals (Johri *et al.*, 1992). Haustoria may develop wall ingrowths of the transfer-cell type, as in *Lobelia dunnii* endosperm (Torosian, 1971) or become very large cells, as in *Grevillea robusta* (Venkata Rao, 1967). Vijayaraghavan *et al.* (1988) suggest that the haustorial cells, which usually

form during embryo development, may increase the nutrient flow from the surrounding tissues into the embryo sac. In the hybrid, during embryo sac development, large nucellar cells which contain very little cytoplasm and large vacuoles containing fibrillar material form in the chalazal channel. These large cells may function as the nucellar haustoria because they connect to the integumentary projections into the chalazal embryo sac, probably increasing metabolite transfer from the integuments to the embryo sac. This may also be the nutrient pathway between the integuments and embryo sac during the the late zygotic and a few-cell embryo stage. The nutrients available in the integuments are translocated via the vascular bundles extending from the maternal tissues.

Synergid haustoria have been reported in many species and may function in increasing nutrient absorption (Johri *et al.*, 1992). Their forms vary among species, for instance, in *Quinchamalium chilense*, synergid haustoria develop long appendages projecting into the stylar base, increasing nutrient absorption into the embryo sac (Johri *et al.*, 1992). In the hybrid, the newly-formed zygote contains many starch which is significantly reduced in the two-cell embryo. Breakdown of this starch apparently provides nutrients for the zygote development. At the two-cell embryo stage, the embryo is enclosed by a thick, fibrillar-containing layer derived from the degenerate synergid. Also, some regions of this layer become irregularly thickened and folded. It is possible that the degenerate synergid possesses haustorial-like functions because the embryo has to compete with the developing endosperm for nutrients transferred from the maternal tissues. This layer may increase absorption of the nutrients from the embryo sac into the embryo. However, this layer disappears during the early globular

embryo stage, indicating a short functional period. The collapse of this layer may result from enlargement of the embryo. This feature has not been reported in other legumes.

The early hybrid embryo lacks a suspensor but remains attached to the micropylar nucellus and the embryo sac wall until late embryo development, as in *A. paniculata* (Souza, 1993). The suspensor has a key role in supporting embryo development as a site of nutrient absorption and hormone synthesis (Cionini, 1987; Johansson and Walles, 1994; Taylor and Vasil, 1995, also see review by Yeung and Meinke, 1993). In the hybrid, it is still questionable whether the micropylar nucellus is functionally comparable to the suspensor. TEM studies show that the micropylar nucellar cells contain abundant stored products but there are no wall ingrowths of transfer cells. One of its functions may be to house the embryo during early development. This needs further investigation.

In the hybrid, the presence of the endothelium during certain stages of embryo development suggests its functions relate to embryo sac nutrition. The endothelium forms from the inner layer of the inner integument during the early globular stage and then disappears during the late globular stage. The endothelial cells possess features similar to those of *Glycine* (Chamberlin *et al.*, 1993, 1994), in that they have dense cytoplasm containing abundant organelles, suggesting that the cells are metabolically active. Autoradiographic studies in *Glycine* ovules reveal that metabolites from the integumentary tissues move into the embryo sac through this layer (Chamberlin *et al.*, 1993). A function of the hybrid endothelium may be similar to that of *Glycine* in that it facilitates nutrient transport from maternal tissues into the embryo sac. The central-

cell wall ingrowths and haustorial-like nucellus disappear when endosperm development is completed at that time. The endothelium appears to be the main nutrient pathway between the integuments and embryo sac.

The function of the endosperm as a nutrient source for the developing embryo has been suggested in many angiosperms (Rau, 1953; Sehgal and Gifford, 1979; Johansson and Walles 1994; Palupi 1996, also see review by Johri *et al.*, 1992) with the exception of some species such as *Pisum* (Marinos, 1970), *Quercus* (Singh and Mogensen 1976) and *Glycine* (Chamberlin *et al.*, 1994). In the hybrid, the developing endosperm acts as a sink and is nutritionally supported by the central cell, digested nucellus and integuments. During late embryo development, when the endosperm is completely developed, the nucellus, endothelium and haustorial-like nucellus degenerate and disappear. The endosperm then becomes a main nutrient source and its degeneration appears to provide nutrition for the growing embryo. In some legumes, such as *Vicia faba* (Johansson and Walles, 1994), the cotyledonary epidermal cells develop wall ingrowths and intercellular spaces to facilitate the transport of the nutrients from degenerated endosperm into the embryo.

CHAPTER 8

General Conclusions and Future Research

The study elucidated many aspects of reproductive biology, and seed and seedling qualities of the *Acacia* hybrid (*A. mangium* Willd x *A. auriculiformis* A. Cunn. x Benth). Both parental species have been introduced to Thailand. The reproductive aspects concentrate on phenology, floral biology, pollination mechanism, fertilization and embryogenesis using light, electron and fluorescence microscopy, and histochemistry. The hybrid shows some reproductive characteristics that are similar to those of the parental species growing either in tropical regions and their natural habitats. Some of the characteristics have not been reported in other acacias. The major reproductive features include:

Phenology

1. Complete development of hybrid flowers and fruits occurs within 50 to 60 days and 140 to 145 days, respectively

2. The hybrid has two peak flowering periods in July and August, and October through mid-November, which coincide with high total rainfall and high temperature. Two peak fruit and seed maturation periods occur in December and from February

through early March when the weather is dry and windy. This may ensure optimum seed dispersal.

Floral biology

1. The hybrid flowers are grouped into a spike containing about 150 flowers (63 to 100% hermaphroditic flowers and 0 to 37% staminate flowers).

2. Flowers are cream colored, fragrant and have no floral nectaries. The style is of the solid type with a smooth, wet stigmatic surface. The ovule is amphitropous and has immature integuments. The anther consists of eight loculi, each bearing only one 16-grain polyad.

3. The hybrid flowers are weakly protogynous. Anthesis is complete at 0500-0600 h but peak female receptivity begins at 0200-0300 and is completed the same day. The stigmatic exudate is lipophilic and secreted from the stigmatic cells by a holocrine mechanism.

4. The pollen to ovule ratio of the hybrid is 1, indicating a moderately efficient pollen transfer system.

Pollination mechanism

1. Honey bees (*Apis mellifera*) and carpenter bees (*Ceratina* sp.) are the most effective pollinators but have different foraging activities.

2. Pollen is the main floral reward and there are a number of floral characteristics which facilitate pollen transfer from the male to the female floral organs.

3. Close spatial and temporal separation of male and female floral organs and behavior of insect pollinators may promote self-pollination in the hybrid.

4. The hybrid has low pollination success due to low pollinator number. An increase in exposure time of flowers to pollinators or pollinator number may increase pollination success but may not affect the rate of pollen deposition on stigmas.

Post-pollination events

1. Hybrid pollen has the highest viability (over 80%) at anthesis but loses most viability within 3 days.

2. *In vivo* pollen germination occurs within a few hours after pollination. All viable polyad grains are capable of germinating due to the presence of a copious stigmatic exudate.

3. A stylar secretion may be triggered by pollination. It contains predominately lipids and carbohydrates secreted from the degenerate transmitting cells. An ovarian secretion occurs but is independent of pollination and mainly contains proteins and carbohydrates probably secreted from the ovarian trichomes.

4. The pollen tubes grow rapidly, up to 16 μm/min, and reach the ovules 7 to 8 hr after pollination. Pollen-tube competition may not occur in the hybrid and the pollen-tube penetration of the ovules is low and appears to be random.

Fertilization

1. The hybrid embryo sac consists of the egg, synergids, central cell and antipodals. These cells differ ultrastructurally and histochemically.

2. One of the two synergids is the site of pollen tube penetration. Its degeneration is triggered by the pollen tube penetration of the nucellus.

3. Polar nuclear fusion is associated with ER, whereas the fusion of the sperm nucleus with the polar nuclei and with the egg occurs by alignment and contact of both nuclei.

4. The hybrid exhibits maternal cytoplasmic inheritance.

Fruit and seed development

1. The hybrid zygote has a two month dormant period. This may be caused by a metabolically inactive zygote, and delays in nutrient resource allocation by the mother plant and delays in embryo sac nutrition from the ovular tissues and endosperm.

2. Proembryo cell divisions are of the *Trifolium* variation of the Onagrad type without formation of a suspensor. Endosperm formation is of the nuclear type.

3. The micropylar nucellus may play a key role in housing the embryo during early development.

4. During early development, the endosperm is nourished by stored products from the central cell, digested nucellus and other maternal tissues through different pathways, including wall ingrowths, haustorial-like endosperm cells, and the endothelium. The endosperm then becomes the main nutrient source for the growing embryo during late embryo development.

5. The mature hybrid embryo consists of an oval, small embryo axis with a well-developed plumule, hypocotyl and radicle, and two large, broad, fleshy cotyledons.

6. Carbohydrates, lipids and proteins are the main seed storage products and are in different embryonic tissues.

7. The mature seedcoat is formed from both the inner and outer integuments and composed of macrosclereids, hypodermal and inner osteosclereids, and multi-layered parenchymatous cells.

Variation in flower, fruit and seed production

1. The number of flowers per spike and floral organs per flower vary significantly among trees but appear intermediate between the parental species.

2. There are significant variations in the number of pods per spike and seed per pod among trees, crown levels and crown quadrants.

Fruit and seed efficiency

1. The hybrid has very low reproductive success (0.0054).

2. In open-pollination, low fruit set (2%) is mainly caused by insufficient pollination (65%) and early fruit abortion (33%). Low seed set (24%) is caused by failure of pollen tubes to penetrate the ovules (over 70%) and by seed abortion.

Seed and seedling quality

1. At maturity, pod and seed water content are about 13% and 6%, respectively. Thousand-seed weight averages 15 gm and viable seeds average 93%.

2. Seed treatments using both cutting and soaking in boiling water for 1 min give high germination percentages (over 80%) but the latter treatment is more practical in the nursery.

3. Development of F_2 hybrid seedlings appears intermediate between the parental species.

4. The 3-month-old F_2 hybrid seedlings have a high survival rate (90%). Their height and diameter growths vary significantly among parental trees but are superior to those of the parental species.

Future research

This study has revealed many reproductive aspects of the hybrid, particularly from pollination to embryogenesis. Compared to the parental species, the hybrid has shown some potential characteristics such as seed quality and seedling performance, suggesting that the hybrid may be used for plantations. The study also elucidate main causes of low seed production, which mostly occur during reproductive process. This provides a useful information for the future research to increase fruit and seed production of the hybrid. Also, based on the techniques used in the this study, there are some major points which remain to be answered. The important future research may include:

1. In open-pollination, the hybrid has shown features typical of gametophytic self-incompatibility, i.e. arrest of the pollen tubes in the style and nucellus, abortion of the male gametes in the embryo sac, and post-zygotic abortion, i.e. early fruit abortion. Whether or not these result from self-pollination needs to be determined using controlled pollinations.

2. Low fruit set is attributed mainly to lack of pollination. An increase in fruit set may be obtained by increasing pollination success using supplementary pollination. Because hybrid pollination is associated with insects, techniques of mass supplemental pollination using domestic insect pollinators, such as bees, needs to be developed.

3. Fruit and seed abortion in the hybrid may be partially attributed to limited maternal nutrient resources. Application of some silvicultural treatments such as fertilization and thinning may increase nutrient levels in the maternal trees and thus fruit

and seed production. Appropriate silvicultural treatments for the hybrid need further investigation .

4. Growth performance of F_2 hybrid seedlings at early developmental stages varies among trees but some characteristics, such as height and diameter growth are superior to the parental species. However, growth performance and other seedling characteristics such as resistance to pest and disease need further investigation, especially when seedlings are outplanted into the field. Outplanting will also show whether undesirable characteristics will be exhibited in F_2 generation at later developmental stages.

5. Results of this study using light, electron and fluorescence microscopy, and histochemistry revealed some reproductive characteristics of the hybrid, which have not been reported in other acacias. Application of new techniques to the hybrid may give insight into sexual reproduction of *Acacia* species, which represent the largest genus of the angiosperms.

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