Embryology of *Swainsona formosa* (Fabaceae): Anther and Ovule Development

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Diterima 22 Juni 2004/Disetujui 7 Februari 2005

Our study showed that floral morphology and anatomy in *Swainsona formosa* were typical of leguminous flower. The anthers were found to be tetrasporangiate, with a 3-layered wall below the epidermis. The wall was comprised of a layer of endothecium, middle layer and secretory tapetum. Pollen grains were triporate and shed at a two-cell stage. The ovules were campylotropous with a zigzag micropyle. Multiple embryo sacs were occasionally found but only one mature embryo was formed in the seed. Rubbing the receptive stigma with fingertip enhanced pollination, resulted in 100% pod formation on treated flowers. During fertilization, the pollen tube entered the embryo sac via one of two existing synergids, destroying the synergid in the process. The endosperm was of nuclear type, and only one or two layers of endosperm left as the seed reached its maturity. The seed coat was composed of a single layer of thick-walled palisade cells on the outside followed by a single layer hypodermal sclereids on the inside.

INTRODUCTION

In general, plant embryology is defined as a study of the developmental process in anthers and ovules leading to the formation of seeds (Prakash 1987). O’Connor (1991) stated that the production of seeds was a critical factor for population maintenance. It ensures the renewal of populations and sustainability of a particular flowering plant (Rost et al. 1998). However, seed production in a species is very dependent upon embryological processes starting from the formation of male and female gametes, to pollination and fertilisation until embryo formation and maturation. Any abnormality at any stage in this process will lead to the failure of sexual plant reproduction, and hence seed production.

There is considerable embryological literature on the Fabaceae to which *Swainsona formosa* belongs. However, despite many botanical and agronomic studies (Thompson 1990, 1993; Jusaitis 1994), there has been no adequate embryological data on this species. In fact, the importance of embryological information is not only in the evaluation of the phylogeny of taxa (Prakash 1987; Tsou 1997; Floyd et al. 1999), but also in the development of a plant breeding programme. The anatomy of anthers and ovules, micro- and megsapogogenesi, development of gametophytes, fertilisation and growth and development of the embryo, endosperm and seed coat are all important aspects in plant embryology (Palser 1975). Furthermore, Prakash (1987) noted that embryological data provides valuable information on many aspects of sexual reproduction, as well as on the histology of developmental sequence in anthers and ovules leading to seed formation in plants. The present study was aimed at investigating the embryological development and sexual reproduction of *S. formosa* in order to reveal characters that are widely regarded as of systematic value.

MATERIALS AND METHODS

Plant Materials. Floral buds, flowers and shoot apices of different stages were collected from glasshouse-grown plants. These materials were fixed in a formalin-propiono-alcohol (FPA) solution for at least 48 hours until required. The FPA was prepared by mixing 90 ml 70% ethyl alcohol, 5 ml propionic acid, and 5 ml formalin (Prakash 2000). Very young floral buds were processed as a whole, while the pistil and anthers of older flowers, petals and sepals were removed prior to fixing.

Dehydration. Before dehydration the specimens were washed in 70% alcohol for 5-10 minutes. The specimens were dehydrated through a series of tertiary butyl alcohol solutions (TBA) for 2 hours in each grade (‘50’, ‘70’, ‘85’, ‘95’, and ‘100’) and for 24 hours in pure TBA (with one change after the first 12 hours).

Infiltration. After dehydration was complete, the specimens were placed in a specimen bottle half-filled with pure TBA and paraplast chips were added until the solution came up to the 3/4 level. The bottle was then kept on top of an oven at 60 °C to dissolve the paraplasts. After the paraplasts were completely dissolved, half of the alcohol-paraplast mixture was poured away and the volume was topped up with molten paraplasts. The molten paraplasts were changed three times at hourly-intervals.

Embedding. The content of each specimen bottle was poured into a paper tray approximately 1 x 1 x 1 cm. Anthers were placed in a vertical position while ovaries and shoot apices that are difficult to place upright due to their larger size were placed horizontally. The entire mass was then cooled until the wax block completely solidified.

Sectioning. The wax block was trimmed to a suitable size and shape and mounted in the specimen holder. All of the sections were cut to a thickness of 12 µm using a rotary microtome (American Optical 820).
Mounting. The ribbon was cut into sections of approximately 4 cm, and transferred into warm water (40 °C) in a warming water bath and left until the sections had wholly straightened out.

A small drop of Haupt’s solution (an adhesive) was placed and smeared evenly over the surface of a frosted microscope slide (76 x 26 mm) until only a barely perceptible film remained. The Haupt’s solution was prepared by mixing 1 g plain gelatin, 2 g phenol crystals and 15 ml glycerin in 100 ml distilled water (Prakash 2000). The ribbon was placed and carefully arranged on the slide. The mounted slide was then put on a warm plate (40 °C) for a few minutes to flatten out any wrinkles in the ribbon and to remove excess water before being cooled down and completely dried in a low temperature oven (40 °C) for 24 hours.

Staining. The dried mounted-slide was placed in histolene for 30 minutes to remove the paraplast followed by the slide being transferred into a histolene-alcohol solution for 15 minutes for clearing. The slide was then transferred to 95% ethyl alcohol for 10 minutes, and then passed through 80%, 70%, and 50% ethyl alcohol for 5 minutes each.

The specimens were stained with 1% aqueous safranin for 24 hours, followed by two rinses in distilled water for 3 minutes each. The slide with the specimen was then passed through 50%, 70%, and 90% ethyl alcohol for 2-4 minutes each before being stained with 0.5% fast-green in 95% alcohol for 10 seconds.

After being stained with safranin and fast-green, the slide was washed in absolute alcohol for 10 seconds, then in a histolene-alcohol solution (1:1) for 15 seconds. It was then placed in histolene I for 15 minutes followed by placing it in histolene II for 48 hours. Finally, the slide was dried in a low temperature oven (40 °C) until completely dry.

Permanent Mounting. A few drops of Euparal (a synthetic resin) were placed on the slide with the stained specimen. Euparal was prepared by mixing 10 ml camsal, 40 ml gum sandarac, 20 ml eucalyptol, 20 ml dioxane, and 10 ml paraaldehyde. This mounting medium has a refractive index of 1.48 (Prakash 2000). A cover slip (No. 1 thickness, 50 x 24 mm) was used to cover the entire specimen. The slide was then dried in a low temperature oven (40 °C) to spread the mounting medium and dry the specimen. When completely dry, the slide was ready for microscopic examination.

Hand Pollination. Flowers were hand-pollinated to aid in fertilization and seed production. Flowers at anthesis were pollinated by rubbing the stigma several times with a fingertip covered with freshly shed pollen grains. In this study both stigma and pollen grains were from the same flower.

RESULTS

Anther Anatomy. Microscopic observation showed that the anthers of S. formosa consisted of four compartments or locules, i.e. the anthers were tetrasporangiate (Figure 1a). The young and mature anther walls in S. formosa, which laid under the single-layered epidermis, consisted of three layers: endothecium, middle layer and tapetum (Figure 2a, b). The epidermis was present throughout anther development. However, the middle layer and tapetum degenerated before or during meiosis leaving only the endothecium and the epidermis (Figure 2c). The endothecium developed fibrous thickening on the radial and inner tangential walls when microspore development was at the uninucleate stage (Figure 2d), and enabled the mature anthers to dehisce and the pollen to be dispersed. Anther dehiscence started from the broken septum, which separated the two sporangia on each side. This broken wall resulted in an opening through which the pollen grains were released (Figure 1b).

Microsporogenesis. At the initiation of meiosis, the microspore mother cells were surrounded by callose (Figure 3a, b), which persisted until the formation of microspores. There was a simultaneous cytokinesis of microspore mother cells following meiosis resulting in the tetrahedral shape of the microspore tetrads (Figure 3b) that were still enclosed in callose.

As the callose disappeared due to dissolution, individual microspores were released into the sporangium, by which time three incipient pores and a thin exine layer became evident on the wall (Figure 4a). The exine layer underwent thickening as the microspore developed into early-binucleate stage (Figure 4b). Furthermore, mitosis took place and the microspore grew into a two-celled pollen grain (Figure 4c). By the time the microspores became mature pollen grains, the tapetum and middle layer were broken down leaving only the fibrous endothecium and the epidermis (Figure 2d).

Ovule. The ovary of S. formosa was round in shape with two locules (ovarian cavity). Each locule consisted of one row of up to 30 ovules. The ovule developed horizontally and the funiculus appeared to be attached half way between the chalaza and the micropyle, forming the campylotropous orientation (Figure 5). The ovules in the ovary developed simultaneously but resulted in non-uniform maturity. Ovules at the apical region of the ovary reached maturity faster than ovules at the basal region.

A single archesporium was present in the ovule. This archesporium originated from one of the hypodermal cells, which divided in a periclinal direction to form a primary parietal cell and a sporogenous cell. The sporogenous cell differentiated into a megasporocyte and the primary parietal cell developed into two nucellar layers above the megasporocyte (Figure 6).

The ovular primordium was dome-shaped and initiated from the marginal placenta as homogenous hemispherical protuberances. The primordia started to undergo curvature before the differentiation of integuments. The outer and inner integuments developed simultaneously from the nucellus at the sporogenous cell stage. Initially, both outer and inner integuments developed in a synchronous manner (Figure 7a, b) but later the outer integument developed more rapidly than the inner integment (Figure 7c) resulting in the enclosure of the inner integment and the nucellus at the megasporocyte stage. Both outer and inner integuments at this stage consisted of two layers of cells. The outer integument continued to grow and completely enveloped the nucellus at the completion
of megasporogenesis (Figure 7d), whereas the inner integument reached only the tip of nucellus. At the two-nucleate stage of the female gametophyte the inner integument reached the micropyle while cells of the outer integument continued to divide anticlinally and periclinally in the micropylar region resulting in a collar 6-8 cells thick at the tip.

The cells in the outer integument continued to divide repeatedly as the ovule grew to maturity. This repeated division resulted in a massive structure of cells growing towards the funiculus and covering the inner integument and the micropylar end of the nucellus. This resulted in a zigzag micropyle (Figure 8). Accumulation of starch took place in the outer and inner integuments in the vicinity of micropyle.

The female gametophyte increased in size at the expense of nucellar tissue, particularly at the sides and in the micropylar region. As a consequence of this, the inner integument on the sides, the single-layered nucellar epidermis at the micropylar end and the hypostase in the chalazal region bordered the mature female gametophyte.

During the development of the female gametophyte, starch grains were deposited in the nucellar epidermis (Figure 9). The presence of starch was abundant in the outer and inner integument as well as in the mature female gametophyte.

An interesting finding in the ovule of *S. formosa* was the presence of more than one embryo sac (Figure 10), however, of a total of approximately 1820 seeds germinated throughout the experiments, only one (0.06%) twin seedling was found.
megaspore underwent successive karyokinesis resulting in 2-nucleate, then 4-nucleate, and finally 8-nucleate female gametophytes. In the mature embryo sac, two synergids, two polar nuclei, and three antipodal cells were found (Figure 11a, b). The polar nuclei laid towards the centre of the embryo sac and were surrounded by starch grains.

**Pollination and Fertilisation.** In this study, pollination was enhanced by rubbing the receptive stigma with a fingertip covered with pollen grains from the same flower. This method of hand-pollination enhanced pollen germination on the stigma and resulted in 100% pod formation. The period of seed development from a successful pollination to harvesting was about 60 days.

**Endosperm and Embryo.** The development of the endosperm in *S. formosa* was nuclear. The primary endosperm nucleus divided following the enlargement of the embryo sac and the accumulation of starch grains. Large starch grains accumulated particularly in the close vicinity of the micropyle. As the zygote continued to grow, starch accumulation proceeded gradually from the micropylar region to the chalazal end.

**Seed Coat.** By the time the cotyledons formed, the outer integument had increased to approximately 8-10 layers of cells (Figure 12a). In mature seeds, the outer layer of the outer integument consisted of thick-walled palisade cells. A layer comprising osteosclereids that are called “hour-glass” cells (Figure 12b) were present in the layer beneath the palisade cells. The remaining integumentary tissues, mainly thin-walled parenchyma, were crushed as the seeds matured.

This was interesting because the occurrence of multiple embryos and germination of twin seedlings are uncommon in Fabaceae.

The megaspore mother cell underwent meiosis leading to the formation of a linear tetrad of megaspores. Of these four cells only the one in the chalazal region functioned, whereas the three micropylar ones degenerated. The functional

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DISCUSSION

The anther characteristics of S. formosa are those typical of a legume flower. In the early stage of anther wall development, the hypodermal cells divided periclinally to form the primary parietal and primary sporogenous cells. The sporogenous cells divided and differentiated into abundant microspore mother cells (microsporocytes) giving rise to a large number of pollen grains, which has been a major problem in the commercialisation of S. formosa as cut flowers (Barth 1990). Meanwhile, the primary parietal cells divided periclinally to form the outer and inner secondary parietal cells. The outer secondary parietal cells underwent further division resulting in the endothecium and one middle layer. The inner secondary parietal cells developed into the secretory tapetum, the food-rich layer of cells (Prakash 1987).

Anther dehiscence occurred before the flower was opened and the stigma was receptive one day before anther dehiscence. This supports the reports of previous authors (Kirby 1996a, b; Williams 1996) that S. formosa was fully self-pollinated. However, self-pollination was often hampered by the presence of the stigmatic cuticle (Jusaitis 1994) that prevents pollen grain germination until the stigmatic cuticle was ruptured. The development of the stigmatic cuticle was also reported in Phaseolus vulgaris (Lord & Webster 1979) and Trifolium pratense (Heslop-Harrison & Heslop-Harrison 1983).

The dicotyledonous type of anther development confirms earlier observations on the Papilionoideae (Davis 1966). The predominance of tapetal cells, simultaneous cytokinesis and two-celled pollen grains indicates that S. formosa closely resembles other members of the family. The tetrasporangiate type of anther is also common in many other legumes except Carmichaelieae (Goodley 1980), which has bisporangiate anthers. The uninucleate tapetal cells recorded in many legumes (Davis 1966; Lim & Prakash 1997), were also found in S. formosa. As with in most members of the family (Prakash 1987), the anther wall of S. formosa showed one endothelial layer and a secretory tapetum. However, in this study only one middle layer was found, whereas in most legumes such as Glycine max and Psophocarpus tetragonolobus, two or three middle layers were reported (Prakash & Chan 1976; Lim & Prakash 1997).

The morphology and development of the ovule confirms earlier observations on the family (Prakash & Chan 1976; Prakash & Herr-Jr 1979; Prakash 1987; Lim & Prakash 1997). Ovules in the apical region of the ovary grew faster than ovules in the basal region. A similar pattern of ovule differentiation was also reported in G. max (Kato et al. 1954) but Prakash and Chan (1976) found that the basal ovule developed faster than the apical ones. The campylotropous arrangement of ovules, as well as the zigzag pattern of the micropyle formed by both outer and inner integuments are common features throughout the family. This ovule arrangement was also found in the members of the Malvaceae and Caryophyllaceae (Tootil 1984). However, as in G. max (Prakash & Chan 1976), the inner integument in S. formosa was slow-growing and completely covered the nucellus only after fertilisation. Starch grains were abundant in the mature embryo sac of S. formosa as in Arachis hypogaea (Reed 1924) and G. max (Prakash & Chan 1976). The presence of starch in the embryo sac seems to be of widespread occurrence in the family (Prakash 1987).

Although there has been no previous record on the occurrence of multiple embryo sacs in the species, this study revealed that polyembryony could happen in S. formosa. Multiple embryo sacs have been reported in a number genera of Mirbelieae, such as Jacksonia, Dillwynia, Oxylobium, Gastrolobium, Nemia, Brachysemia, and Jansonia (Cameron & Prakash 1994). This finding is the first recorded in the genus so far. A very low incidence of twin seedlings (0.06% throughout the work presented here) suggests that only one mature embryo is normally formed in the seeds, and polyembryony is not a common feature of S. formosa. This also indicates that although polyembryony could theoretically occur in the ovule, only one mature embryo is usually formed in the seed.

Swainsona formosa is a self-compatible plant, however, as with most papilionoid legumes (Shivanna & Owens 1989) pollination is often hampered by the presence of a stigmatic cuticle that prevents pollen germination until the stigmatic cuticle is ruptured. Rubbing the stigma with finger tip has been an effective way to peel-back the stigmatic cuticle compared to just touching the undisturbed stigma with pollen (Jusaitis 1994).

Upon germination, the pollen tubes travelled down the stylar canal and grew along the placenta towards the nucellus through the micropyte. The pollen tube entered the embryo sac via one of the synergids, destroying the synergid in the process. The other synergid degenerated soon after fertilisation occurred (Prakash & Chan 1976; Prakash 1987).

As in most legumes, the endosperm of S. formosa is of the nuclear type (Prakash 1987). The zygote enlarged and underwent successive divisions resulting in an early globular embryo. Mature seeds had only one or two layers of endosperm as the embryo consumed the endosperm during its development. The mature embryo had two equal cotyledons. The embryo in the mature seed consumed almost all the endosperm, and the reserve food was transferred to the cotyledons. Though the development of S. formosa endosperm is of typical legume, a considerable variation in the organization of the endosperm in the family has been recorded in other Australian legumes (Cameron & Prakash 1994).

The organization of the seed coat of S. formosa is similar to that in most legumes. Corner (1951) described the seed coat of leguminous plants as developing entirely from the outer integument of the ovule through cell division, enlargement and modification of the wall. Until the mature embryo sac stage the outer and inner integuments consisted of two layers of cells. The cells of the inner integument divided anticlinically and underwent radial elongation becoming 2- or 3-layered until these cells disappeared at the globular embryo stage. Meanwhile, the outer epidermal cells of the outer
integument also divided anticlinally and differentiated into radially elongated cells. The layer beneath this did not show any differentiation. The two cell layers of the outer integument enlarged and acquired wall thickenings during food reserve deposition in the cotyledons.

This study revealed that the testa was typically differentiated into a layer of palisade thick-walled columnar cells on the outside and a mesophyll with a layer of hypodermal sclereids (the so-called hour-glass cells). A similar composition was also noted for the seed coat of *G. max* (Vaughn 1970; Prakash & Chan 1976). However, in *P. tetragonolobus* the layer of hour-glass cells was not distinguished in the mesophyll of the seed coat (Lim & Prakash 1997). The inner integument did not take part in the formation of the seed coat and was usually crushed in the mature seed (Prakash 1987).

It is clear that anther and ovule development in *S. formosa* are of typical leguminous flower, but minor modifications occur. Further investigation is necessary to complete the picture of detailed embryology and seed development in *S. formosa as a fundamental aspect of its breeding programme.

REFERENCES


