DEVELOPMENT AND GERMINATION OF *Sandersonia aurantiaca* (HOOK.) SEEDS

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ABSTRACT

Sandersonia aurantiaca (Hook.) has recently become an important horticultural crop through its economic value for export of its cut flowers and tubers. Little information however is available on seed structure, morphology, development and propagation. The main objectives of this study were to investigate the pattern of seed development, to find satisfactory methods of improving the seed germination and to assess possible mechanisms of seed dormancy of *Sandersonia aurantiaca* (Hook.).

Seed development was investigated by fixing plant material in FAA solution, embedding in paraffin, and staining with safranin-fast green. A series of sections were examined and photographed under a microscope. Both embryo and endosperm development in *Sandersonia* show close similarity to development in *Allium fistulosum* (Alliaceae). Embryo development passes through early globular, late globular, elongated spheroidal and linear embryo development stages. Endosperm development conforms to the Nuclear type. Freely-growing walls between the endosperm nuclei may be associated with the embryo sac wall as projections. The structure of the mature seeds is very similar to that of *Iris* (Iridaceae) seeds. The small, linear embryo is embedded in the endosperm which constitutes most of the seed volume. Such small, linear embryos may be one reason for embryo dormancy in *Sandersonia* seed. A special structure (a conical or cylindrical protuberance) is observed in the inner part of the seed coat, which may combine with a lignified layer (and perhaps including the endosperm) to contribute to the coat-imposed dormancy in this species.

Eighty five treatments were firstly used to improve the germination percentage of *Sandersonia* seed. Only the treatment in which seeds scarified firstly with sandpaper for 1 min and then nicked near the radicle end showed increased germination from 0 to 10.6 % by 30 days, at 20° C. Based on this result, 31 new treatment methods were designed in germination experiment 2. Water uptake patterns, allelopathic effect on lettuce seeds and embryo rescue of *Sandersonia* seed were also studied for assessing the possible mechanisms of dormancy.

The findings of the present study suggest that the *Sandersonia* seeds have double dormancy. The dormancy mechanism is located in both the seed coat and the embryo and it consists of at least two steps that must be activated in sequence before germination can occur. The first step can be activated prematurely by scarifying and nicking the seeds, thus allowing the seed coat to become permeable to water, oxygen or to reduced mechanical restriction. The second step can be activated directly by GA_3 which stimulates embryo growth. This germination-promoting technique has great potential for *Sandersonia* for improvement of the germination percentage of seeds from 0 to about 70 %, but development on a commercial scale needs further studies.

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LIST OF ABBREVIATIONS

6-BA	6-benzyladenine
ABA	abscisic acid
AVOVA	An Analysis of Variance
C_2H_4	ethylene
ca	apical cell
cb	basal cell
DAA	days after anthesis
DAP	days after pollination
DW	dry weight
FAA	Formalin-alcohol-glacial acetic acid solution
GA	gibberellic acid
IAA	indole-3-acetic acid
ISTA	International Seed Testing Association
LS	Linsmaier and Skoog
LSD	Least Significant Difference
MPD	morphophysiological dormancy
MS	Murashige and Skoog
NZ	New Zealand
ppm	parts per million
PAS	periodic acid-schiff's reagent
RH	relative humidity
TBA	tertiary butyl alcohol
TTC	2,3,5-triphenyl tetrazolium chloride
WC	water content

Chapter 1

GENERAL INTRODUCTION

Sandersonia aurantiaca (Hook.) is a monotypic species in the genus and is a deciduous monocotyledonous perennial herb, related to *Gloriosa* and *Littonia*. *Sandersonia* has been placed in the Colchicaceae family. It is a tuberous plant with clear orange flowers and occurs naturally in South Africa (Natal and Cape provinces) at an altitude of 600-2000 m in areas of high summer and low winter rainfall (Mathew 1978, Brundell and Reyngoud 1985). Each attractive flower is 2-2.5 cm long and 1.3 cm in diameter. It is puffed into the shape of a bell and slightly constricted at the mouth where it is flushed with yellow (Eliovson 1955). The texture is smooth, shiny and yet papery. Each single flower hangs down from a curved, wiry flower-stalk, and there are several that spring at intervals from the stem, just below the upper leaves (Plate 1.1). The leaves are soft in texture, bright green, and almost 10.2 cm long, 1.9 cm wide at the broadest part and they taper to a thread-like tip. *Sandersonia* plants usually have fork-shaped tubers with a single growing point on each tip. Marble shaped tubers can be formed when any tuber is grown in poor conditions and may be formed at end of the first season from seed germination (Brundell and Reyngoud 1985). During the growing season the original tuber dies after producing usually two joined (daughter) tubers, one of which is dominant. *Sandersonia* flowers have excellent keeping quality, and if placed in a preservative solution, flowers will continue to grow, young flower buds develop and open normally (Tjia 1988). *Sandersonia* tuber extracts contain about 0.9% colchicine, and can potentially be utilized as source for commercial colchicine (Finnie and van Staden 1991).

The unique bright orange flowers with their distinct shape and good vase life have made *Sandersonia* a sought-after cut flower on the international market (Eason and Webster 1995). The production of *Sandersonia* cut flowers and tubers for export increased in New Zealand during the 1980s (Warren 1988). *Sandersonia* became the rising star of the NZ export flower industry in 1992/1993 (Flower NZ July 1993) and rapidly climbed to the number three position in the cut flower export stakes (Fig. 1.1a).

Until the year ended June 1995 *Sandersonia* exports totalled $5.62 million (Fig. 1.1b) and 99.8% of their fresh cut flowers were exported to Japan (Flowers NZ October 1995).

In South Africa *Sandersonia aurantiaca* is a protected species which is rarely seen growing and which is forbidden to be picked. New Zealand obtained its stock over 78 years ago. Donald Ross brought the first tubers into New Zealand after a trip which took him to South Africa (Warren 1988). New Zealand is favorably placed with *Sandersonia* stock plant material they have built up over the years. *Sandersonia* usually flowers outdoor in late November and December from tubers planted in the spring (Clark 1994). It requires a damp friable acid soil and will not tolerate lime. Fertilizer needs to be applied and soil test levels of pH: 5.5-6.5, calcium: 10, potassium: 20, phosphorous: 50 and magnesium: 30 obtained good results for producing tubers (Warren 1988). However, low nitrogen levels throughout the growing season tend to result in smaller tubers with good formation or shape and less marble (secondary tuber) formation (Boundy *et al.* 1996). *Sandersonia* tubers require a period of cool storage. The ideal cool storage parameters are a temperature of 4°C for 90-day duration (Clark 1995). Desirable pre-sprout temperatures are 20-23°C (Clark 1994). *Sandersonia* tubers are susceptible to a host of fungi that can rot and destroy tubers. Several different fungicides can be used to dip the tubers before planting, e.g. Benlate 0.5 g/l, Thiram 2.0 g/l or Nu-film 2 ml/l (Boundy *et al.* 1996). To control pests and diseases the plants need to sprayed once a week during harvesting. It is preferable to use an insecticide and an upturned wand, mainly for control of thrips, moths and other small insects which could go up into the bells (Warren 1988).

Sandersonia can also be propagated from seeds, but seed germination is considered to be difficult taking up to two years. Some methods have been developed to achieve germination, and while there are a few cryptic clues such as 'chilling', or 'cold winters', these are not readily known (Warren 1988).

In recent years research scientists have studied a number of aspects of *Sandersonia* biology and horticulture. These have included (1) year-round flower production techniques for export (Catley 1994a, Catley and Halligan 1994); (2) growth

habit (Warren 1988, Catley and Davies 1994, Boundy *et al.* 1996), (3) growth media (Flowers NZ November 1993, Boundy *et al.* 1996), (4) tuber quality (Clark *et al.* 1995, Steens 1995, Catley *et al.* 1996); (5) vegetative propagation (from tubers) (Brundell and Reyngoud 1985) and *in vitro* propagation (Finnie and van Staden 1989); (6) effect of storage duration and storage temperature on tuber dormancy and sprouting (Clark 1994 and 1995); (7) development and senescence of flowers (Eason and Webster 1995, Eason and de Vre 1995); (8) leaf anatomy (Baijnath 1988), leaf nutrient levels and imbalance symptoms (Ryan 1993), (9) morphology of the carpel (Sterling 1975); (10) effect of temperature, light and growth regulators on flower initiation, flower quality and stem length of *Sandersonia* (Catley 1994b, Catley and Davies 1994, Davies *et al.* 1994); (11) new *Sandersonia* hybrids (Flowers NZ June 1995), (12) disease control (Ryan 1993, Steens 1995, Boundy *et al.* 1996), and (13) isolation of colchicine (Finnie and van Staden 1991). However, very little information is available on seed structure, morphology, development and propagation. Studies on seed development and germination are thus necessary and important for giving us more information about *Sandersonia* seed biology, and more information for generating plants for export. In this thesis I have concentrated study on *Sandersonia* seeds with the main objectives being:

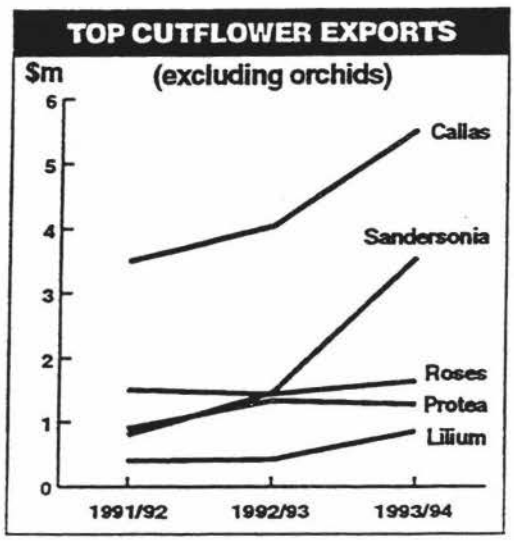
a. To describe early seed development in the ovary and the structure of mature seeds of *Sandersonia aurantiaca.*

b. To investigate and characterize the embryo, endosperm (or perisperm), and testa development of *Sandersonia aurantiaca* seeds.

c. To find satisfactory methods for breaking seed dormancy and improving seed germination percentage of *Sandersonia aurantiaca.*

d. To discuss the possible mechanism of dormancy of *Sandersonia aurantiaca* seeds.

a



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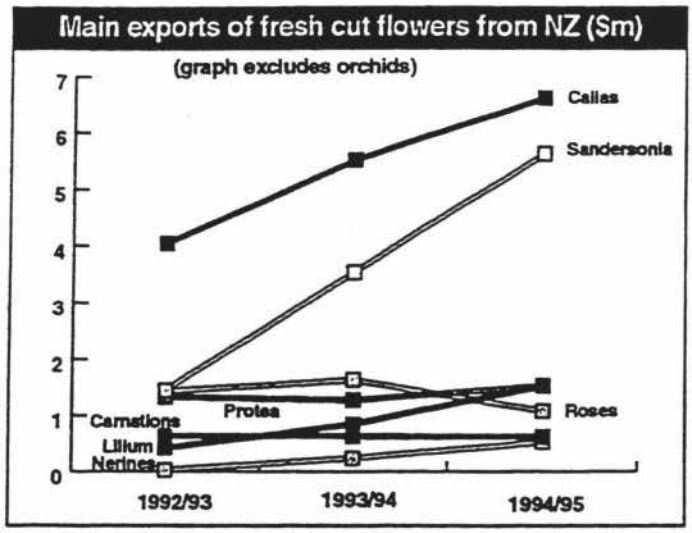


Figure 1.1 Main exports of fresh cut flowers from New Zealand. All figures are f.o.b for years ended June 30. a) 1991/1992–1993/1994; b) 1992/1993–1994/1995 (Source: *Flowers New Zealand August 1994 and October 1995*).

Plate 1.1 The cut flowers of *Sandersonia aurantiaca* (Hook.) (Supplied by E R Morgan, Crop & Food Research Ltd. at the Levin Research Centre).

Chapter 2

LITERATURE REVIEW

2.1 SEED DEVELOPMENT

Sandersonia is a monocotyledon plant and belongs to the family Colchicaceae. It was recently removed from the family Liliaceae to the family Colchicaceae (Finnie and van Staden 1989). The closest relatives of Colchicaceae genera are undoubtedly genera within the Liliaceae (Dahlgren *et al.* 1985), and some authors still place it in the Liliaceae family. According to Dahlgren *et al* (1985), the flowers in both families have many similar morphological features, e.g., (1) flowers are generally trimerous, actinomorphic, hypogynous and bisexual; (2) microsporogenesis is successive; (3) the pistil is tricarpellary, syncarpous and trilocular, and the stigma is 'Dry' or 'Wet'; (4) each locule contains several to many anatropous ovules; (5) the endosperm generally contains somewhat thick-walled, pitted cells with aleurone and fatty oils, but no starch; (6) the embryo is relatively short and little differentiated.

A seed is an embryo surrounded by an envelope of stored food and a protective seed coat. It develops from the ovule as a consequence of double fertilization. Bewley and Black (1982) indicated: "In almost all cases the following can be recognized as the fertilized ovule develops: (1) the testa -- the product of one or both integuments of ovule; (2) the perisperm -- derived from the nucellus; (3) the endosperm -- produced as a result of fusion between one male generative nucleus and the two polar nuclei to form the triploid endosperm nucleus; (4) the embryo -- the result of fertilization of ovum by a male nucleus." To provide the essential background for these statements and help to explain the development of *Sandersonia* seed in this study, attention will be concentrated on the pattern of ovary and ovule, the development of endosperm, embryo and testa, and the structure of mature seeds.

2.1.1 Ovary, Ovule and Embryo Sac

2.1.1.1 Ovary

Angiosperm flowers display a great variety of forms, but in general they are composed of the same basic elements: the perianth, androecium and gynoecium (Rudall 1987). The perianth is the outer sterile part, with whorls of sepals (calyx) and petals (corolla); the androecium is made up of stamens, which are the site of microsporogenesis; and the central gynoecium, where megasporogenesis occurs, consists of one or more carpels. In *Sandersonia* flowers have a fused perianth (Eason and Webster 1995), the ovary is superior and generally tricarpellate (Sterling 1975). In cross section, there is a central hole and/or distinctly marked sutural margins, but there are no unequivocally open sutures and deep sulci extended inward in the ovary along the septal boundaries of the carpels (Sterling 1975).

2.1.1.2 Ovule

The ovule is the forerunner of seed (Bhatnagar and Johri 1972, Bouman 1984). A normal ovule has a stalk called the funiculus by which it is attached to the placenta (Bhatnagar and Johri 1972, Fahn 1974, Bouman 1984). The ovular envelopes, the integument (outer and inner integument), enclose a massive nucellus. Generally five types of mature ovules have been recognized (Maheshwari 1950, Bhatnagar and Johri 1972, Dahlgren *et al.* 1985). These are orthotropous, anatropous, amphitropous, campylotropous, and hemianatropous. The ovule in Liliaceae is hemianatropous, anatropous, or orthotropous, and bitegmic with the micropyle formed by the inner integument (Davis 1966), but in Colchicaceae it is anatropous to weakly campylotropous (Dahlgren *et al.* 1985). *Sandersonia* ovaries have numerous, plagiotropic, anatropous ovules with bitegmic structure (Sterling 1975).

Depending upon the extent of development of the nucellus, the ovule may be tenuinucellate or crassinucellate (Maheshwari 1950, Bhatnagar and Johri 1972, Dahlgren *et al.* 1985). In the tenuinucellate ovule, the archesporial cell develops directly into a megaspore mother cell. The archesporial cell is surrounded by the epidermis only. Normally, a crassinucellate ovule is one where the archesporial cell cuts off a parietal cell

and its derivatives make the megaspore cells deep-seated. Sometimes the tissue formed by periclinal divisions of the nucellar epidermis is called the nucellar cap. If this is the case in ovules without parietal tissue some embryologists refer to such ovules as "pseudocrassinucellate" (Dahlgren *et al.* 1985), but no special term is employed for crassinucellar ovules which develop a nucellar cap (Davis 1966). The ovule is either tenuinucellate or crassinucellate in Liliaceae (Davis 1966), or in Colchicaceae (Dahlgren *et al.* 1985). The nucellus often degenerates at an early stage, but may persist, especially at the chalazal end, and act as a storage tissue called perisperm (Bhatnagar and Johri 1972, Rudall 1987).

Sometimes, in the lower part of the nucellus, just below the embryo sac, a group of cells which may or may not contain dense cytoplasm and may or may not have thickened walls has been found. These are called a hypostase (Bhatnagar and Johri 1972). Many functions have been attributed to the hypostase: (1) it may act as a barrier tissue for stopping the encroachment of the embryo sac (Venkateswarlu and Prakasa Rao 1972); (2) it may connect the vascular supply with the embryo sac and facilitate transport of nutritional material (Tilton 1980); (3) it may be responsible for the production of certain enzymes or hormones; and (4) it may play a role in the water balance of dormant seeds, or have a protective function in mature seeds (Bouman 1984).

A structure which appears to be associated with directing the growing pollen tube toward the micropyle is referred to as an obturator (Maheshwari 1950). The commonest type is the funicular obturator, which is a local swelling of the funiculus. A detailed ultrastructural study of this tissue in *Caltha* was made by Peterson *et al.* (1979), who characterized the cells which had thick outer cell walls with electron-dense wall ingrowths, numerous dictysomes, and secreted material outside the cell walls, as transfer cells according to the presence of deeply staining cytoplasm. The transfer cells are supposed to be involved in the short-distance transport of metabolites and/or the secretion of chemotropic substances for the growth of the pollen tube (Bouman 1984). Several species in the Liliaceae have been found to possess hypostase and obturator structures. *Ornithogalum caudatum* has both a hypostase and an obturator (Tilton and Lersten 1981). Green onion (*Allium fistulosum*) is a species in the Alliaceae (and

assigned to the Liliaceae by some authors) and has also been reported to have a hypostase at the chalazal end (Xiang-Yuan 1987).

2.1.1.3 Embryo Sac

The commonest and most widely distributed embryo type in monocotyledons and also in dicotyledons is Polygonum (Normal) type (Willemse and van Went 1984, Dahlgren *et al.* 1985, Raghavan 1986). In this type of embryo sac, the lowest (chalazal) megaspore undergoes three divisions to form an eight-nucleate embryo sac with one egg cell accompanied by two synergids at the micropylar end and three antipodals at the chalazal end (Dahlgren *et al.* 1985). The remaining two nuclei (the polar nuclei) may fuse to form a central nucleus. Several other types of embryo sac development which are characterized by being composed of more or fewer cells, and by aberrant ploidy-level of some of their nuclei have also been reported in monocotyledons. The Allium Type (a bisporic type) occurs in Alliaceae, Alismataceae, Amaryllidaceae, Hyacinthaceae, Liliaceae and Trilliaceae etc., the Endymion Type (a bisporic type) in Convallariaceae, Hyacinthaceae etc., the Fritillaria Type (a tetrasporic eight-nucleate type) in the Liliaceae (David 1966, Dahlgren and Clifford 1982, Dahlgren *et al.* 1985). Further types, the Clintonia, Adoxa and Drusa types (all tetrasporic) have also been found in Liliaceae (Davis 1966, Dahlgren and Clifford 1982). The embryo sac formation in the Colchicaceae conforms to the Polygonum type (Dahlgren *et al.* 1985).

The synergid cells may be hooked or beaked, and usually show a vacuole at the micropylar end, and a nucleus at the chalazal end. Sometimes, the synergids also show a filiform apparatus (Bhatnagar and Johri 1972). The synergids in the Liliaceae have three types: (1) hooked and exhibit the filiform apparatus, (2) only hooked, or (3) only filiform apparatus. The central cell is the largest cell of the megagametophyte and lies between the antipodals at the chalazal end and the egg apparatus at the micropylar end (Tilton and Lersten 1981). Three types of behaviour of the polar nuclei are known (Davis 1966): (1) fusion occurs prior to fertilization, (2) the polar nuclei remain distinct although closely associated, and fusion occurs only after one has been fertilized, and (3) the polar nuclei and the male gamete fuse simultaneously. In the Liliaceae family the polar nuclei fuse before fertilization and the secondary nucleus moves close to the antipodals (Davis

1966). Antipodals seem to be the most variable cells of the megagametophyte, but they are always located at the chalazal pole (Tilton and Lerston 1981). The antipodals may degenerate without being delimited into cells and may become large multinucleate cells or even polyploid (Bhatnagar and Johri 1972). Multinucleate (Eunus 1949) and multicellular (cited in Tilton and Lersten 1981) antipodal cells have been reported in some species of Liliaceae. The egg cell in most species shows a similar organization, characterized by distinct polarity and partially surrounded by a cell wall (Willemse and van Went 1984). The wall is thickest at the micropylar region of the cell, and gradually thins toward the chalazal region.

2.1.2 Endosperm Development

The endosperm is usually triploid, formed by fusion of one male nucleus with two female polar nuclei (Maheshwari 1950, Bhatnagar and Johri 1972, Vijayaraghavan and Prabhakar 1984, Dahlgren *et al.* 1985, Rudall 1987). Soon after fertilization, the primary endosperm nucleus initiates a series of nuclear divisions to form endosperm nuclei. The growth of the endosperm precedes the growth of the embryo (Dnyansagar 1957, White and Williams 1976, Raghavan 1986). According to whether a wall is formed or not in early development, the development of endosperm is classified as three main types: Nuclear, Cellular and Helobial type (Maheshwari 1950, Bhatnagar and Johri 1972, Vijayaraghavan and Prabhakar 1984, Raghavan 1986, Rudall 1987).

In the Nuclear-type development, the early cell divisions are unaccompanied by cell wall formation, and the nuclei are initially free in the cytoplasm of embryo sac, usually surrounding a central vacuole (Rudall 1987). Later, centripetal wall formation takes place. The cellularization begins in the micropylar region and extends to the chalazal region. The embryo sac eventually becomes completely cellular (Bhatnagar and Johri 1972).

The Cellular endosperm is characterized by absence of a free-nuclear phase, and division of the primary endosperm nucleus and subsequent divisions are quickly followed by wall formation. A noteworthy feature of Cellular endosperm is the formation of

haustoria which are much more varied than in the Nuclear type (Bhatnagar and Johri 1972, Raghavan 1986).

Helobial endosperm formation occurs only in monocotyledons (Rudall 1987), or both in dicotyledons and monocotyledons (Vijayaraghavan and Prabhakar 1984). The first division of the primary endosperm nucleus is accompanied by the formation of a small chalazal chamber and a large micropylar chamber (Rudall 1987). Later the micropylar chamber becomes multicellular following an initial free nuclear phase, while the chalazal chamber remains undivided or undergoes a limited round of divisions to produce a cluster of free nuclei (Raghavan 1986).

Much research has been made on endosperm development in the Liliaceae family since 1933. Davis (1966) reported that the endosperm formation in the Liliaceae was Nuclear or Helobial type. In the latter, the small chalazal chamber is usually ephemeral and 2-4 nucleate. Many free-nuclear divisions take place in the large micropylar chamber before walls develop and tissue becomes cellular. Earlier investigations on endosperm development in *Asphodelus tenuifolius* (Liliaceae) revealed that endosperm development was of the Helobial type (Maheshwari 1933). Wall formation took place only in the micropylar chamber and the growth of the endosperm was centripetal (Eunus 1952). Apart from this Chitralekha and Bhandari (1992) recently reported that wall formation was initiated simultaneously at both the extreme ends of the chamber and gradually proceeded to the central part. The first-formed cell walls are laid down perpendicular to the embryo sac wall; such anticlinal walls originate either as wall projections from the embryo sac wall or as cell plates following nuclear divisions. These extend inwards and fuse laterally with adjacent walls to produce open compartments. The centripetal extension of these compartments and repeated divisions within them result in completely cellular endosperm.

The endosperm development of *Allium fistulosum* (Green Onion) is the Nuclear type (Xiang-Yuan 1987). In the endosperm, cell formation starts simultaneously at the micropylar and the chalazal ends of embryo sac when the embryo is in the late globular stage. The first anticlinal walls arise from the cell plates without phragmoplasts between the free interphase nuclei resulting in a layer of open cells. The first periclinal walls are initiated as a result of normal cytokinesis of the first formed open cells, and are

associated with phragmoplasts and cell plates. The subsequent cell divisions give rise to the endosperm cells centripetally until those from the opposite sides of the embryo sac meet.

2.1.3 Embryo Development

The main stages in development of the embryo are (a) proembryo, and (b) embryo proper (late embryogeny). Generally, the globular stage marks the transition from proembryogeny to late embryogeny in angiosperms (Romberger *et al.* 1993). After fertilization, the zygote undergoes a resting period. The time taken for the zygote to undergo its first division varies with different species (Maheshwari 1950, Fahn 1974, Sporne 1974, Raghavan 1986).

In general, the nucleus, which constitutes only a small fraction of zygote volume, is near the chalazal end of the cell, while a large vacuole occupies the micropylar end (Natesh and Rau 1984, Romberger *et al* 1993). The first division of the zygote is transverse, rarely vertical or oblique, and asymmetric in most angiosperms (Maheshwari 1950, Bhatnagar and Johri 1972, Romberger *et al* 1993). Two cells are produced as a result of transverse division. The smaller cell, which is more densely cytoplasmic and near the cavity of the embryo sac, is the apical cell (*ca*), the larger cell, which is vacuolar and faces the micropyle, is the basal cell (*cb*). After first division of the zygote into an apical and a basal cell, the apical cell may divide longitudinally, obliquely, or transversely. The six main types of embryogeny have been described by Natesh and Rau (1984), based on Schnarf and Johansen's system of classification and are shown in Fig. 2.1. The Asterad and Onagrad types are common in monocotyledons (Davis 1966).

There is no essential difference between the monocotyledons and the dicotyledons regarding the earlier stages in embryogeny up to the proembryo stage (Maheshwari 1950, Bhatnagar and Johri 1972, Natesh and Rau 1984). Then, the globular stage, the four-celled (quadrant-stage), apical tier shows quadrant-based differences in development between the two groups. In most dicots, the progeny of two diametrically opposite quadrant cells produces the two cotyledon primordia, while the progeny of the other two quadrant cells is involved mainly in initiating the epicotyl. In

monocots, cells of two adjacent quadrants typically are the progenitors of the cells of the single cotyledon, while the progeny of the two remaining adjacent quadrant cells construct the epicotyl (Lakshmanan 1972).

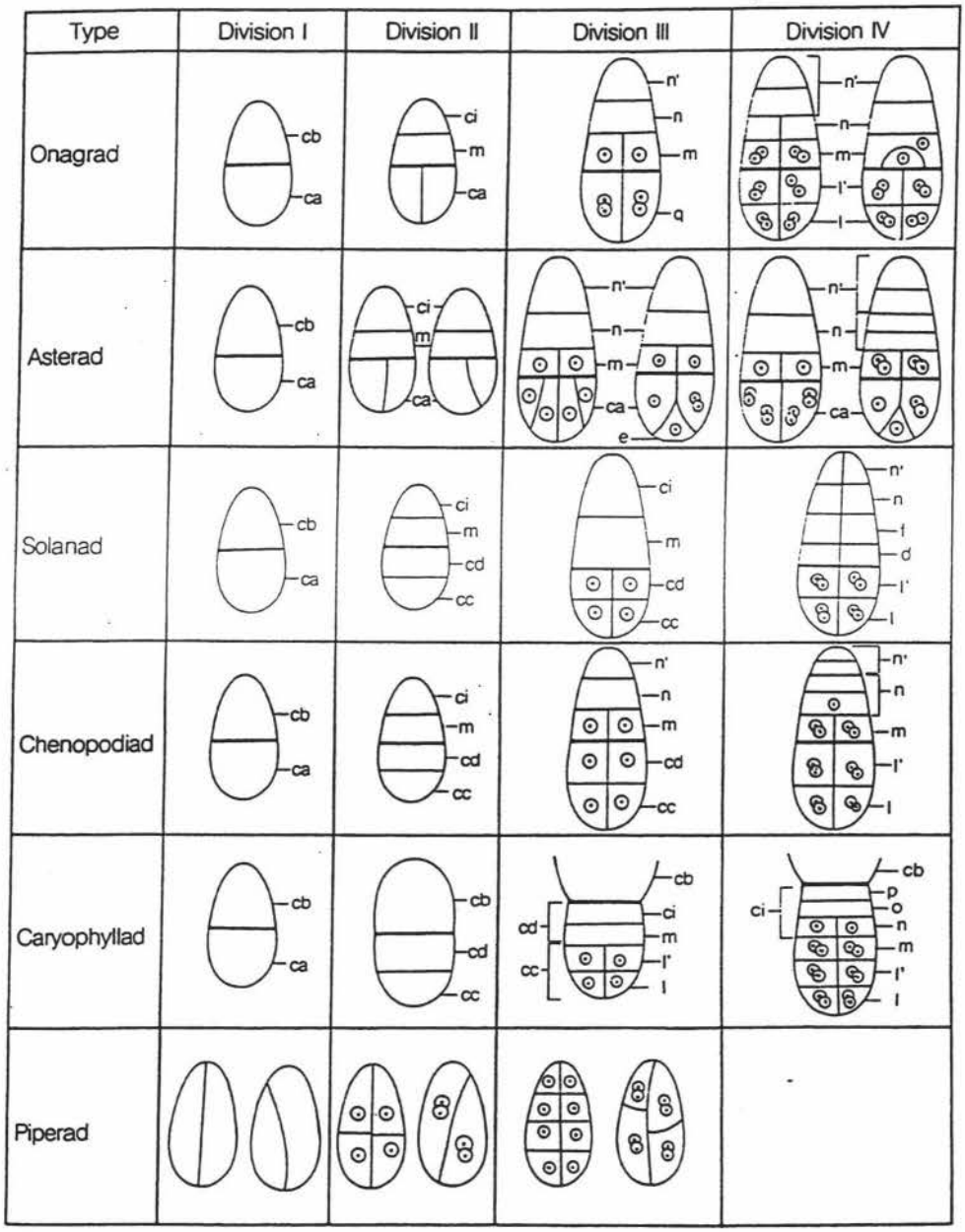


Figure 2.1 Schematic representation of main types of embryogeny; based on Schnarf and Johansen's system of classification (Source: Natesh and Rau 1984).

Davis (1966) reported that several types of embryogeny in Liliaceae were assigned as Onagrad type (*Erythronium, Lilium*), Asterad type (*Heloniopsis, Muscaria*), Caryophyllad type (*Dipcadi*), and Chenopodiad type (*Asphodelus*). Adventive polyembryony had also been described in *Funkia* and *Smilacina,* but in *Erythronium* and *Tulipa* the embryo apex underwent cleavage, and in *Iphigenia* and *Lilium* an extra embryo had been attributed to fertilization of a synergid.

The embryogeny of *Muscari comosum*, a representative of the family Liliaceae, was described by Soueges (1932) (cited in Maheshwari 1950). The early stages of proembryo development are shown in Fig. 2.2. After the first division of the zygote, the basal cell *cb* and the terminal cell *ca* undergo several divisions either transversely or longitudinally and produce the octants (Fig. 2.2 A-E). Finally, the tier *q* gives rise to the cotyledon, *m* to the hypocotyl and stem tip, *n* to the initials of root, *o* to the root cap, and *p* to the suspensor (Fig. 2.2 F, G) (Maheshwari 1950).

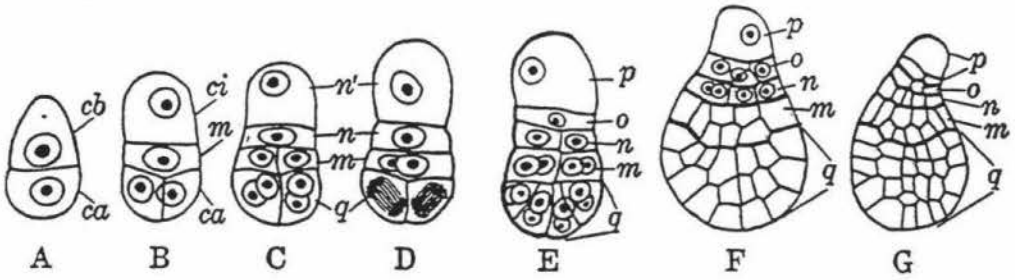


Figure 2.2 Development of embryo in *Muscari comosum* (Source: Maheshwari 1950).

The embryo development of green onion (*Allium fistulosum* L.) conforms to the Asterad type passing through the proembryo, globular, spheroidal, concave, stick-shaped, curved stage and then to mature embryo (Xiang-Yuan 1987). The persistent synergid is present till the late globular stage.

After the first mitotic division of the zygote, the embryo often differentiates into the embryo proper and the suspensor (Murray 1988). Suspensors in angiosperm embryos vary greatly in size, shape and number of cells (Bhatnagar and Johri 1972,

Natesh and Rau 1984, Raghavan 1986). The suspensor attains full development in the proembryo, then begins to degenerate and is completely obliterated by the growing embryo (Romberger *et al* 1993). Angiosperm suspensor systems have several possible functions: (1) mechanical action in pushing the embryo into the endosperm (Maheshwari 1950); (2) absorption of metabolites and nutrients from parental tissues and translocation of them to the growing embryo (Yeung 1980); and (3) elaboration of growth-regulating substances and nutrients and translocation of them to the embryo (Yeung and Sussex 1979, Natesh and Rau 1984).

2.1.4 Seed Coat

The variation in structure of the seed coat is related to the specific features of the ovule, such as number and thickness of integuments and pattern of vascularization, and to the developmental changes in the integuments during seed maturation (Esau 1977). Most angiosperm seed coat derives from the two integuments or single integument of the ovule. In bitegmic seeds the term 'testa' is correctly applied only to the outer layer, formed from the outer integument, the part formed from the inner integument being the tegmen (Rudall 1987). As the ovule matures into a seed, the integuments undergo conspicuous changes. Mostly there is reduction in thickness and disorganization but sometimes additional layers may be formed, and hence the thickness may be increased (Bhatnagar and Johri 1972).

Seed coats may be complex multilayered tissues, or simply enlarged ovule walls (Rudall 1987). In small seeds none or hardly any cell divisions take place after fertilization, and the seed coat is formed largely through the elongation and differentiation of integumentary cells. Large ovules develop into large seeds, often with complicated seed-coats (Boesewinkel and Bouman 1984). In this case, the cells of both integuments divide after by anticlinal division and more cell-layers by periclinal division (Corner 1976). Because the integuments are usually built up of cell layers, the differentiated elements in the seed-coat are mostly also arranged in layers. Several cell types contributing toward the differentiation and build up of the seed-coat may be parenchyma cells, tannin cells, crystal cells, mucilage cells, cork cells and sclerenchyma or collenchyma cells (Boesewinkel and Bouman 1984).

2.1.5 Seed Structure

Seeds typically consist of three components: embryo, endosperm (sometimes perisperm), and seed coat. Both embryo and endosperm are the products of double fertilization, whereas the seed coat develops from the maternal, ovular tissues (Rudall 1987). Boesewinkel and Bouman (1984) indicated that several parts of the seed could be recognized from the outside to the inside. The seed coat of bitegmic seeds consists of testa (outer integument) and tegmen (inner integument). Each integument has its own opening, both opening from the micropyle. The raphe is the prolongation of the funicle running along the seed and ends at the chalaza. The scar resulting from the disconnection of the seed from the funicle is called the hilum. The perisperm is a food storage tissue and comes from the nucellus, especially at the chalazal end. In most seeds there is another storage tissue called endosperm. Two types of seeds have been classified according to whether their endosperm is completely used up by the developing embryo (exendospermous seeds) or not (endospermous seeds). The embryo is situated opposite the micropyle. Within the embryo there are hypocotyl (stem portion), two cotyledons (in dicots) or one cotyledon (in monocots), plumule (embryonic bud) and radicle (rudimentary root) (Kozlowski and Gunn 1972).

The external morphology of seeds is quite variable and can be described by shape, color and seed coat surface. Common seed shapes are ellipsoid, globose, lenticular, oblong, ovoid, reniform and sectoroid (Kozlowski and Gunn 1972). The shape of the seed usually depends on shape of the ovule. According to Davis (1966), about 80% of the families of angiosperms have anatropous ovules which give rise to the normal seeds. In this type of seed the hilum is situated close to the micropyle, and the embryo is straight (Boesewinkel and Bouman 1984). Brown, grey and pale colors are the most common in seeds. Brown and black make up over half of the seed color (Boesewinkel and Bouman 1984). The surface of the seed in Angiosperm may be smooth, wrinkled, ribbed, punctate, reticulate, glabrous, pulpy, hairy, or have markings resembling fingerprints (Bhatnagar and Johri 1972).

The internal morphology of seeds is also important. Martin (1946) divided seeds into three divisions and a number of subdivisions according to size of embryo in relation to endosperm, and differences in size, shape and position of embryo in seed (Fig. 2.3).

The basal and axile divisions are related to the monocotyledons (Dahlgren and Clifford 1982). In the former, the embryo is usually relatively small and restricted to the lower part of the seed, and seeds are medium to large in size. In the latter, the embryo is small relative to the total seed size, central (axile) straight or variously curved, and seeds are minute to large in size.

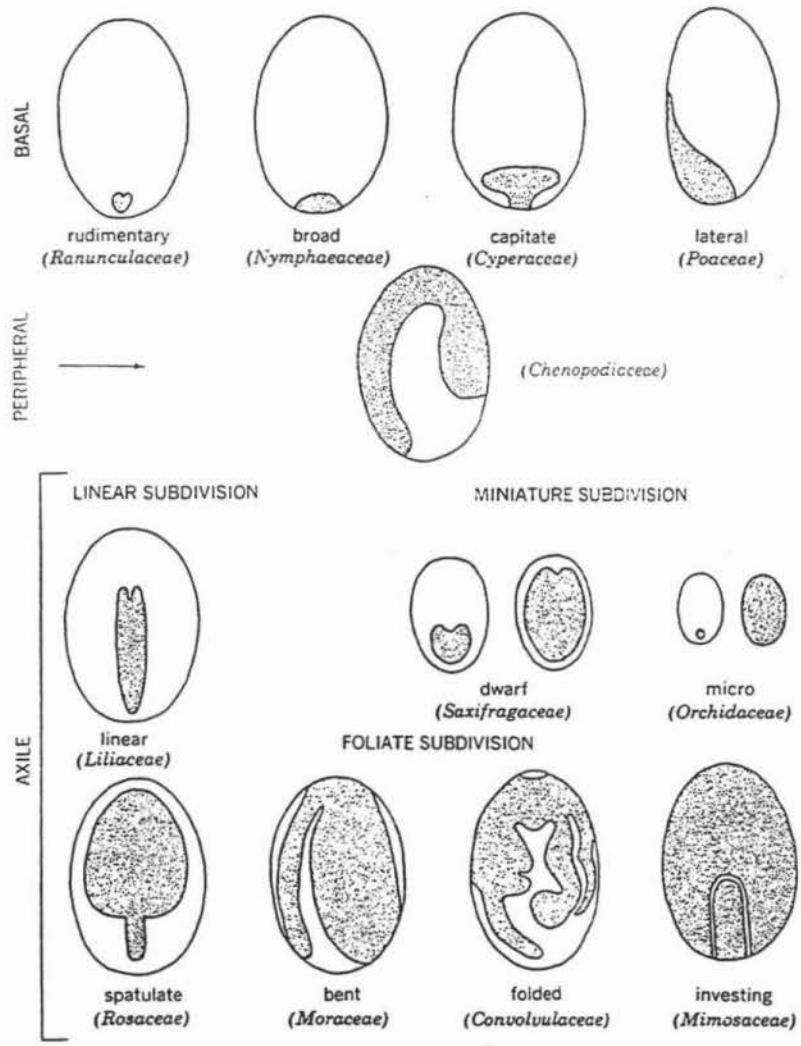


Figure 2.3 Diagrams illustrating typology of seeds based on size, shape, and position of embryo as seen in longitudinal sections of mature seeds (Source: Esau 1977).

In Colchicaceae, the seeds are generally globose, but some are ovoid. The testal part of the seed coat consists of few to many cell layers, which contain starch in the unripe seed. The outer epidermis is retained or compressed (to collapsed) and often contains the red-brown pigment phlobaphene. The tegmen is variably compressed, with or without the pigment phlobaphene or other yellowish red pigments. The endosperm generally contains somewhat thick-walled, pitted cells with aleurone and fatty oils, but no starch. A small, basal, straight, relatively short, elongate or rarely subglobose embryo is enclosed in the endosperm (Dahlgren *et al.* 1985). The detail of seed development of *Sandersonia* (Colchicaceae) from a description of ovule structure prior to fertilization through to seed maturity is a major study focus of this thesis.

2.2 SEED DORMANCY AND GERMINATION

Seed dormancy is defined as "the absence of germination of an intact, viable seed under germination favouring conditions within a specified time lapse" (Hilhorst 1995). Most seeds undergo a period of dormancy after being dispersed from the parent plant. This dormancy may be of short duration or up to many decades (Atwater and Vivrette 1987). The ability of seeds to retain viability for prolonged periods of time without germinating is one of the most important adaptive properties of plants (Nikolaeva 1977). They prevent germination during unfavourable conditions and increase the longevity of seeds in the soil (Fenner 1985).

The phenomenon of seed dormancy cannot be divorced from the processes of seed development and germination. Seed dormancy is initiated during seed development, the process being influenced by the parental and zygotic genotype (Simpson 1990). The genotype will interact with the different environmental and physiological phenomena which occur from anthesis to dispersal, producing varying degrees of dormancy (Jain 1982, Nooden *et al.* 1985). During the first half of seed development most seeds become germinable, marking the completion of histodifferentiation of the embryo (Hilhorst 1995). The final phase of seed development involves the loss of water, cessation of reserve synthesis and accumulation of metabolic substances (Kelly *et al.* 1992), which bring seeds from the developing stage to a propagating stage. Seeds which do not exhibit dormancy will germinate when supplied with a suitable temperature,

adequate moisture and oxygen after dispersal. However, in dormant seeds the period between desiccation and germination is extended, and the seeds do not germinate even when placed under favourable conditions. According to Bewley and Black (1994), the relationships between dormancy and germination are shown in Fig. 2.4. Here the additional complexity of secondary dormancy which may exist in some seeds is shown in relation to primary dormancy. The scheme is an over-simplification however as many kinds of dormancy are known.

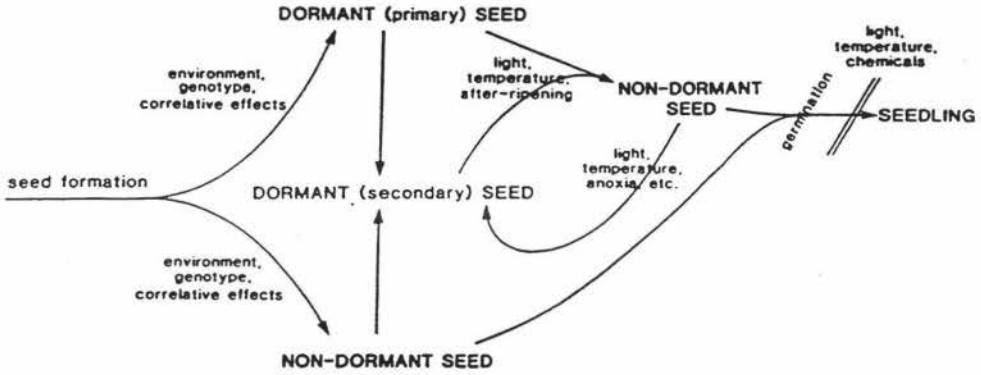


Figure 2.4 Dormancy and germination (Source: Bewley and Black 1994).

2.2.1 Classification of Types of Seed Dormancy

Over the years many classifications of seed dormancy types have emerged (e.g., Nikolaeva 1977, Lang *et al.* 1987). Nikolaeva (1977) introduced no less than 15 types of dormancy while Lang *et al.* (1987) even found 54 terms in the literature. In an attempt to clarify the confusing situation they proposed three main classes of dormancy with at least 15 sub-classes. These classifications are mainly based on the variety of factors that may impose dormancy.

A generally accepted distinction is that between embryo dormancy, in which case

removal of the seed coat does not permit embryos to germinate normally, and coat-imposed dormancy, where the seed coat (but often including endosperm, pericarp, or extrafloral organs) exerts constraints that the embryo cannot overcome and the embryo germinates when excised (Bewley and Black 1994). Both types of dormancy exist simultaneously or successively in some species. For example, in apple seed embryo dormancy predominates, but a contribution is made by the covering tissues–endosperm and testa (Bewley and Black 1994).

Seeds are also said to have primary dormancy when they are dispersed from the parent plant in a dormant state, and secondary dormancy which is induced in mature nondormant seeds (Karssen 1982, Bewley and Black 1994). This classification is based on timing rather than endogenous or exogenous dormancy-controlling factors. It may be meaningful because primary dormancy is essentially related to seed development and maturation whereas secondary dormancy can only occur after seed dispersal (Hilhorst 1995).

2.2.2 Embryo Dormancy

Embryo dormancy is defined as the situation when a viable, mature embryo of a dormant seed fails to germinate, even after it has been separated from the rest of seed. In this case, many detailed studies suggest that the control of embryo dormancy involves (a) the cotyledons, and (b) germination inhibitors (Bewley and Black 1982). The morphologically immature embryos, in which the control of dormancy obviously has an anatomical basis, are also considered in the context of embryo dormancy because they do not germinate when isolated. In embryo dormancy there is usually a requirement for hormonal, temperature and/or light treatments (Kelly *et al.* 1992).

2.2.2.1 Cotyledons and Embryo Dormancy

Amputation of the cotyledons often allows the embryonic axis of the dormant embryo to germinate and grow. In this way, dormancy is partially or completely broken in *Corylus avellana* (hazel) (Jarvis 1975) and *Euonymus europaeus* by excising one cotyledon, and in *Fraxinus excelsior* by cutting off two (reviewed in Bewley and Black 1994). Seed dormancy of *Spartina alterniflora* could be broken by surgically altering the scutellum

(98% and 44% germination in seeds with part of the scutellum removed and the scutellum punctured, respectively) (Plyler and Carrick 1993). Removal of the cotyledons from embryos of freshly harvested *Acer velutinum* fruits allows more rapid germination of the embryonic axes (Pinfield and Stutchbury 1990). Applied ABA strongly suppressed germinative capacity in intact embryos and isolated embryonic axes from freshly harvested fruits. These experiments indicate that cotyledons exert an inhibitory effect on the germination of the embryonic axis. This has frequently been ascribed to a chemical germination inhibitor--ABA that is produced in the cotyledons (Pinfield and Stutchbury 1990, reviewed in Bewley and Black 1994) or is present in the cotyledons, but derived from the other tissue such as testa and pericarp (Jarvis 1975).

2.2.2.2 Germination Inhibitors

Dispersal units of many species contain some substances called inhibitors which can inhibit germination. Inhibitors of different chemical classes have been found in seeds of many species, some in the embryo and some in the seed coat (see Table 2.1). The most widespread and important inhibitor is abscisic acid (ABA), found not only in embryos but also in the covering tissues (see Table 2.1). Correlations can be seen between the depth of embryo dormancy and the concentration of ABA. For example, the more deeply dormant embryos of the Lovell cultivar of peach (*Prunus persica*) contain approximately 1.5 μg ABA/g dry weight, whereas the less dormant embryos of the Tetela cultivar have only 0.2 μg ABA/g dry weight (Diaz and Martin 1972). However, it is often found that there is no clear correlation between the retention of ABA into maturity and the depth of dormancy. For instance, the nondormant Great Lakes cultivar of lettuce contains relatively high amounts of ABA (reviewed in Bewley and Black 1994). The mechanism of ABA regulated dormancy induction is largely unknown but appears to be associated with cell wall properties (Hilhorst 1995).

In addition to ABA, other inhibitors isolated from many species are unsaturated lactones such as coumarin, and various phenolic substances. The phenolic compounds include a wide range of plant substances which possess in common an aromatic ring bearing one or more hydroxyl substituents (Harborne 1973). They tend to be water-soluble, and located in the cell vacuole. Chow and Lin (1991) reported that the

phenolics in the sarcotesta played an important role during dormancy and germination of papaya (*Carica papaya* L.) seed. Acid hydrolysis of papaya seed sarcotesta releases a number of ether soluble phenolics. These phenolic fractions inhibit germination and seedling growth of lettuce seed. There are several hypotheses regarding the inhibitory effects of phenolic compounds on seed germination. Willemsen and Rice (1972) suggested that the quantity of auxin in the seed may be regulated by phenols via the IAA-oxidase system. Monohydric phenolics acted as cofactors to the IAA-oxidase system and accelerated decarboxylation; dihydric phenols or polyphenols with at least one free hydroxyl group in the *ortho* position on the aromatic ring inhibit decarboxylation of IAA. Marbach and Mayer (1974, 1975) hypothesized that during dehydration of seeds, oxidation of phenolic compounds in seed coats catalyzed by catechol oxidase might render the seed coat impermeable to water. Taylorson and Hendricks (1977) suggested that consumption of oxygen by seed coat phenolics during germination could limit oxygen supply to embryos.

Table 2.1 Some seeds containing germination inhibitors (Source: Bewley and Black 1994)

Species	Location	Inhibitor
Acer negundo	Pericarp	ABA
Avena fatua	Not determined	ABA
		Short-chain fatty acids
Beta vulgaris	Pericarp	Phenolic acids
		cis-Cyclohexene-1,2-dicarboximide
		Inorganic ions
Corylus avellana	Testa, embryo	ABA
Eleagnus angustifolia	Pericarp, testa	Coumarin
	Embryo	Coumarin
Fraxinus americana	Pericarp	ABA
	Embryo	ABA
Medicago sativa	Endosperm	ABA
Prunus domestica	Embryo	ABA
Rosa canina	Pericarp, testa	ABA
Taxus baccata	Embryo	ABA
Triticum spp.	Pericarp/testa	Catechin, tannins

2.2.2.3 Embryo Immaturity

Morphological dormancy, or under-development of the embryo is widely encountered in plants of many families (Martin 1946): Aquifoliaceae, Araliaceae, Magnoliaceae, Papaveraceae and Ranunculaceae, etc. These primitive families have medium to large seeds containing non-starchy endosperm and under-developed embryos. Such embryos are sometimes considered in the context of embryo dormancy because they do not germinate when isolated (Bewley and Black 1982). Under-developed embryos are either rudimentary (globular to ovaloblong) or linear (several times longer than broad) (Martin 1946, Atwater and Vivrette 1987), in some cases poorly differentiated, and must grow and develop before being ready for germination. Seeds exhibiting morphological dormancy are more frequently met in tropical plants. However, they occur also in plants of temperate zones, for example, in certain *Aconitum* species (Nikolaeva 1977).

In the majority of cases, the under-development of the embryo is associated with the presence of the physiological mechanism of inhibition, this giving rise to morphophysiological dormancy types (Nikolaeva 1977). At the time of seed dispersal, under-developed embryos lacking physiological dormancy grow as soon as seeds are placed under suitable temperature, light/dark, and moisture conditions (Baskin and Baskin 1990). Thus, germination is delayed only by the small size of the embryo, and seeds have morphological dormancy (Nikolaeva 1969). In contrast, growth of physiologically dormant embryos is delayed until environmental conditions are appropriate for both dormancy loss and embryo growth (Nikolaeva 1969). Baskin and Baskin (1994) reported that seeds of *Delphinium tricorne* Nutt had under-developed linear embryos which were also inhibited by physiological dormancy. The embryos must grow from about 0.5 to 2.4 mm before seeds can germinate, and this growth occurs only at low temperatures by 10-12 week of stratification. Another example is freshly matured seed of *Osmorhiza claytonii* which exhibits a type of morphophysiological dormancy. Embryos do not grow in seeds for 24 weeks at 30°/15° or 5° C. However, in seeds given 12 weeks at 30°/15° and then 12 weeks at 5° C, embryo length increases 1246%. 2 to 12 weeks of warm followed by 24 weeks of cold stratification results in 80%-98% germination of fresh seeds (Baskin and Baskin 1991).

2.2.3 Cost-Imposed Dormancy

Seed dormancy in the majority of species is imposed by the structures surrounding the embryo; these are often referred to as the seed coat (Bewley and Black 1982). The structures responsible for imposing and maintaining dormancy vary from species to species. They include the glumes, palea and lemma, the pericarp, testa, perisperm and endosperm. Seed coats and surrounding structures may influence the ability of a seed to germinate through interference with water uptake, gas exchange, diffusion of endogenous inhibitors, or by mechanical restriction of embryo growth (Ikuma and Thimann 1963, Mayer and Shain 1974). Prevention of germination may be due to the action of one or more of these mechanisms (Bewley and Black 1994). In these cases, the dormancy could be broken by complete removal of the seed coat, perforation, scarification and even exposure to concentrated sulfuric acid (reviewed in Bewley and Black 1982).

2.2.3.1 Water Uptake by Seeds

The uptake of water by seeds is an essential, initial step toward germination. The total amount of water uptake during imbibition is generally quite small and may not exceed two or three times the dry weight of the seed (Bewley and Black 1994). For subsequent seedling growth, which involves the establishment of the root and shoot systems, a larger and more sustained supply of water is required. Under optimal conditions the uptake of water by seeds shows three stages (Fig. 2.5). In phase I, imbibition is probably very rapid and the water content of seeds increases to 80 to 120% dry weight (Hartmann *et al.* 1990) regardless of whether the seed is dormant or nondormant, viable or nonviable because the water potential of a mature dry seed is much lower than that of the surrounding moist substrate and can exceed -100 Mpa (Bewley and Black 1994). Phase II is a slow (lag) period of water uptake in which the value of water potential of seeds does not exceed -1 to -1.5 Mpa (Bewley and Black 1994). This phase is characterized by enzyme activation and synthesis (Hartmann *et al.* 1990). Only germinating seeds enter phase III which is concurrent with radicle elongation. The increase in water uptake is initially related to the changes that cells of the radicle undergo as they extend, marking the completion of germination (Bewley and Black 1994).

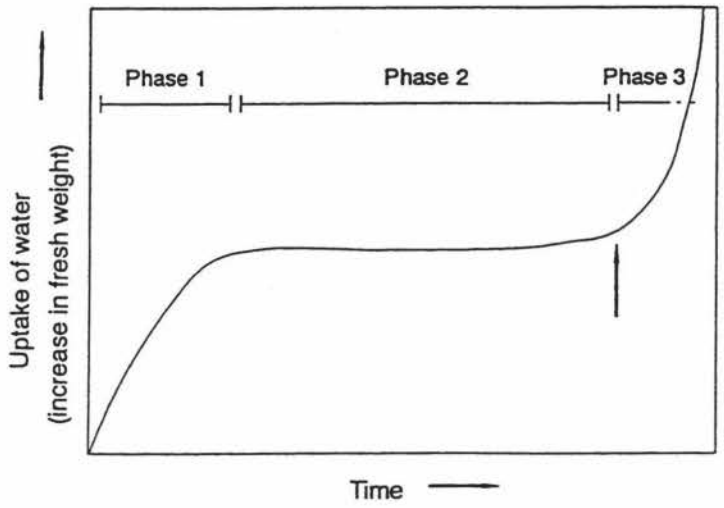


Figure 2.5 Triphasic pattern of water uptake by germinating seeds. Arrow marks the time of occurrence of the first signs of radicle protrusion (Source: Bewley and Black 1994).

2.2.3.2 Seed Impermeability

Kelly *et al.* (1992) indicated that the impermeability of the seed coat to water and/or gases and mechanical restriction of the embryo is achieved structurally and/or chemically. Structurally, impermeability is imposed as the seed shrinks during maturation, the strips of thickenings in the upper part of each epidermal cell, become pressed together until they occlude the cell lumen entirely (van Staden *et al.* 1989). Compression of the palisade layer reinforces the impermeability to water as well (Graaff and van Staden 1983, Werker *et al.* 1979). Chemically, the deposition of hydrophobic substances in the palisade cells, such as callose, lipid, suberin, and the conversion of hydroxyphenolics to insoluble lignin polymers, ensure that the seeds remain impermeable to water and/or gases (Kelly *et al.* 1992). A structure called the "light-line" which is located in palisade cells of seeds of Leguminosae also contributes to the impermeability of the seed coat (Serrato Valenti *et al.* 1989). In some cases the impermeable barrier includes the surface as well as the palisade layer. For example *Rhynchosia minima* impermeability is ensured by a waxy/lipoidal surface material, a hemicellulose/cellulose barrier, followed by the palisade cells containing cellulose fibrils, arabinans, phenolic compounds and tannins (Ramgaswamy and Nandakumar 1985). The testa in the Leguminosae has the hilum,

micropyle, and strophiole which also contribute to impermeability of seed coat to water (Bewley and Black 1994).

Detailed investigations to determine which region of the coat is responsible for preventing water entry have been carried out mainly in the Leguminosae, but work has also been done in other families such as the Cannaceae, Convolvulaceae, Chenopodiaceae, Malvaceae, Papilionaceae and Liliaceae (Nikolaeva 1977, Bewley and Black 1982). Dormancy of *Kosteletzkya virginica* (L.) Presl. (Malvaceae) seeds is primarily due to the impermeability of the seed coat to water (Poljakoff-Mayber *et al.* 1992). The impermeable structure is assumed to be the palisade layer of the seed coat. The percentage of seeds capable of imbibition and germination increases with increasing time of storage at low temperature. The field germination rate of *Cistus albidus* (Juhren 1966) and *C. monspeliensis* (cited in Corral *et al.* 1990) (Cistaceae) are shown to be increased by 10 times in burnt areas. Laboratory experiments carried out with both species revealed the presence of dormancy imposed by the seed coats, which implied water impermeability. Scarification and high temperatures (80-100°C for 5 to 30 minutes) were the most effective methods in breaking dormancy in the seed of *C. albidus* and *C. monspeliensis* (cited in Corral *et al.* 1990).

The several layers of tissue surrounding the embryo might limit the capacity for gaseous exchange by the embryo. First, entry of oxygen may be impeded; second, escape of carbon dioxide may be hindered (cited in Bewley and Black 1994). For instance, the removal of the seed coat of *Cynoglossum officinale* L. results in nearly complete germination of innately dormant seeds. The seed coat does not substantially retard water uptake nor does it contain any water soluble germination inhibitor, but the O_2 uptake of seeds increases approximately six-fold upon removal of the seed coat (Qi and Upadhyaya *et al.* 1993). The increase in O_2 uptake induced by seed coat removal is due to both an increase in seed respiration and a high level of non-respiratory O_2 consumption (Qi and Upadhyaya *et al.* 1993). Freshly harvested seeds of numerous cereals from temperate climates, such as wheat, barley (reviewed in Come *et al.* 1988) and oat (Corbineau *et al.* 1986), germinate well between 0° C and 10° C or 15° C, but their germination is increasingly difficult at higher temperatures. This is due to a special dormancy which mainly results from an inhibition exerted by the coverings of the grain

(seed coat + pericarp) and by the glumellae when they remain attached to the caryopsis as in the case of barley or oat (reviewed in Come *et al.* 1988). These structures may inhibit germination at relatively high temperatures by depriving the embryo of oxygen, probably through the utilization of available oxygen in the enzymatic oxidation of phenolic compounds (Lenior *et al.* 1986, Corbineau *et al.* 1986). Some examples show that coat-imposed dormancy could be removed by removing the tissues surrounding the embryo, puncturing the seed coat or supplying high oxygen. (Table 2.2).

Table 2.2 Some treatments that remove coat-imposed dormancy (Source: Bewley and Black 1994)

Species	Tissue removal	Puncturing coat	High oxygen
Acer pseudoplatanus	+++ (testa)	+++	–
Avena fatua	+++ (hull)	–	++
Betula pubescens	+++ (pericarp, testa, endosperm)	+	++
Hordeum spp.	+++ (hull)	++	++
Oryza sativa	+++ (hull)	++	++
Phacelia tanacetifolia	+++ (endosperm)	–	++
Triticum spp.	+++ (pericarp, testa)	+	+++
Xanthium pennsylvanicum	+++ (testa)	–	+++

[a]+++, Strong dormancy-relieving effect; ++, moderate effect; +, slight effect; –, no effect.

2.2.3.3 Mechanical Restraint

When the seeds imbibe and still do not germinate, the seed coat or surrounding tissues such as endosperm or perisperm (Bradford 1990, Groot and Karssen 1987, Ni and Bradford 1993, Sanchez *et al.* 1990) may be preventing germination mechanically.

In *Stachys alpina* L. seeds, the dormancy can only be overcome by exogenous applications of gibberellins, or by the removal or perforation of the seed coat (Pinfield *et al.* 1972). The seed coat of *Onopordum nervosum* Boiss, especially in the palisade cells layer (macrosclereids) could represent an important mechanical barrier to radicle expansion, and the resistance of the seed coat decreased with increasing imbibition time (Perez-Carcia and Pita 1989). Mechanical scarification of iris seeds at the micropylar end allows for radicle protrusion and germination (Blumenthal *et al.* 1986). A pressure

of 135 atmospheres is necessary in *Iris lorteti* seeds for radicle protrusion, while in *Iris atropurpurea*, 77 atmospheres is sufficient.

In some species, the tissue that is responsible for coat-imposed dormancy is endosperm. A rather good example provided by dormant cultivars of the lettuce seed (*Lactuca sativa*). Encasing the embryo of lettuce is a two-celled layer of endosperm, characterized by thick-walled cells and dense cytoplasm (Jones 1974). The endosperm envelope restrains the germination process, delaying radicle emergence under optimal conditions or preventing germination under inhibitory conditions (Bradford 1990). Ikuma and Thimann (1963) proposed that red light relieved endosperm restriction on the embryo in photosensitive lettuce varieties by triggering the production of cellulolytic enzymes in the embryo, weakening the endosperm layer. Nabors and Lang (1971) also studied the effect of the lettuce endosperm on restriction of radicle emergence and found that this restriction could be overcome by increasing the growth rate of the embryo. Pavlista and Haber (1970) proposed that both the mechanical force of the growing embryo pushing against the endosperm, and the weakening of the endosperm were necessary for lettuce seed germination. Structural modifications of lettuce seeds in restricted areas of endosperm opposite the radicle tip (micropylar endosperm region) were observed prior to radicle emergence (Georghiou *et al.* 1983). Similar modifications of the micropylar endosperm region were seen after red light exposure (Psaras *et al* 1981) and GA_3 treatment (Psaras and Georghiou 1983). These observations suggest that structural modifications in the micropylar region of the endosperm are involved in the completion of the germination process. Weakening the endosperm tissue around the radicle tip prior to radicle emergence has been related to the regulation of germination in pepper (*Capsicum annuum* L.), tomato (*Lycopersicon esculentum*), and *Datura* seeds (Watkins and Cantliffe 1983, Groot and Karssen 1987, Sanchez *et al.* 1990).

In muskmelon (*Cucumis melo* L.) seeds, embryos are enclosed in an envelope of tissue consisting of a layer of endosperm and a multi-cell-layered perisperm by which the embryos, and particularly the axes, are prevented from attaining full hydration (Welbaum and Bradford 1990). When the perisperm and testa are removed, the external pressure is relieved and axis water content is increased by 50 %. They hypothesize that growth of

the embryonic axis may be initiated by weakening or separation of cells in the region of the perisperm opposite the radicle tip.

2.2.3.4 Interference with Diffusion of Endogenous Inhibitors

Examples of coats with inhibitors are given in Table 2.1. Frequently, the inhibitor has been detected by its action on wheat or oat coleoptile growth, or by its effect on germination of a different species, such as lettuce or cress (Bewley and Black 1982). In some cases it has been possible to show directly that the influence of the enclosing structures in dormancy is connected with the presence of inhibitors. Embryos of *Iris* germinate readily when isolated, though the intact seed exhibits dormancy. The tissue responsible is the endosperm. That inhibitors are involved is strongly suggested by the observation that the germination is considerably retarded if excised embryos are in contact with pieces of endosperm (reviewed in Bewley and Black 1982). A rather similar approach has been taken with dormant seeds of papaya (*Carica papaya* L.) (Chow and Lin 1991). When removed from the sarcotesta, or leached with tap water these seeds germinate.

Germination inhibitors occur within the inner tissues as well as in the outer coverings of the seed (Table 2.1). It is conceivable that seed coat impairs the escape of these inhibitors by acting either as a completely impermeable barrier or to reduce the rate of outward diffusion (Bewley and Black 1982). Dormancy in most strains of *Avena fatua* is generally imposed and maintained by the hull (lemma and palea). When naked caryopses are held on a wet substratum, dormancy is relieved and rapid germination ensues. However, if only part of the hull is removed from one side, to leave the caryopsis in a boat-shaped portion, germination occurs only if the caryopsis is in contact with the wet paper and not if the unit is placed caryopsis uppermost with the hull in contact (Bewley and Black 1994). In another example, the coat of the deeply dormant upper seed of *Xanthium pennsylvanicum* (polymorphic seeds) acts by preventing the escape of inhibitors from the embryo (Wareing and Foda 1957). The embryo contains two water-soluble inhibitors which diffuse out from the naked embryo but not from the intact seed when these are held on a wet substratum. Therefore the testa acts as an effective barrier against the outward movement of the inhibitors.

2.2.4 Laboratory Techniques for Breaking Seed Dormancy

Although the mechanism of dormancy breaking and induction is still imperfectly understood, effective laboratory techniques for breaking dormancy of seeds have been developed. Seeds of many species can be effectively removed from their dormancy in the laboratory by using different treatments, such as after-ripening, temperature, light, chemical, mechanical and hormone. These methods have been used for many years, but no simple method has been found suitable for all species. Sometimes, the termination of dormancy is influenced by more than one factor (Bewley and Black 1994). For example, dormancy of many light-requiring seeds is also broken in darkness by chilling, by alternating temperatures, by exposure to nitrate in the soil, and by after-ripening in the "dry" or near-dry state.

2.2.4.1 After-ripening

Table 2.3 Removal of dormancy by dry after-ripening

Species	Alternative period of dry storage	Reference
Acer negundo	7-8 months	Nikolaeva 1969
Ambrosia trifida	12 months reduces required cold period by 70 %	Davis 1930
Avena fatua	25° C for 18 months	Adkins & Ross 1981
Avena fatua (Line M73)	3-16 months depend on temperature and relative humidity	Foley *et al.* 1993
Betula pubescens	12 months	cited in Bewley & Black 1982
Bromus secalinus	1 month	Steinbauer & Grigsby 1957
Dioscorea composita	9 months	Viana & Felippe 1990
Helianthus tuberosus L.	More than 27 months	Lim & Lee 1989
Helichrysum apiculatum	20 weeks	Willis & Groves 1991
Hordeum spp.	3-9 months	cited in Crocker & Barton 1953
Hygrophila auriculata	28° C for 4 months	Amritphale *et al.* 1995
Hyptis suaveolens Poit.	8 months	Wulff & Medina 1971
Kosteletzkya virginica L.	storage at 5° C	Poljakoff-Mayber *et al.* 1992
Lactuca sativa	12-18 months	cited in Bewley & Black 1982
Oryza sativa	2-3 months	Roberts 1962
Rumex crispus	60 months (approx. 50 % of seeds released)	Cavers 1974
Sporobolus cryptandrus	> 48 months	Toole 1941
Stylidium graminifolium	over 24 weeks	Willis & Groves 1991
Triticum aestivum	3-7 months	cited in Bewley & Black 1982
Wahlenbergia stricta Sweet	64 weeks	Willis & Groves 1991

Dormant seeds of some species slowly lose their dormancy by the process of after-ripening, e.g. barley requires as little time as a few weeks or *Rumex crispus* as long as 60 months (Bewley and Black 1994). The process of after-ripening takes place only in seeds which have a low water content. Some species which need after-ripening to break seed dormancy are shown in Table 2.3. Dormancy of *Ambrosia trifida* (Davis 1930), *Acer semenovii* and *Fraxinus excelsior* (Nikolaeva 1969) is not totally terminated by dry after-ripening but instead the requirement for chilling is much reduced. Similarly in *Rumex crispus* 5 years of after-ripening removes dormancy in only about 50 % of the seeds in a population but the remainder display enhanced sensitivity to alternating temperature and light (Cavers 1974).

2.2.4.2 Stratification

Hydrated seeds of many woody and herbaceous species are released from dormancy when they experience relatively low temperature -- in the range 1°-15° C (Bewley and Black 1982). For the former, an one to 12 months treatment at 1°-5° C might be necessary in order to overcome dormancy (ISTA 1985). In the latter, including seeds for agriculture, vegetable production, flower production and grass seeds, a much shorter period is usually required, and the optimum temperature seems to be somewhat higher (Aamlid 1992). This low-temperature experience is variously described as chilling, cold after-ripening or stratification.

Chilling is effective in a range of dormancy types. It is commonly required by seeds of woody species which have embryo dormancy, such as *Acer saccharum, Corylus avellana, Crataegus* spp., *Pyrus* spp. and many genera in the Rosaceae (Nikolaeva 1969) as well as species which have coat-imposed dormancy, for example, *Acer ginnala, A. negundo* (Nikolaeva 1969) and *A. pseudoplatanus* (Thomas *et al.* 1973). Some successful examples in which dormancy of seeds is broken by chilling are shown in Table 2.4. Most chilling-responsive species are satisfied by a single period of cold, but in those which have double dormancy the radicle and shoot appear to have different requirements for low temperature (Stokes 1965). In *Caulophyllum thalictroides* and *Trillium erectum*, for example, radicle emergence occurs after chilling but the shoot does not appear until after a second period of low temperature has been experienced.

Table 2.4 Some successful examples for breaking seed dormancy by stratification

Species	treatments	germination (%)		Reference
		Before	After	
Acer velutinum Boiss.	5° C in moist sand for 110 days	0	75	Pinfield & Stutchbury 1990
Arum maculatum L.	6° C for 1 year	0	> 70	Pritchard *et al.* 1993
Bidens laevis	5° C at least for 8 week with plenty of oxygen and light	-	>80	Leck *et al.* 1994
Delphinium tricorne Nutt	5° C for 38-40 weeks	2	96-100	Baskin & Baskin 1994
Delphinium tricorne Nutt	5° C for 14 weeks, 14 h light/d, germinated at 15/6° C in darkness	2	74	Baskin & Baskin 1994
Discaria toumatou	Fully imbibed seeds at 4° C for 8 weeks	2	> 90	Bannister & Jameson 1991
Echinacea angustifolia	3-5 C for 2-3 weeks	48	80	Parmenter *et al.* 1996
Eustoma grandiflorum	3 °C at least for 11 days	0	98	Ecker *et al.* 1994
Helianthus tuberosus L.	2.5° C for 70 days	0	> 85	Lim & Lee 1989
Helipterum albicans DC.	4° C for 6 weeks in darkness	50	90	Willis & Groves 1991
Lactuca serriola	15° C in darkness	0	66	Small & Gutterman 1992
Panicum virgatum L. "Alamo"	Stratification for 4 weeks	52	94	Tischler *et al.* 1994
Penstemon dolius	4 weeks at 10/20° C + 12 weeks at 1° C	0	57	Meyer *et al.* 1995
Penstemon goodrichii	4 weeks at 10/20° C + 12 weeks at 1° C	1	53	Meyer *et al.* 1995
Penstemon moffattii	4 weeks at 10/20° C + 12 weeks at 1° C	0	98	Meyer *et al.* 1995
Penstemon acuminatus	1° C for 12 weeks	0	94	Meyer *et al.* 1995
Penstemon pachyphyllus	1° C for 12 weeks	15	92	Meyer *et al.* 1995
Penstemon confusus	1° C for 12 weeks	1	96	Meyer *et al.* 1995
Penstemon utahensis	1° C for 12 weeks	3	86	Meyer *et al.* 1995
Petunia hybrida	0-5° C for 15 days	-	73	Gonzalez & Villalobos 1988
Pinus koraiensis	Presoaking seeds in a initial 70°C, then at room temperature for 6 d, +3° C for 3 months	3.3	63	Qi, Bilan & Chin 1993
Pinus taeda L.	2° C for 35 days	19	76	Schneider & Gifford 1994
Pittosporum eugenioides	4° C for 12 weeks	0	>90	Bannister & Jameson 1991
Pittosporum obcordatum	4° C for 8 weeks	0	>90	Bannister & Jameson 1991
Pyrus serotina Rehd cv. Niauli	4° C for 21 days, germinated at 25° C	0	63	Lin *et al.* 1994

2.2.4.3 Other Effects of Temperature on Dormancy

In the field, dormant seeds are commonly subjected to fluctuating temperatures, for example, low night temperatures and high daytime temperatures. The promotive effect of alternating temperatures on seed germination has been known for at least 100 years. For example, von Liebenberg (1884, reviewed in Aamlid 1992) established that Kentucky bluegrass germinated better when kept at 20°C for 19 h and at 28°C for 5 h each day than at a constant temperature of either 20°or 28°C. Thus, such temperature alternations are frequently used in laboratories for seed dormancy breakage. For example, breaking dormancy in *Rumex obtusifolius* is obtained by alternating temperatures. High germination percentages occur in 16 h at 25°-35°C, 8 h at 1.5°-15°C; or 8 h at 25°-35°C, 16 h at 1.5°-20°C (Totterdell and Roberts 1980). Seven native Australian herbaceous species were studied by Willis and Groves (1991) for their seed germination. They suggested that the germination in two species – *Helichrysum apiculatum* and *Bulbine bulbosa* was strongly temperature-dependent. For instance, the optimum temperature regime for germination of *H. apiculatum* was 20°/10°C, at which 94 % of seeds germinated, significantly more than at either 15°/5°C (31% germination) or 35°/25°C (23% germination). Similarly, at the 35°/25°C extreme, no *B. bulbosa* seeds germinated, compared with 90% germination at the optimum regime of 20°/10°C. At 25°/15°C and 15°/5°C, germination was limited to 44% and 50%, respectively.

In a few species, relatively high temperatures can break, or assist in breaking, dormancy. Seeds of *Hyacinthoides non-scripta* require several weeks at 26°-31°C followed by a germination phase at 11°C (cited in Bewley and Black 1994). *Osmorhiza claytonii* require 2-12 weeks at 30°/15°C warm stratification prior to chilling at 5°C for 24 weeks (Baskin and Baskin 1991). After-ripening of dry seed at high temperatures also reduces dormancy in wild oats (Simpson 1990), wheat (Hagemann and Chia 1987) and barley (cited in Aamlid 1992). Heating at 30°-35°C (ISTA 1985) is therefore the most common way of overcoming dormancy in cereals, but similar results have been reported for Kentucky bluegrass (Phaneendranath and Funk 1981) and *Helichrysum apiculatum* as well (Willis and Groves 1991).

2.2.4.4 Light

Light is an extremely important factor for releasing seeds from dormancy. Almost all light-requiring seeds have coat-imposed dormancy (Bewley and Black 1994). Seeds of some species are promoted to germinate by very short exposures (minutes or seconds) to white light (e.g., lettuce); whereas others require intermittent irradiation (e.g. *Kalanchoe blossfeldiana*). Some seeds whose dormancy and germination are associated with light are shown in Table 2.5. In nature, white light (i.e. sunlight) breaks dormancy, but it is known that the wavelengths in the orange/red region (660 nm) of the spectrum are most effective, whereas the wavelengths in the far-red region (730 nm) have an inhibitory function (Bewley and Black 1994). The reversible promotion and inhibition of germination of light sensitive seeds by alternate exposures to red and far-red light are two facets of the operation of the phytochrome system (Bewley and Black 1982).

2.2.4.5 Chemicals

Some examples in which seed dormancy can been broken by chemicals are given in Table 2.6. The mechanism by which these promote germination is usually unknown but possible modes of action include anaesthetics in relation to membranes, respiratory inhibitors, nitrate, nitrite, methylene blue in relation to the pentose pathway, and oxygen in connection with various oxidation processes (Bewley and Black 1994).

2.2.4.6 Hard-Coated Seeds

Several artificial methods are used to soften the hard, impermeable seed coats of the Leguminosae, Cannaceae, Malvaceae, Convolvulaceae and other family to release them from dormancy (Bewley and Black 1982). These include treatment with concentrated sulfuric acid or ethanol, mechanical scarification, freezing, heating, radiation, percussion and pressure (Rolston 1978).

Treatment with sulfuric acid is one of the commonest and most effective methods. Table 2.7 shows the increase in germination after sulfuric acid treatments with a number of species reported by some authors. The use of concentrated sulfuric acid is not favoured by all workers for safety reasons.

Table 2.5 Effect of light on breaking seed dormancy of some species

Species	treatments	germination (%)		Reference
		Before	After	
Acaena novae-zelandiae T.Kirk.	Germinating at 15° C with 18 h light/d	0	90	Gynn & Richards 1985
Caesulia axillaris Roxb.	Red light for 10 min	0	59	Naidu & Amritphale 1994
Caesulia axillaris Roxb.	Soaking for 120 h + far-red light for 10 min	0	23	Amritphale 1993
Celmisia angustifolia	Germinating at 20° C with light	15	54	Scott 1975
Celmisia coriacea	Germinating at 20° C with light	18	72	Scott 1975
Celmisia gracilenta	Germinating at 20° C with light	24	77	Scott 1975
Celmisia lyallii	Germinating at 20° C with light	9	37	Scott 1975
Chrysocephalum apiculatum (Labill)	45.1±6.0 μM m^{-2} s^{-1} light, 10-12/d	0.05	56.9	Bunker 1994
Datura stramonium	Daily light	<30	>80	Benvenuti 1995
Datura ferox	Below 5 cm in soil, at 32/ 12° C	13	68	Reisman-Berman *et al.* 1991
Elaeagnus mollis	1000 lx or nature light for 3 days, at 15-25° C	38	84-92	Du *et al.* 1989
Eragrostis curvula (Schrad.)	Soaking for 24 h + red light for 2 min, at 29° C	38	96	Toole & Borthwick 1968
Eragrostis curvula (Schrad.)	Soaking for 24 h + far-red light for 2 min, at 29° C	38	59	Toole & Borthwick 1968
Fagopyrum esculentum Moench.	Germinating at 20°C 16h /30°C 8h with light	41	66	Samimy 1994
Gaultheria antipoda	Germinating at 20°C with light	11.5	84	Bannister 1990
Gaultheria depressa	Germinating at 20°C with light	0	39	Bannister 1990
Hyalosperma glutinosum ssp.	45.1±6.0 μM m^{-2} s^{-1} light, 10-12/d	0.05	31.7	Bunker 1994
Hygrophila auriculata	10 min red light, germinating in darkness at 28°C	22	68	Amritphale *et al.* 1995
Lactuca sativa L. cv. Grand Rapids	Soaking for 2 h, then red light for 1.5 min	0	98	Bewley & Black 1982
Lactuca serriola L.	Continuous white light at 20°C	0	98	Small & Gutterman 1992
Lactuca serriola L.	5 min red light 3 and 6 h after start of imbibition	0	92	Small & Gutterman 1992
Lactuca serriola L.	Red / Far-red / Red at 20°C	0	90	Small & Gutterman 1992
Nigella damascena L.	Soaking for 2 days, germinating at red light	41.2	52.5	Rudnicki & Kaukovirta 1991
Pernettya macrostigma	4°C for 26 days, then germinating at 20°C with light	0	57	Bannister 1990
Waitzia acuminata Steetz	45.1±6.0 μM m^{-2} s^{-1} light, 10-12/d	7.4	34.5	Bunker 1994

Table 2.6 Some successful examples for breaking seed dormancy by chemicals

Species	treatments	germination (%)		Reference
		Before	After	
Caesulia axillaris Roxb.	10^{-5} M NaN_3 for 120 h + 10 min red light	0	91	Naidu & Amritphale 1994
Caesulia axillaris Roxb.	10^{-4} M Salicylhydroxamic azide for 120 h + 10 min red light	0	89	Naidu & Amritphale 1994
Caesulia axillaris Roxb.	Soaking in 5 mM KNO_3 for 5 days + 3×10 min far-red light	8	42	Singh & Amritphale 1992
Caesulia axillaris Roxb.	Soaking in 5 mM NH_4Cl for 5 days + 3×10 min far-red light	8	43	Singh & Amritphale 1992
Capparis spinosa L	20 min H_2SO_4, germinating in 0.2 % KNO_3	0	57	Sozzi & Chiesa 1995
Cassia sieberiana DC	95 % ethanol for 9 h	0-5	40	Todd-Bockarie *et al.* 1993
Cinnamomum camphora L. Sieb	15 % H_2O_2 for 25 min, germinated at 22° C+12 h light / d	11	58	Chien & Lin 1994
Celmisia coriacea	1 % H_2O_2, at 20° C in darkness	2	80	Scott 1975
Clitoria ternatea	Hoagland solution, at 28° C	48	80	Cruz *et al.* 1995
Gloriosa superba L.	Soaking 24 h+4000 ppm Thiourea 36 h	12	68	Suparna *et al.* 1993
Hordeum vulgare L.	25 mM glutathione for 24 h	60	96	Fontaine *et al.* 1995
Hordeum vulgare L.	2×10^{-2} M H_2O_2 for 24 h	8	99	Fontaine *et al.* 1995
Oryza sativa	53 mM acetic acid at pH 4.8 for 24 h	9	92	Cohn *et al.* 1987
Oryza sativa	20 mM propionic acid pH 4.8 for 24 h	9	98	Cohn *et al.* 1987
Oryza sativa	0.5 mM NaN_3 24 h, pH 4	6	86	Cohn & Hughes 1986
Oryza sativa	0.5 mM KCN 48 h, pH 8	6	94	Cohn & Hughes 1986
Oryza sativa	10 mM NH_2OH-HCl 72 h, pH 7	6	95	Cohn & Hughes 1986
Oryza sativa	10 mM $NaNO_2$ 4 h, pH 3	6	99	Cohn & Hughes 1986
Panicum virgatum L. "Alamo"	1.5 M Chloroethanol for 15 min	52	88	Tischler *et al.* 1994
Paulownia tomentosa	Soaking in 10^{-3} M $Na_2[Fe(CN)_5NO]$ for 3 days in darkness, then 5 min red light	2	75	Giba *et al.* 1994
Paulownia tomentosa	Soaking in 5×10^{-3} M KNO_3 for 3 days in darkness, then 5 min red light	2	62	Giba *et al.* 1994
Paulownia tomentosa	Soaking in 10^{-2} M $K_3[Fe(CN)_6]$ for 3 days in darkness, then 5 min red light	2	61	Giba *et al.* 1994
Paulownia tomentosa	Soaking in 5×10^{-3} M $K_2Ir\ Cl_6$ for 3 days in darkness, then 5 min red light	2	83	Giba *et al.* 1994
Pyrus serotina Rehd cv. Niauli	200 μm thidiazuron at 25° C	0	41	Lin *et al.* 1994
Spartina alterniflora Loisel.	10 mg/l Fusicoccin	0	88	Plyler & Carrick 1993

Table 2.7 Some successful examples for breaking seed dormancy by mechanical treatments

Species	treatments	germination (%) Before	After	Reference
	Acid scarification			
Adansonia digitata L.	95 % H_2SO_4 for 6-12 h	15	90	Danthu *et al.* 1995
Adansonia digitata L.	HNO_3 for 10 min	0	86	Esenowo 1991
Astragalus granatensis	H_2SO_4 for 20 min and 2 h	-	90	Angosto Trillo & Matilla Carro 1993
Calopogonium mucunoides	H_2SO_4 for 10 min, germinated at 28	20	75	Cruz *et al.* 1995
Canavalia brasiliensis	H_2SO_4 for 75 min, germinated at 28	20	80	Cruz *et al.* 1995
Cassia sieberiana	98% H_2SO_4 for 45 min	0-5	93	Todd-Bockarie *et al.* 1993
Leucaena leucocephala	H_2SO_4 for 20-30 min, germinated at 28	3	100	Cruz *et al.* 1995
Lotus corniculatus var. japonicus	H_2SO_4 for 10-15 min	4	89	Kondo 1993
Panicum virgatum L. "Alamo"	H_2SO_4 for 10 min	52	92	Tischler *et al.* 1994
Vernonia galamensis Less.	H_2SO_4 for 5 min	35	84	Teketay 1993
	Other mechanical treatments			
Adansonia digitata L.	Removing seed coat approx. 5-10 mm^2	15	68	Danthu *et al.* 1995
Astragalus mongholicus Bunge	Rapid freezing (-20°C) wet seeds for 24 h+ thawing in 40° C water bath for 5 min	20	75	Shibata *et al.* 1995
Canavalia brasiliensis	Hot water (80°C) for 30 min, germinated at 28°C	20	50	Cruz *et al.* 1995
Cassia sieberiana DC.	Scarifying the seed coat with a commercial mill for 4 min	0-5	95	Todd-Bockarie *et al.* 1993
Cassia sieberiana DC.	Nicking the seed coat	0-5	96	Todd-Bockarie *et al.* 1993
Cassia sieberiana DC.	Rotating the seeds in a coffer grinding for 2 sec	0-5	66	Todd-Bockarie *et al.* 1993
Cistus ladanifer L.	Dry heat 100° C for 30 min	23	90	Corral *et al.* 1990
Cistus salvifolius L.	Dry heat 100° C for 30 min	4	38	Corral *et al.* 1990
Crotalaria sericea Retz.	Grinding with quartz sand in a mortar for 5 min	0	100	Saha & Takahashi 1981
Crotalaria sericea Retz.	Decoated	0	80	Saha & Takahashi 1981
Crotalaria sericea Retz.	Hot water (65 °C) for 10 min	0	70	Saha & Takahashi 1981
Eremophila maculata	Excised seeds from the fruit	28	98	Richmond & Ghisalberti 1994
Geleznowia verrucosa Turcz.	Nicking seeds at or near the radicle, at 23/15° C	<2	55-60	Paytner & Dixon 1991
Leucaena leucocephala	Chipping testa approx. 1 mm^2, at 20° C	24	88	Gosling *et al.* 1995
Lomandra drummondii	Leaching seed for 24 h + pericarp	40	80	Plummer *et al.*1995
Rhodanthe manglesii Lindley	Piercing the pericarp and testa to expose a portion of the embryo	38.5	63.9	Bunker 1994
Spartina alterniflora Loisel.	A portion of the scutellum removed	0	98	Plyler & Carrick 1993
Terminalia tomentosa	Removing seed coat + soaking 24 h	0	97	Negi & Todaria 1995

Mechanical scarification is used widely in laboratories (Table 2.7) for reduction of seed impermeability and mechanical restraint. These include removing seed coat, nicking seeds, pricking seed coat with a shape needle, scarifying seed coats with sandpaper, or with a commercial mill or a coffee grinder.

The best-known effects in nature are high temperatures and temperature fluctuations (reviewed in Bewley and Black 1982). Various types of heat treatment have been used to overcome hard-seededness with varying success. Softening of the coat by temperature fluctuations occurs in *Trifolium subterraneum*, *Stylosanthes humilies* and *Lupinus varius* but only when the seed coats are relatively dry (Quinlivan 1968). In *Lupinus* these fluctuations cause the strophiole of the coat to crack and water is allowed to enter. High temperatures can also act on this region of the hard testa. It has long been known that immersion in boiling water for a few minutes leads to a permanent increase in the subsequent permeability of the coat to water (Rolston 1978). Other areas of the hard coat may be affected by heating. Hot water disrupts the chalazal plug of cotton, and dry heat cracks the micropylar region of *Rhusovata* and the seed coat of various legumes (Rolston 1978). These effects of high temperature are thought to account for the stimulation of germination by fire. Several leguminous species in forests break dormancy after being subjected to fire (Purdie 1977).

2.2.4.7 Hormones

The growth regulators (hormones), gibberellin (usually gibberellic acid GA_3, GA_4, and GA_7), cytokinin (usually kinetin, benzyladenine), and ethylene, variously affect seed dormancy (Bewley and Black 1994). Some growth regulators that break seed dormancy are shown in Table 2.8.

The growth regulators with the widest spectrum of activity are the gibberellins (GA). In the international Rules for Seed Testing, GA_3 is recommended for promoting germination of dormant cereals (ISTA 1985). The general role of GA in overcoming dormancy in cereals is its action in mobilization of endosperm reserves (Simpson 1990). GA promotes the synthesis of amylase and other hydrolytic enzymes in the scutellum and aleurone tissue, and these enzymes are, in turn, responsible for breakdown of starch. In

Table 2.8 Some successful examples for breaking seed dormancy by plant hormones

Species	treatments	germination (%)		Reference
		Before	After	
Adansonia digitata	100 mg Etherl/l, germinating at 25° C	0	70	Esenowo 1991
Avena fatua L.	after-ripening at 25°C, for 12 months +Ethylene 10^3 µl/l at 15° C	49	78	Adkins & Rose 1981
Avena fatua L.	after-ripening at 25°C, for 12 months +Ethrel 10^3 µl/l at 15 °C	42	97	Adkins & Rose 1981
Caesulia axillaris Roxb.	Soaking in 10^{-4} M C_2H_4 for 120 h, then 10 min red light	0	68	Amritphale 1993
Capparis spinosa L.	20 min H_2SO_4, + 100 ppm GA_{4+7} for 90 min	0	68	Sozzi & Chiesa 1995
Cyclopia intermedia	5 µl/l C_2H_4 for 24 h	53	100	Whitehead & Sutcliffe 1995
Cyclopia intermedia	100 µm GA_3	53	96	Whitehead & Sutcliffe 1995
Cyclopia intermedia	100 µm BA	53	90	Whitehead & Sutcliffe 1995
Fagopyrum esculentum Moench.	Germinating in 500 mg /l ethephon at 20°C 16h /30°C 8h with light	41	97	Samimy 1994
Helipterum albicans DC.	500 ppm GA_3	52	92	Willis & Groves 1991
Hygrophila auriculata	10^{-3} M GA_3 in darkness at 28°C	22	55	Amritphale *et al.* 1995
Hygrophila auriculata	10^{-5} M C_2H_4 in darkness at 28°C	24	73	Amritphale *et al.* 1995
Gloriosa superba L.	Soaking 24 h + 36 h 300 ppm GA_3	12	59	Suparna *et al.* 1993
Gloriosa superba L.	Soaking 24 h + 36 h 200 ppm BA	12	34.5	Suparna *et al.* 1993
Gloriosa superba L.	Soaking 24 h+36 h 100 ppm Ethrel	12	54	Suparna *et al.* 1993
Lactuca serriola L.	100 mg/l GA_3	0	87	Small & Gutterman 1992
Leucochrysum fitgibbonnii	500 mg/l GA_3	18.4	69.6	Bunker 1994
Leucochrysum stipitatum (F. Muell.)	500 mg/l GA_3	24.2	65.7	Bunker 1994
Meconopsis paniculata	100 mg/l GA_3 at 20° C with 10 h light /d	0	>85	Sulaiman 1993
Meconopsis simplicifolia	100 mg/l GA_3 at 20° C with 10 h light /d	0	>60	Sulaiman 1993
Muntingia calabura L.	210 ppm GA_3	2	48	Laura *et al.* 1994
Petunia hybrida	150 µg/ml GA_3	-	71.3	Gonzalez & Villalobos 1988
Rhodanthe moschata Wilson	500 mg/l GA_3	16	75.6	Bunker 1994
Rhodanthe stricta Wilson	500 mg/l GA_3	0.5	31.5	Bunker 1994
Stylidium graminifolium Swartz ex Willd	500 ppm GA_3	<10	>30	Willis & Groves 1991
Vernonia galamensis Less.	100 µm CA_3	35	99	Teketay 1993

some experiments, GA has been successfully applied as a last remedy to overcome dormancy, after treatments like alternating temperature, light or KNO_3 have failed (Williams 1983a, b, Schonfeld and Chancellor 1983). The concentration of GA required to elicit maximal germination depends on how deep the dormancy is (Aamlid 1992). A solution containing 500 ppm GA_3 is normally used in seed testing, but 1000 ppm may be justified in some cases (ISTA 1985). In some species where gibberellin is poorly effective on the intact seed, an effect can be induced by various treatments of the seed coat such as mild scarification, pricking or scratching (Hsiao 1979).

Cytokinins are less widely effective, and even when they do act, they often induce abnormal germination; e.g. in lettuce, the cotyledons tend to emerge from the seed before the radicle (Bewley and Black 1994). Cytokinins have been reported to overcome secondary dormancy in wild oats (Tilsner and Upadhyaya 1985), primary dormancy in *Pyrus malus* (reviewed in Bewley and Black 1994).

Ethylene is also limitedly effective, and many species remain unaffected (Bewley and Black 1994). But some species such as canarygrass (Landgraff and Junttila 1979) and *Cyclopia intermedia* (Whitehead and Sutcliffe 1995) are observed to have enhanced germination of seeds after treatment with ethylene.

Jasmonic acid and its methyl ester have been found to have the stimulatory effects on germination of apple embryos (Ranjan and Lewak 1992). When dormant apple embryos are cultured for 10 days in 20 μm jasmonic acid and methyl-jasmonate solution with light, the germination percentage of embryos increases from 39 to 56 or from 39 to 69, respectively (Ranjan *et al.* 1994).

2.2.5 Embryo Rescue by Embryo or Ovule culture

Tissue culture is a term used to indicate the aseptic culture *in vitro* of a wide range of excised plant parts (Hartmann *et al.* 1990). Embryo culture is the excision of embryos from individual ovules and their eventual germination in aseptic culture (Collins and Grosser 1984, Hu and Wang 1986, Pierik 1987, Yeung *et al.* 1981). This technique is used to rescue embryos that would have aborted within the seed before maturity (Hartmann *et al.* 1990). This is a common consequence of many interspecific hybrid

crosses. The other major use of embryo culture is to induce prompt germination of mature dormant seeds (Henny 1980, Dunwell 1986, Arrillage *et al.* 1992) and as a tool to investigate possible mechanisms of dormancy (Pinfield *et al* 1990). Examples where embryo rescue removes dormancy include *Maranta leuconeura* (Henny 1980), *Acer platanoides* and *A. pseudoplatanus* (Pinfield *et al* 1990), *Fraxinus ornus* and *Sorbus domestica* (Arrillage *et al.* 1992) and *Triticum aestivum* (Morris and DeMacon 1994).

Cultured ovules are useful to rescue embryos that abort at a very young stage if not separated from the plant (Dunwell 1986, Goldy and Amborn 1987, Ramming 1985, Spiegel-Roy *et al.* 1985). Embryos at very early stages of development are only able to be excised with great difficulty, and their nutritive requirements are more complex than those of mature embryos. Ovules on the other hand, can easily be sterilized and generally will grow in a basal medium of inorganic salts, sucrose, and sometimes hormones (Hartmann *et al.* 1990). This technique was developed first in barley (reviewed in Dunwell 1986) and has now been extended to a number of other species (Table 2.9).

Table 2.9 Species successful in ovule culture

Species	Reference
Beta vulgaris	Cited in Dunwell 1986
Gerbera jamesonii	Cited in Dunwell 1986
Gossypium Hirsutum	Stewart 1981
Impatiens	Arisumi 1980
Prunus salicina L.	Ramming 1985
Tulipa L.	Custers *et al.* 1995
Vitis spp.	Goldy & Amborn 1987
Vitis vinifera L.	Aguero *et al.* 1995
Hybrids between *Brassica campestris* and *B. oleracea* L.	Diederichsen & Sacristan 1994
Hybrids between *Festuca* and *Lolium*	Lee & Han 1989

Chapter 3

ANATOMICAL AND MORPHOLOGICAL STUDIES OF SEED DEVELOPMENT IN *Sandersonia aurantiaca* (HOOK.)

3.1 INTRODUCTION

Although *Sandersonia aurantiaca* has recently become an important cut flower crop in New Zealand, a systematic study of seed development has not been reported in the literature. The *Sandersonia* flower is located in the axil of the upper leaves and is borne on a slender pedicel 2-3 cm long (Mathew 1978). Six perianth segments are fused together to form an inflated urn-shaped, pendulous, bright orange flower, 2-2.5 cm long, which has the mouth facing downwards and six prominent spurs on the base pointing upwards (Mathew 1978). The tips of the segments are just free and curl outward. Sterling (1975) studied the morphology of the carpel in *Sandersonia* and indicated that the pistils were generally tricarpellate and had many ovules. The ovules are mostly plagiotropic, anatropous and essentially bitegmic, with inner integuments often nearly fused with nucellar remnants. The pistils in some specimens showed a small obturator (a funicular proliferation in the form of a protuberance which brings the stigmatoid or transmitting tissue into close proximity to the micropyle). Flower buds of *Sandersonia* are visible 3 weeks after planting with numbers increasing until week 6, and flowers open between weeks 7 and 9 (Brundell and Reyngoud 1985). The dry weight of the flowers continues to increase until week 11, then declines. The development and senescence of *Sandersonia* flowers have been described by Eason and Webster (1995). They assigned twelve stages of development and senescence according to flower size, shape, a reduction of fresh weight and of soluble protein, loss of pigment, wilting and necrosis of the tepals. The senescence of *Sandersonia* flowers is insensitive to ethylene and is accompanied by changes in transcription and translation of nucleic acids (Eason and de Vre 1995).

Seed formation follows as a result of pollination and fertilization of the ovule, which is the embryonic seed (Corner 1976). The cells of the ovule are small, thin-walled

and isodiametric. They have large nuclei and a few small vacuoles. After fertilization the cells renew their growth, divide, enlarge, and differentiate in a programmed process resulting in a mature seed composed of an embryo together with storage tissues and covering structures (Corner 1976). In addition to the morphological changes, seed development is accompanied by physiological and biochemical changes, such as loss of water, accumulation of metabolic substances, and even induction of dormancy. While the above is a general description of seed development in angiosperms, the details of morphological, physiological and biochemical changes of *Sandersonia* seeds during developmental periods are unknown and no detailed developmental studies exist on the embryo, endosperm and seed coat. The present investigation was undertaken to provide a detailed account of seed development in *Sandersonia* to fill this gap in our knowledge. The purpose of the study is to investigate the development of the embryo, the endosperm and the seed coat of this species. In addition, any changes of cell and tissue structure in the above structures were also of interest as they may be associated with relationships between seed structure and dormancy.

3.2 MATERIALS AND METHODS

3.2.1 Plant Materials

Sandersonia plants were supplied by Crop & Food Research Ltd. at the Levin Research Centre. The plants were grown in a glasshouse at the Department of Biology and Biotechnology, Massey University. The temperature varied between 26-32°C during the day and 15-20°C at night, and a natural photoperiod was experienced by the plants. The plants were watered 4 times per day for approximately 3 min.

Flowers opened from the bottom to the top on each stem continuously. Anther dehiscence occurred after the flowers had opened for two days. Freshly opened flowers were pollinated by touching each stigma with a small camel hair brush which was full of fresh pollen from the dehiscing anthers in the same plant.

Several unpollinated ovaries were collected on day 0 (the day before pollination), and pollinated ovaries were removed from inflorescences of the plants on day 1, 4, 5, 6,

7, 8, 9, 10, 12, 14, 21, 28, 35, 42, 49, 56 and a final full-maturity stage. The materials were immediately placed in formalin-alcohol-glacial acetic acid solution (FAA) for study of seed development. Some of the ovaries, e.g. the day 0, 1, 8, 14, 21, 28, 35, 42, and 59, were also used for measurement of ovule diameters. Two flower buds were also collected 1 week before flower opening and used for investigation of flower and early seed development in the ovary.

3.2.2 Experiment Methods

3.2.2.1 Measurements

The collected ovaries were dissected under a dissecting microscope and the diameters of ovules were measured with a calibrated eyepiece micrometer under a light microscope at the day 0, 1, 8, 14, 21, 28, 35, 42 and 59 after pollination, respectively.

3.2.2.2 Paraffin Embedding and Section Preparation

The specimens were fixed in a mixture of 4% formalin, glacial acetic acid, and 70% ethyl alcohol (FAA) in proportions 5: 5: 90 at younger stages (before and including day 14 after pollination), or 40% formalin, glacial acetic acid, and 70% ethyl alcohol (FAA) in proportions 10: 5: 85 (Berlyn and Miksche 1976) at older stages for at least 48 hours. The materials were washed in 50% ethyl alcohol before dehydration. Johansen's ethyl alcohol/tertiary butyl alcohol (TBA) method was used for dehydration. The series of solution of water, ethyl and tertiary butyl alcohols were prepared as described by Johansen (1940). After 3 changes of pure TBA over 24-36 hours, TBA/paraffin oil (50:50) was used for linking TBA to pure wax. After 3 repeats of melted wax over 24 hours at 62°C, the specimens were removed from the oven and embedded in small tin-foil embedding 'boats'.

Microtoming was carried out according to the procedures outlined by Johansen (1940). Sections were cut 10-12 μm in thickness on a Reichert ultramicrotome and mounted on microscope slides with 10% white resin glue. Before sections could be stained, paraffin was removed by xylol, xylol was removed by alcohol. The sections

were stained in 1 % safranin solution for 24-48 hours and counterstained in 0.5% fast green for several seconds (Johansen 1940). D.P.X. mountant was used to mount the sections.

3.2.2.3 Examination

Longitudinal and some transverse sections of ovules at different stages of development and transverse sections of the flower buds were viewed under a light microscope. Several developmental stages of ovule, embryo, endosperm and testa were examined and photographed with a Zeiss compound microscope. The images were recorded using Kodak EKTAR 25 color negative film. Magnification shown in the plates is the light microscope magnification only.

3.3 OBSERVATIONS

3.3.1 Flower, Ovary and Ovule

The *Sandersonia* flowers are bisexual with a fused perianth which consists of about 7-8 layers of parenchymatous cells arranged in a uniform manner (Plate 3.1). There are six stamens in two whorls. The ovary is superior, tricarpellate, syncarpous, and trilocular with axile placentation. There are three segregated styles with stigmas on the top of the ovule. The ovary has distinctly marked sutural margins (Plate 3.2) and open sutures which extend inward in the ovary along the septal boundaries of the carpels. A central hole can be seen in a few of sections. Each carpel contains numerous ovules, and each ovule is attached to the placentation by a funiculus (Plate 3.2).

The ovules are anatropous, crassinucellate and bitegmic (Plate 3.3). The micropyle is formed by both outer and inner integuments (Plate 3.3). The inner integument is composed of two layers of cells except in the micropyle region, where more layers are observed. The outer integument is 4-5 cell layered at the apex and more layered at base (Plate 3.3). Later the outer integument becomes a hood-shaped envelope. An obturator is formed on the concave side of the funiculus and intervenes in a gap of the outer integument (Plate 3.3). The structure known as the nucellar cap is located at the micropylar region and persists till the globular embryo stage (about 14

DAP, Plates 3.3, 3.7, 3.10, 3.11, 3.12, 3.13 and 3.22 A, B, D). A well developed bowl-like hypostase, which is a group of nucellar cells that lose their cytoplasm, nuclei or contain deformed nuclei and become thick-walled, surrounded by one to two layers of transfer cells, is conspicuous in the chalazal region and does not disappear until later stages (about 42 DAP, Plates 3.2, 3.3, 3.13, 3.14, 3.15 and 3.25). A vascular bundle can be found at 21-28 DAP and passes through the strophiole inside the outer integument, along the ovule towards the chalazal region and terminates at the base of the hypostase (Plates 3.4 and 3.17).

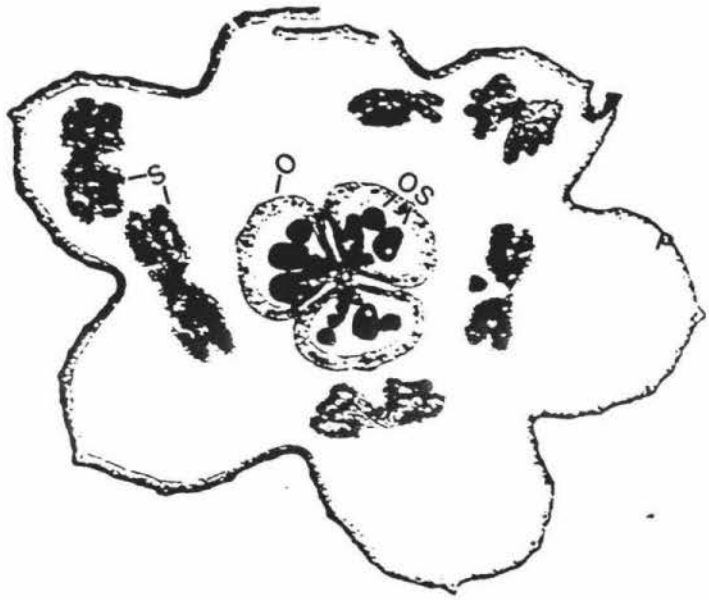


Plate 3.1 Transverse section of flower bud at 1 week before flower opening (×14). p, perianth; os, ovules; o, ovary; s, stamens.

3.3.2 Ovule Growth

Ovule extension over the period between pollination and seed maturity is plotted in Fig. 3.1. Mean ovule diameter plotted with time forms a sigmoidal curve. The ovules grow from 0.39 mm at pollination to an average 2.34 mm in diameter at 56 DAP. The lag phase of the curve is present before the first division of zygote (Fig.3.1, Table 3.1). The exponential growth phase begins at about 8 DAP when both embryo and endosperm development start. The rate of extension of the ovule decelerates when the embryo develops into the late globular stage and the embryo sac is full of endosperm cells, at about 21 DAP (see Plate 3.17).

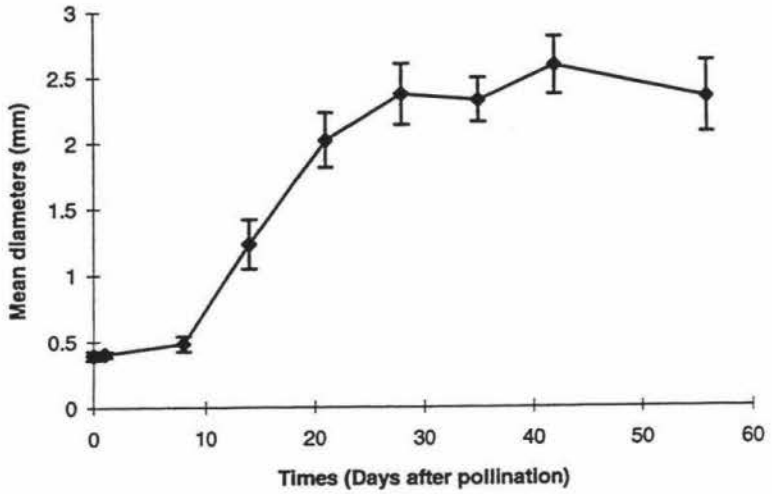


Figure 3.1 Changes of mean ovule diameter in seed development of *Sandersonia*. The values are the mean diameter of 60 ovules ± mean standard deviation.

3.3.3 Embryo Sac

The type of embryo sac development appears to conform to the Polygonum type. The chalazal megaspore of a linear tetrad of megaspores develops into a megasporocyte (Plate 3.5). Before pollination (day 0), only a bi-nucleate embryo sac could be found (Plate 3.6). After pollination, the egg cell, synergids, polar nuclei and antipodal cells can be observed, but they are not always arranged in the same plane in the embryo sac and were not observed together.

A clear distinction in morphology was not observed between the egg cell and the synergids before fertilization. The synergids seems to be triangular in shape and have denser cytoplasm (dark stained) without vacuoles (Plate 3.7). Both synergids disappear before the first division of the zygote. The egg cell is nearly the same size as the synergids and has less denser cytoplasm and one to several vacuoles (Plates 3.7 and 3.8). The nucleus is located at the micropylar pole before fertilization (Plates 3.7 and 3.8), and then transferred to the chalazal pole (Plate 3.9). After pollination, the two polar nuclei are in close contact (Plate 3.10). About 7 days after pollination (DAP), they fuse to form the secondary nucleus (Plate 3.11). The antipodal cells are located at the chalazal end (Plate 3.12). After fertilization, they enlarge and often degenerate before the initiation of cellularization of endosperm (Plate 3.13).

Plate 3.2 Transverse section of ovary at 1 DAP (×40). os, ovules; es, embryo sac; h, hypostase; f, funiculus.

Plate 3.3 Longitudinal section of anatropous ovule at 1 DAP with micropyle (in outer integument region), obturator and hypostase (×160). oi, outer integument; ii, inner integument; ob, obturator; h, hypostase; nu, nucellar cap; es, embryo sac; m, micropyle.

Plate 3.4 Part of longitudinal section of developing seed at 28 DAP with a vascular bundle (×40). s, strophiole; vb, vascular bundle; en, endosperm; ts, transfer cells.

Plate 3.5 Functional and degenerated megaspores (collected at 1 week before flower opening, ×400). fm, functional megaspore; dm, degenerated megaspores.

Plates 3.2-3.5

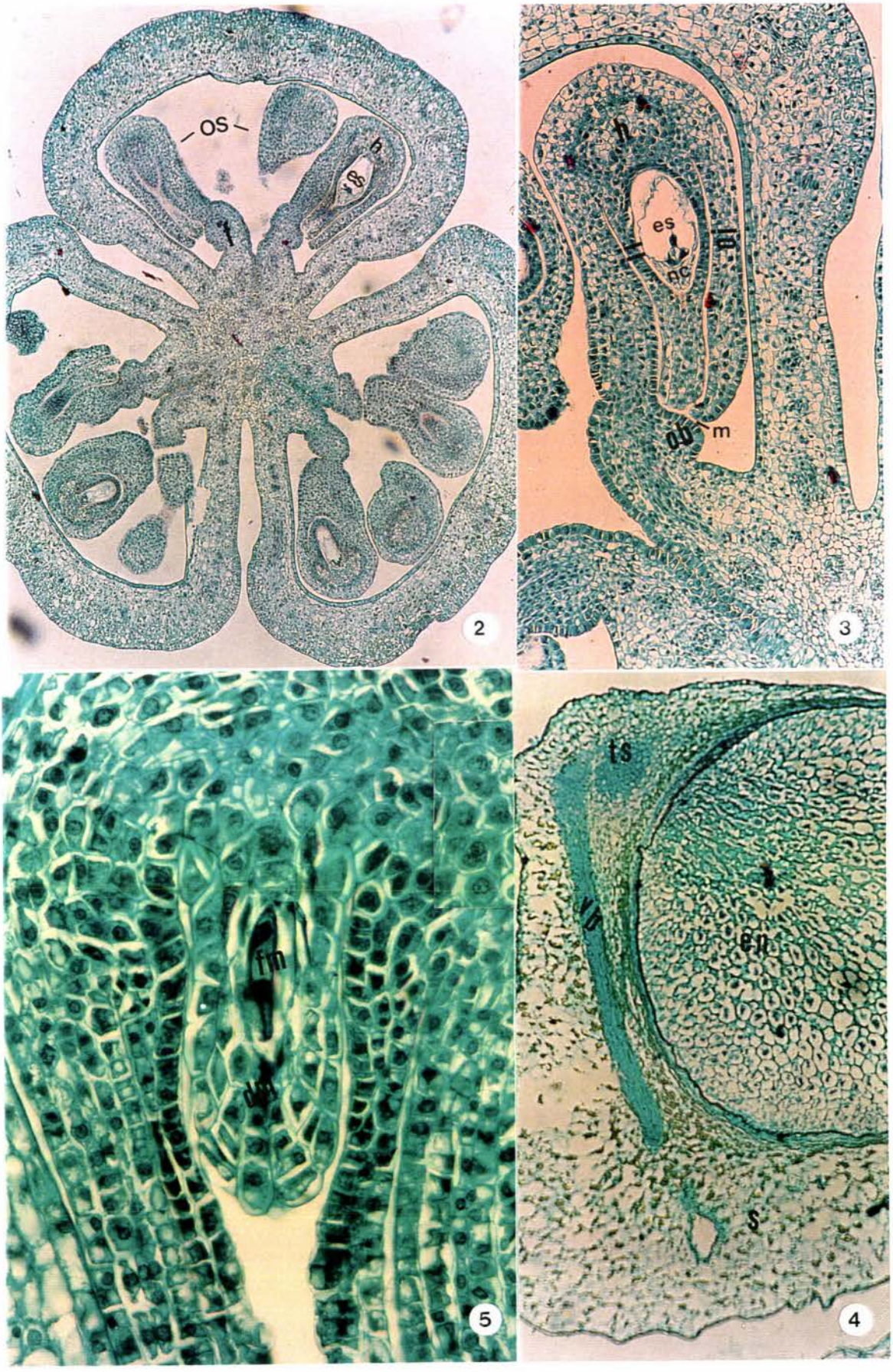


Plate 3.6 Bi-nucleate embryo sac at 0 DAP (×640).

Plate 3.7 Embryo sac at 1 DAP (×400). s, synergid; e, egg cell; c, central cell; nc, nucellar cap.

Plate 3.8 Egg cell at 1 DAP with a nucleus at micropylar pole (×400).

Plate 3.9 Zygote at 8 DAP with a nucleus at chalazal pole (×400).

Plates 3.6-3.9

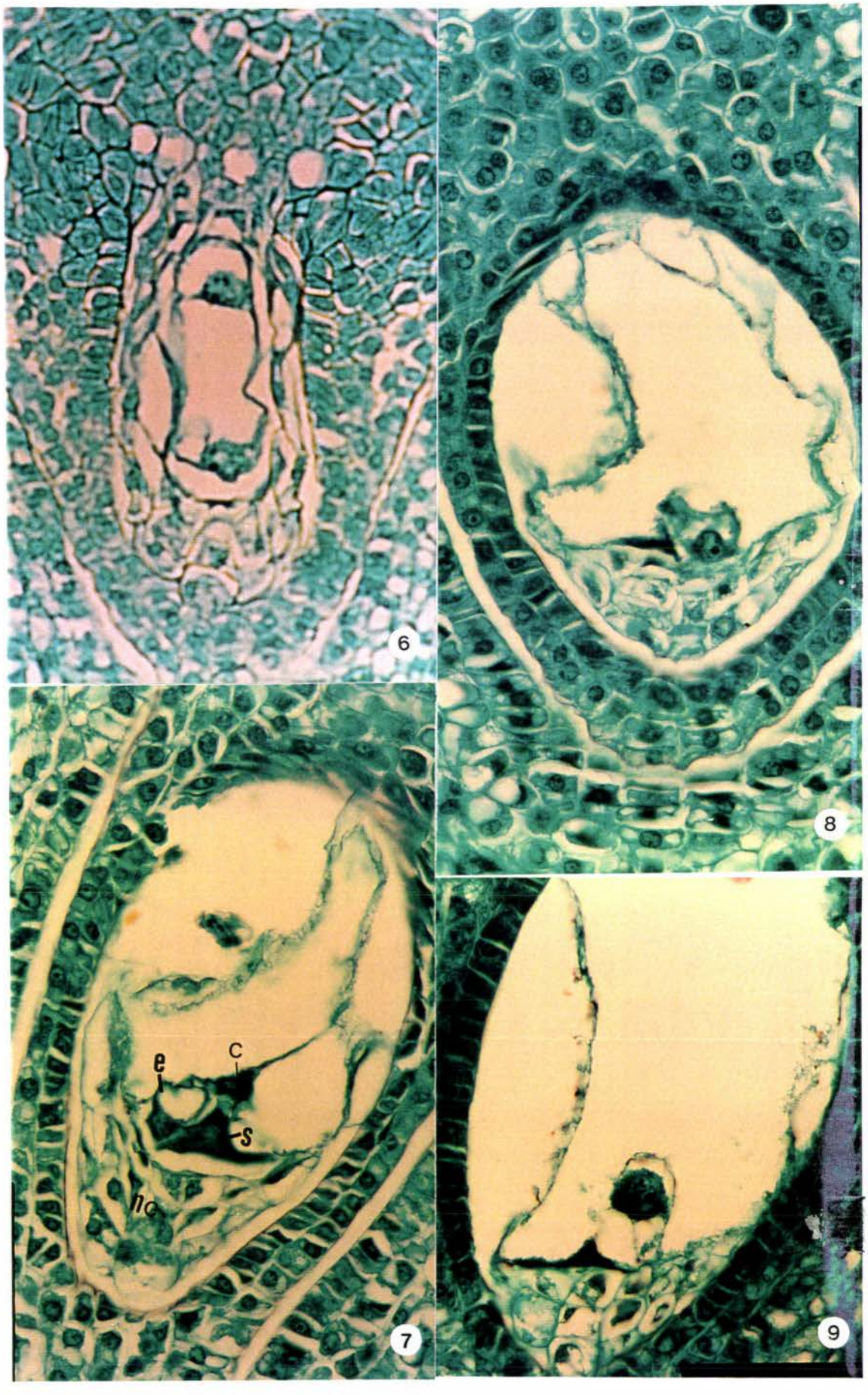


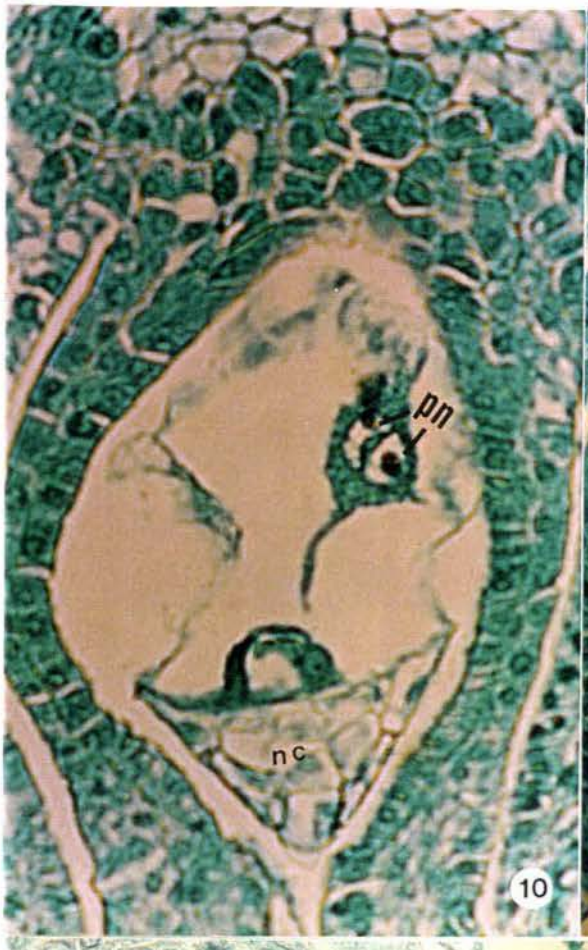
Plate 3.10 Two polar nuclei adjacent to each other at 4 DAP (×640). pn, polar nuclei; nc, nucellar cap.

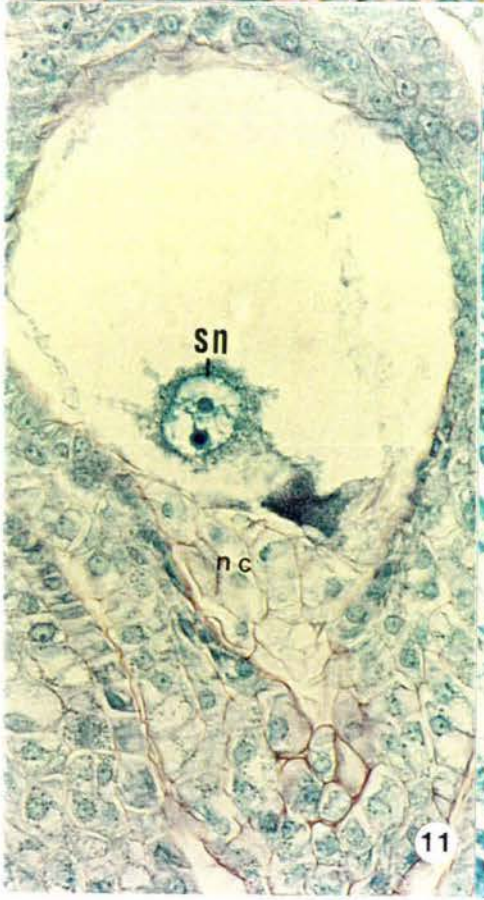
Plate 3.11 Two polar nuclei fusing to form the secondary nucleus at 7 DAP (×400). sn, secondary nucleus; nc, nucellar cap.

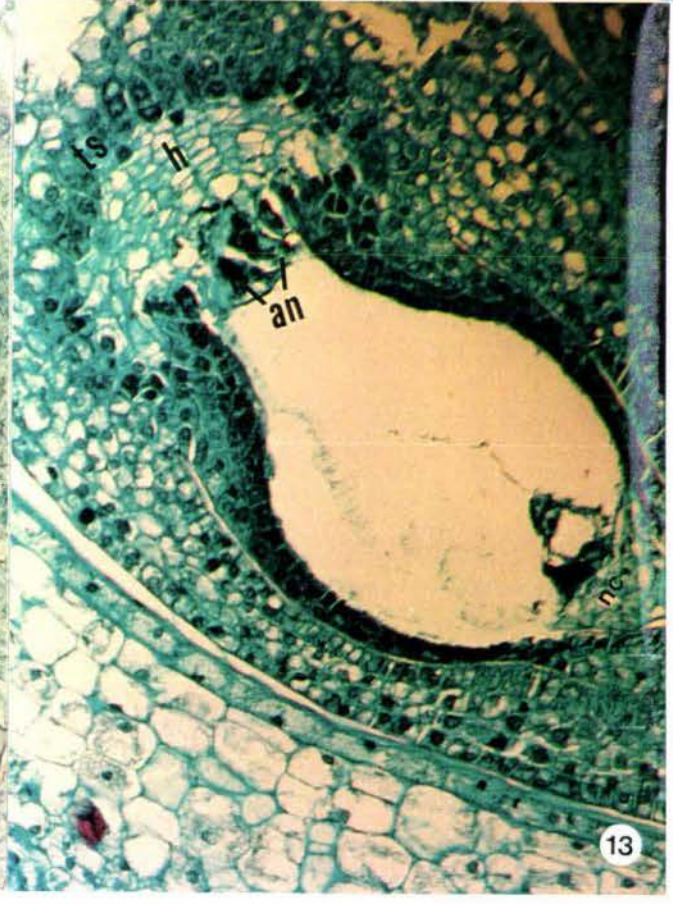
Plate 3.12 The antipodal cells at 1 DAP (×400). an, antipodals; nc, nucellar cap.

Plate 3.13 Enlarged antipodals at 10 DAP (×200). an, antipodals; h, hypostase; ts, transfer cells; nc, nucellar cap.

Plates 3.10-3.13







3.3.4 Development of Endosperm

The development of the endosperm is of the Nuclear type. The primary endosperm nucleus undergoes divisions soon after its formation. After several divisions, most of the coenocytic nuclear endosperm and cytoplasm is situated in the chalazal end of embryo sac close the hypostase. Small numbers of free nuclei of endosperm are also distributed in a thin layer of peripheral cytoplasm. The remainder of the embryo sac is occupied by a large central vacuole (Plate 3.14).

Endosperm cell wall formation begins at about 14 DAP during the early stage of embryogenesis. Wall formation is initiated at the chalazal end and the edges of embryo sac (Plate 3.15), and grows inward and toward the micropylar end (Plates 3.15, 3.18 and 3.19). The entire embryo sac becomes fully cellular and the central vacuole disappears at about 21 days after pollination when the embryo develops into the late globular stage (Plate 3.17). At first, endosperm cells form at the chalazal end and in a layer along the periphery of the embryo sac (Plates 3.15 and 3.18). The first anticlinal walls may be associated with the embryo sac wall and project into the central vacuole (Plate 3.15). As the process of wall formation proceeds, a band of cellular endosperm in several cell thickness forms around the edge of the embryo sac and the central vacuole decreases in size (Plates 3.18 and 3.19). As more endosperm cells are formed, the number of endosperm nuclei increases by karyokinesis and cell plates form between the daughter nuclei (Plate 3.16). Finally, the whole area of the embryo sac from chalazal to micropylar region becomes filled with cellular endosperm (Plate 3.17).

In a mature seed, endosperm cells are well developed and possess thickened walls (Plate 3.20). The endosperm occupies much of the seed volume (Plate 3.21). At the later embryo stage, many small bodies are present in the cytoplasm of the endosperm cells (Plate 3.20) but the iodine test for starch did not reveal the presence of starch granules. These small bodies may contain aleurone and fatty oils (Dahlgren *et al.* 1985) and are also found in the cells of ovary wall (plate not shown), outer integument (Plate 3.22 E) and suspensor (Plate 3.22 D, F) .

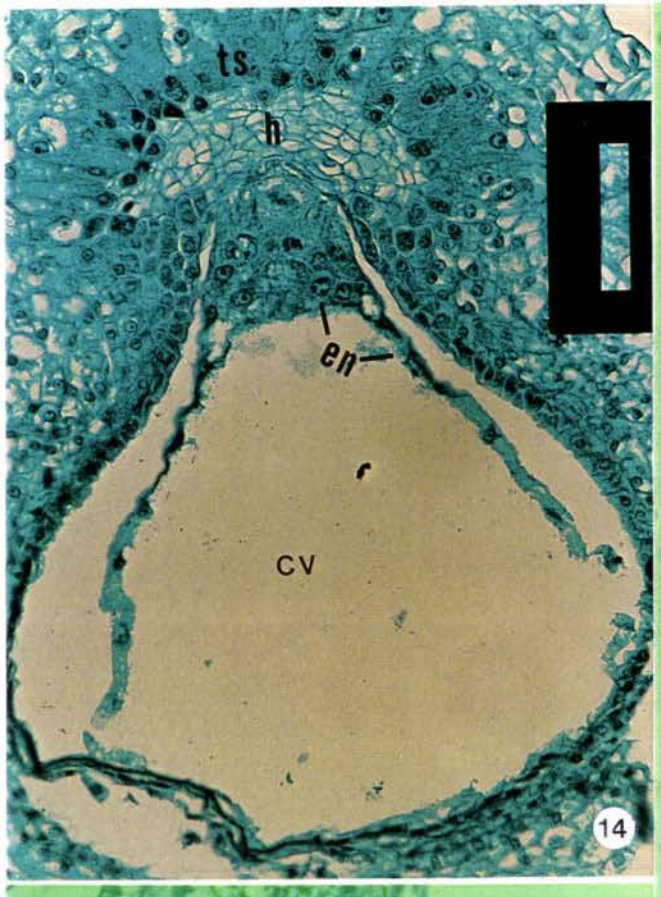
Plate 3.14 Longitudinal section of embryo sac showing most of the coenocytic nuclear endosperm and cytoplasm situating in the chalazal end and peripheral zone of embryo sac at 14 DAP (×200). h, hypostase; cv, central vacuole; en, coenocytic nuclear endosperm; ts, transfer cells.

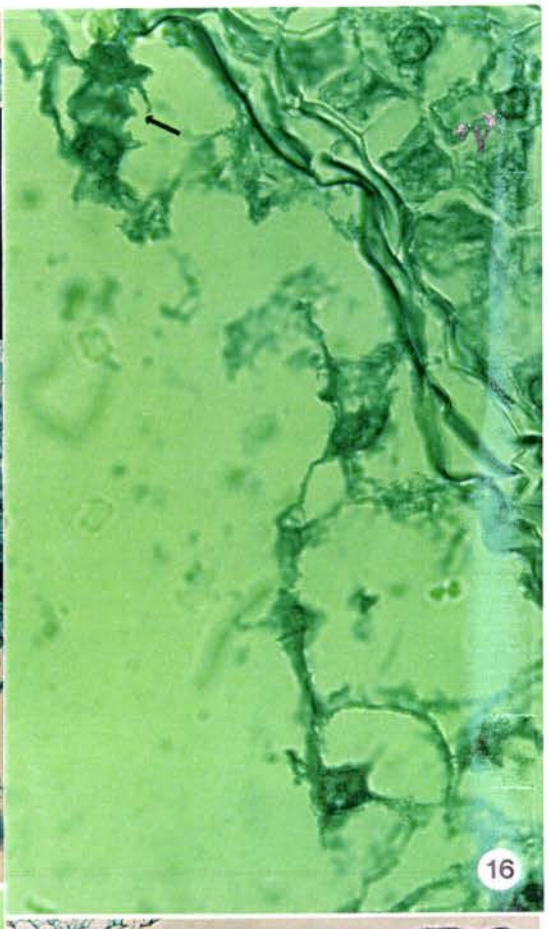
Plate 3.15 14 DAP showing initiation of freely growing walls in between the endosperm nuclei at the charazal end and the edges of embryo sac; note crooked and irregularly growing walls associated with embryo sac wall (arrows) (×160). h, hypostase; ts, transfer cells.

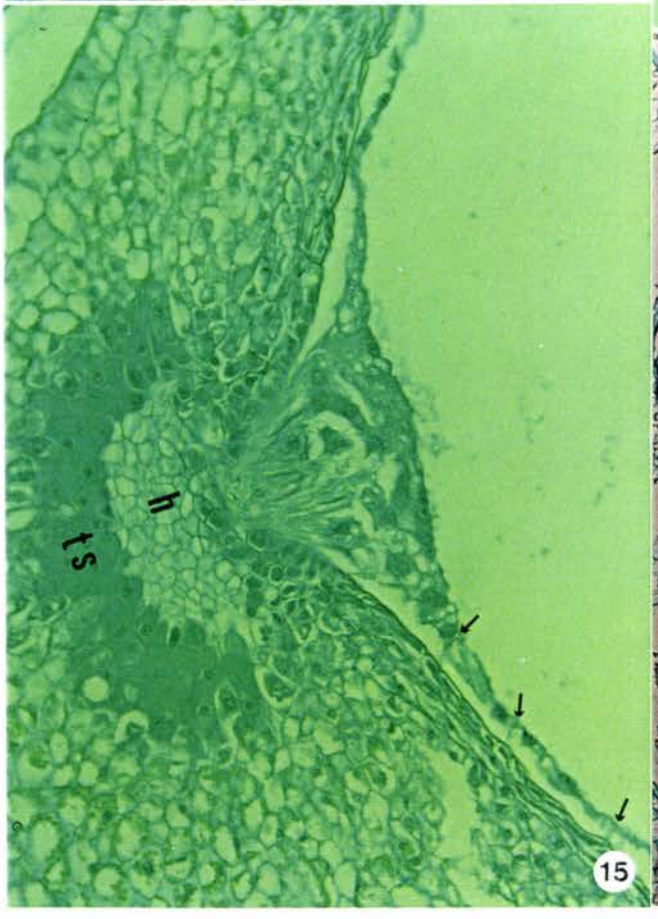
Plate 3.16 Part of cellular endosperm (14 DAP) showing the division of a cell through cell plate (arrow) formation following karyokinesis (×640).

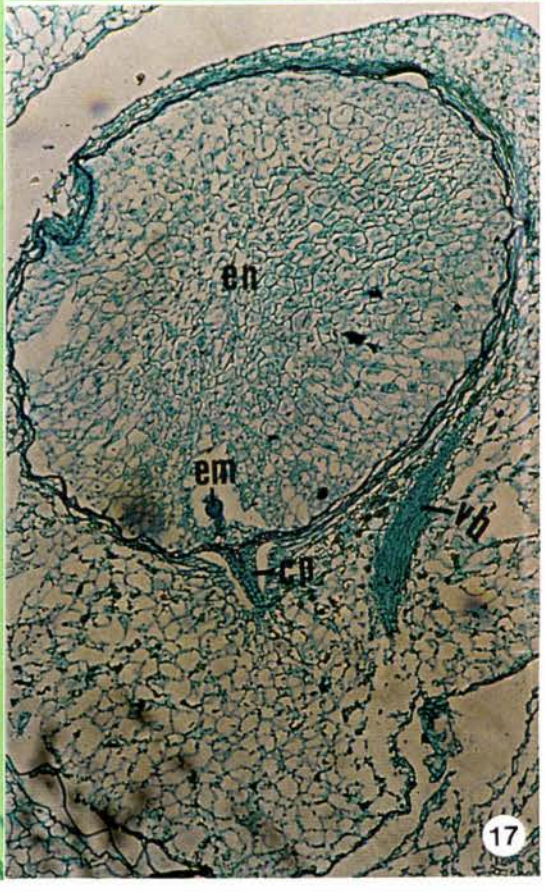
Plate 3.17 Completely cellularized endosperm (21 DAP, ×40). em, embryo; en, endosperm; vb, vascular bundle; cp, cylindrical protuberance.

Plates 3.14-3.17







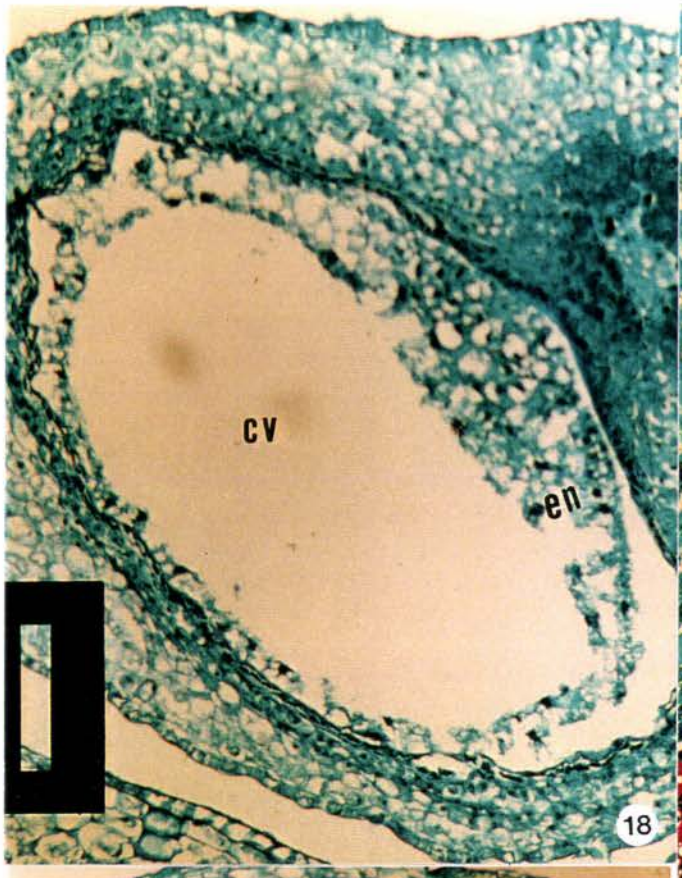


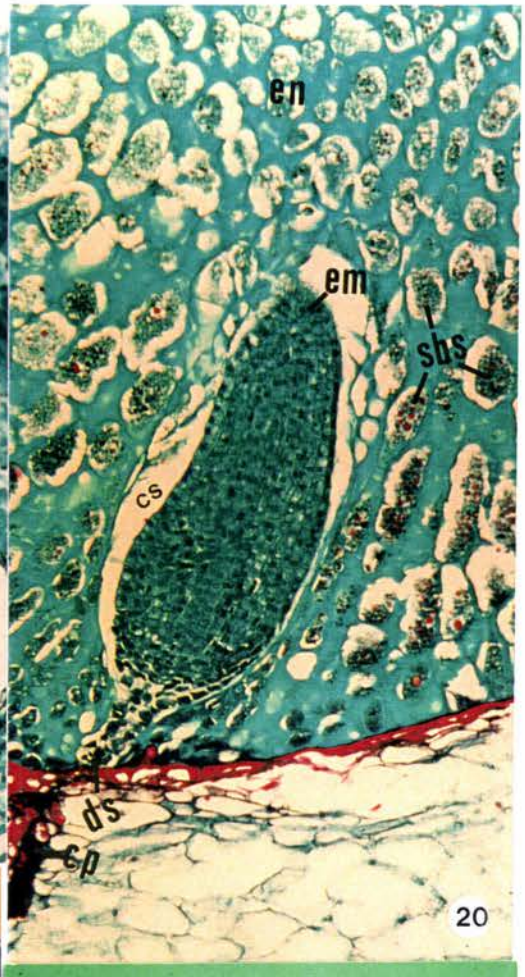
Plates 3.18 and 3.19 17 DAP showing one to several layers of cellularized endosperm cells and a reduced central vacuole (×100). cv, central vacuole; en, cellularized endosperm; em, embryo; cp, cylindrical protuberance.

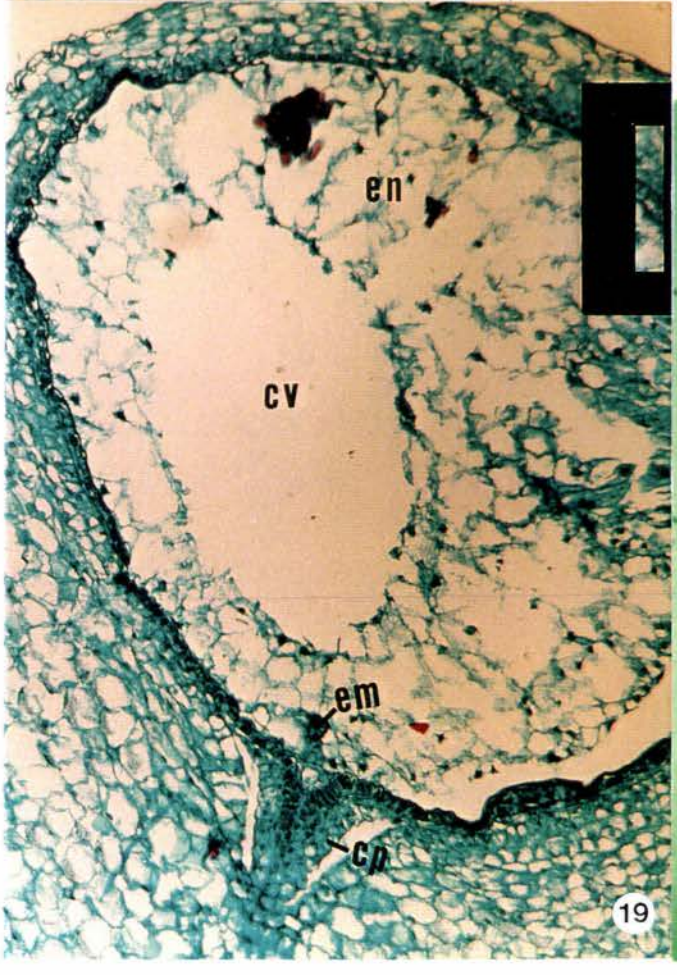
Plate 3.20 Linear embryo with a degenerating suspensor surrounded by a clear space and endosperm cells (49 DAP, ×100). em, embryo proper; ds, degenerating suspensor; en, endosperm; cs, clear space; sbs, small bodies.

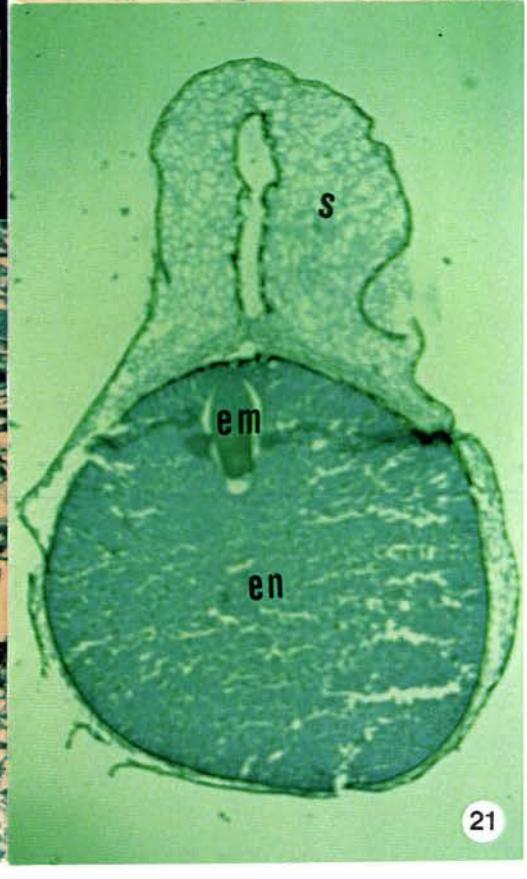
Plate 3.21 Median longitudinal section of a mature seed (56 DAP, ×25). em, embryo; en, endosperm; s, strophiole.

Plates 3.18-3.21









3.3.5 Development of Embryo

About seven to eight days after pollination, the zygote is at metaphase (Plate 3.9). The zygote begins division at about 9 days after pollination. The first division of the zygote is transverse resulting in a smaller apical and a larger basal cell (Plate 3.22 A). This is followed by another transverse division of the basal cell, so that the three-celled embryo consists of an apical cell and two basal cells (Plate 3.22 B). The proembryo at 14 days after pollination has a 4 to 8-celled embryo proper and a 8 to 9-celled suspensor (Plate 3.22 C, D). About 18-21 DAP, the embryo develops into the late globular stage (Plate 3.22 E). The cotyledonary primordia appear at about 28 DAP, giving the embryo its distinctive elongated spheroidal shape (Plate 3.22 F). The growing point of the shoot forms at the lateral side of embryo at about 35-42 days after pollination (Plate 3.22 G, H). The embryo in a mature seed (56 DAP) shows a prominent radicle, a small embryonic axis, shoot apex and a large cotyledon (Plate 3.22 I).

The suspensor, which is produced by a series of divisions of the basal cell, at the base of the radicle end of embryo near the micropyle is observed from 14 days after pollination and remains to 49 DAP. It comprises one or two tiers of cells and a large basal cell (Plates 3.20, 3.22 D, E, F).

3.3.6 Development of Seed Coat

The ovule in *Sandersonia* is bitegmic. At anthesis (day 0) the outer integument consists of 4-5 layers of parenchyma, and the inner one contains 2 layers (Plate 3.3). The seed coat of a mature seed differentiates into three layers: the epidermis, subepidermal parenchyma (middle layer), and the lignified layer (Plate 3.24). The epidermis is uniseriate and most of the cells are square or rectangular in shape (Plate 3.24). Inside the epidermis, several layers of large parenchyma cells are differentiated in the middle cell layer (Plate 3.24). Both epidermis and subepidermis come from the outer integument. The lignified layer may be derived from both outer and inner integuments and shows a high degree of lignification.

The differentiation of the seed coat is initiated at about 12 days after pollination (Plate 3.23 A). At this time, the seed is about 1.0 mm in diameter (Fig. 3.1); the outer

Plate 3.22 A-E

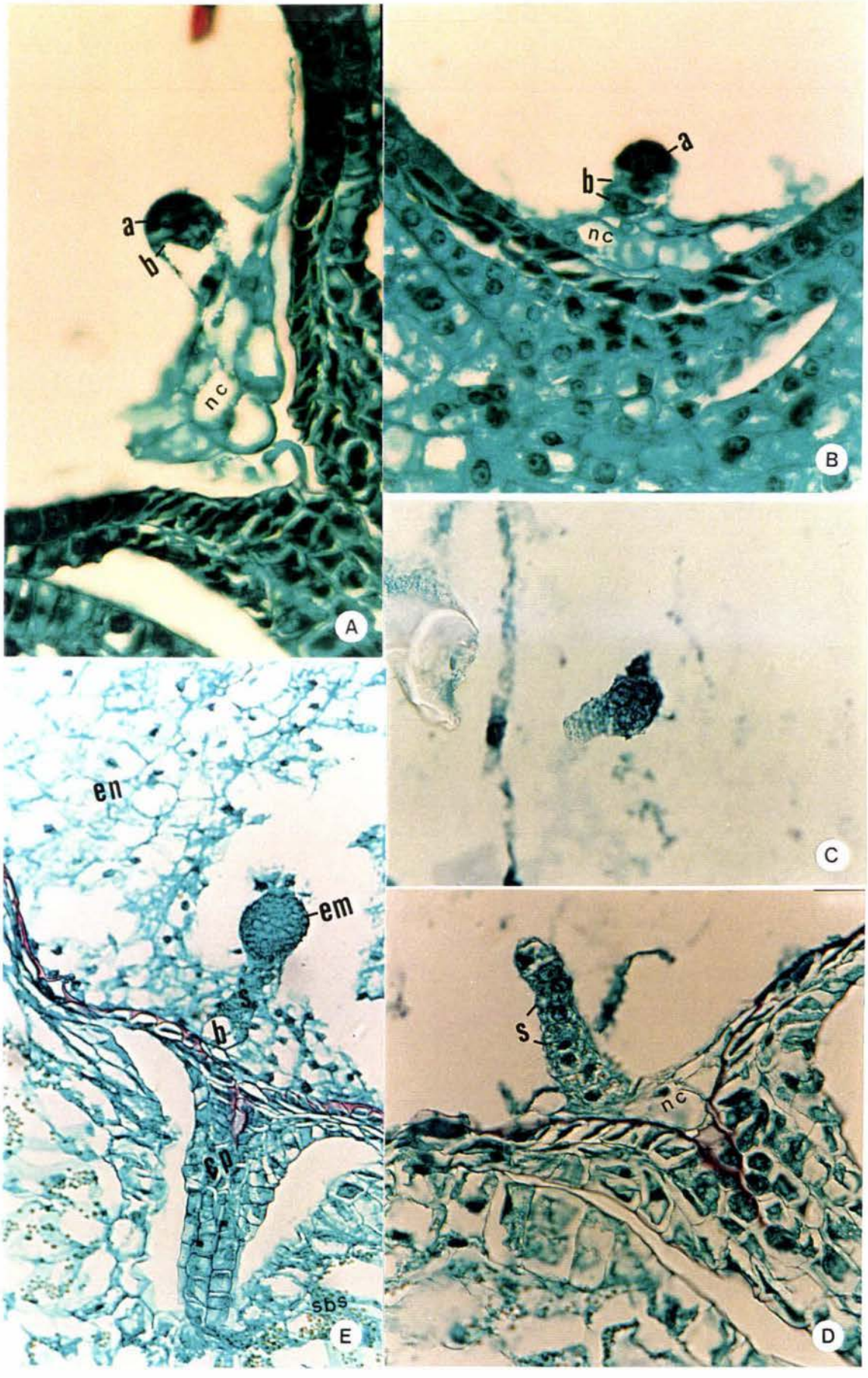


Plate 3.22 Stages in the development of the embryo. a, apical cell; b, basal cell; em, embryo proper; s, suspensor; en, endosperm; cp, cylindrical protuberance; sa, shoot apex; cot, cotyledon; r, prominent radicle; ea, embryonic axis; cs, clear space; nc, nucellar cap; sbs, small bodies. (A) Two-celled proembryo at 9 DAP (×400). (B) Three-celled proembryo at 9 DAP (×400). (C) Eight-celled embryo proper at 14 DAP (×400). (D) A eight to nine-celled suspensor at 14 DAP (×400). (E) Late globular embryo surrounded by endosperm cells at 21 DAP (×200). (F) Elongated spheroidal embryo with a suspensor at 28 DAP (×400). (G) Longitudinal section of linear embryo at 42 DAP (×200). (H) Transverse section of the embryo which was shown in (G) (×400). (I) Linear embryo in a mature seed (56 DAP, ×200).

Plate 3.22 F-I

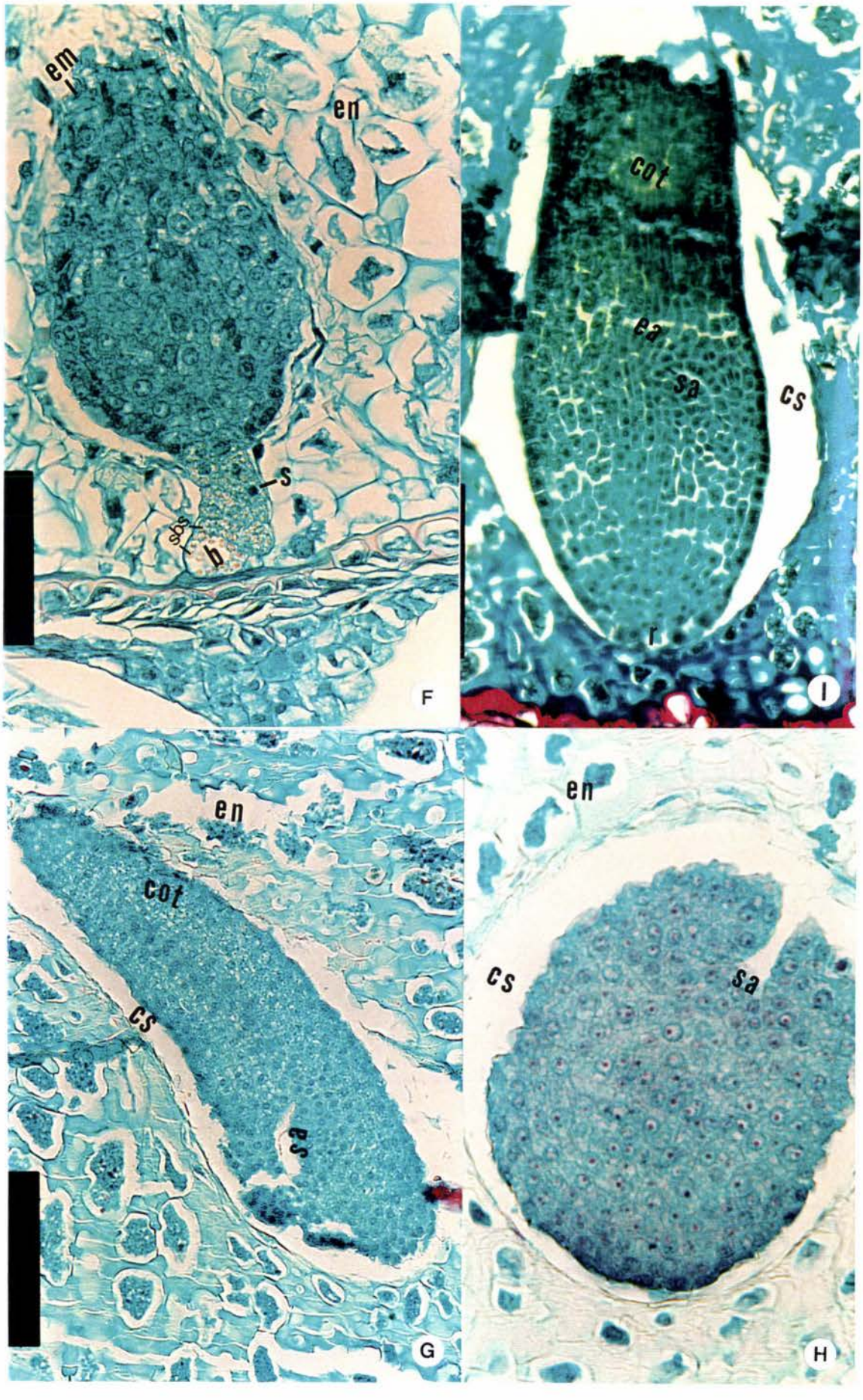
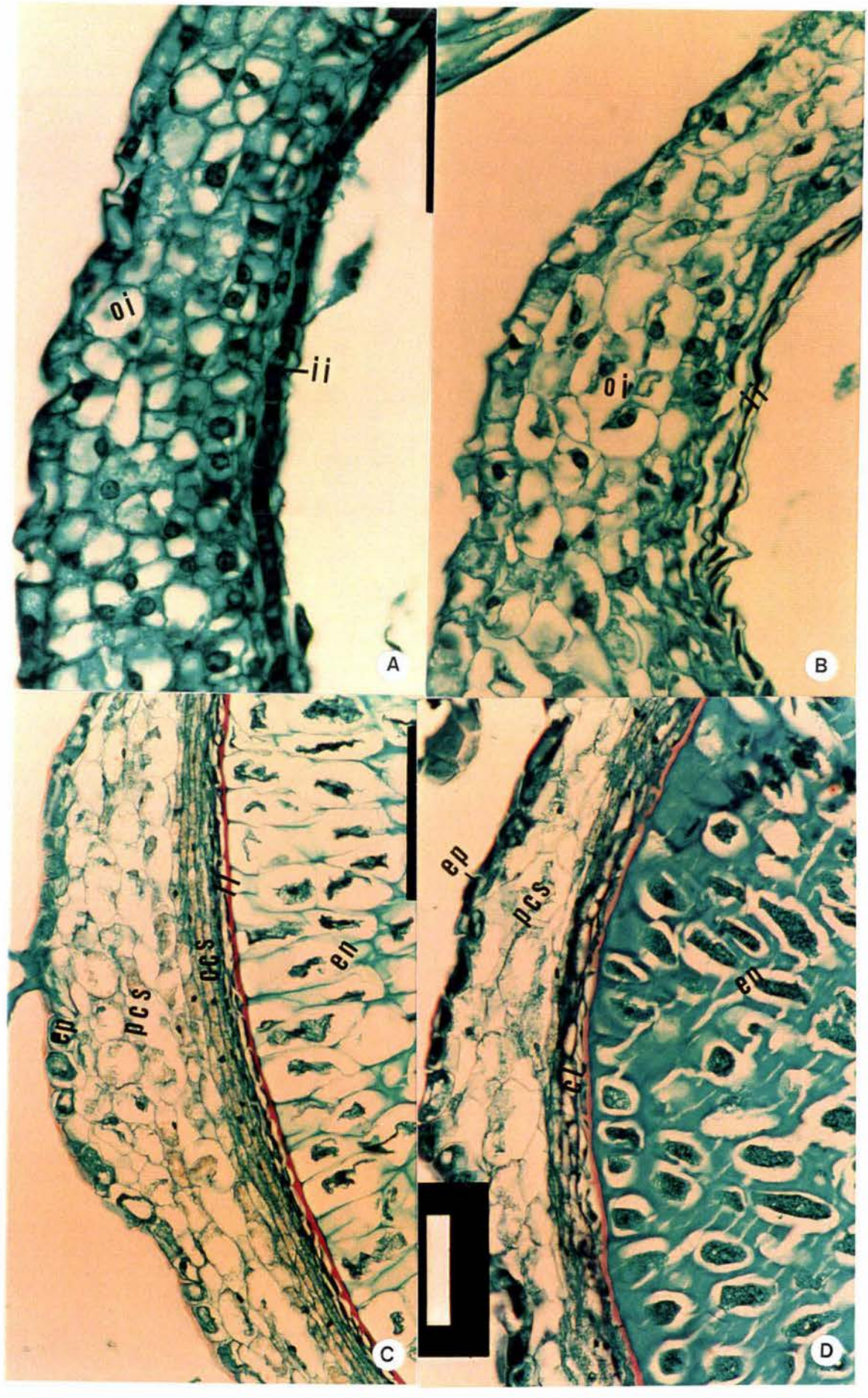


Plate 3.23 Stages in the development of the seed coat. oi, outer integument; ii, inner integument; en, endosperm; ep, epidermis; pcs, parenchyma cells; ccs, compressed cells; ll, lignified layer; cl, compressed cells + lignified layer. (A) A younger seed coat showing inner integument cells with horizontal elongation (12 DAP, ×400). (B) Developing seed coat shows that the cells of outer integument become enlarged and more vacuolated, while the inner ones are crushed and initiate lignification (14 DAP, ×400). (C) Seed coat at 28 DAP with four types of cells (×200). (D) Seed coat at 42 DAP (×200).

Plate 3.23 A-D



integument shows that the cells are arranged in an organized fashion and have nuclei and denser cytoplasm; the inner integument cells show horizontal elongation, denser cytoplasm without vacuoles (Plate 3.23 A). At approximately 14 DAP, the cells of the outer integument become enlarged and show more vacuolation, while the inner ones are crushed and begin lignifying (Plate 3.23 B). At 28 DAP, four types of cells can be recognized in the seed coat: (1) the epidermis consists of one layer of cells, (2) large parenchyma cells in several layers in which the cells enlarge and lose their contents, (3) several layers of cells show compression in thickness and horizontal elongation, and (4) a lignified layer which is derived from the damaged cells of the inner integument (Plate 3.23 C). After 42 DAP, the cells in (3) may degenerate or be fused with those in (4), and show a high degree of lignification (Plate 3.23 D). In mature seed (56 DAP), these lignified cells develop into a mechanical barrier covering the endosperm (Plate 3.24).

3.3.7 Seed Structure

The mature seed is globose with a strophiole around the micropyle and is dark brown in color. Diameter varies from 2.07 mm to 2.61 mm (Fig. 3.1) and the surface is rough. The seed coat of a mature seed differentiates into three layers: the epidermis, the subepidermal parenchyma (middle layer), and the lignified layer (Plate 3.24). Both epidermis and subepidermis come from the outer integument. The lignified layer is derived from both outer and inner or only inner integuments, which is stained red in color by safranin suggesting lignification (Plate 3.24) (Johansen 1940). At the micropylar end the outer integument increases in thickness forming the strophiole. The embryo is linear and very small, only 0.8 mm in length and 0.25 mm in diameter and embedded in the endosperm (Plate 3.21). The mature embryo shows a prominent radicle, a small embryonic axis, shoot apex and a large cotyledon (Plate 3.22 I). The endosperm constitutes most of the seed volume (Plate 3.21) and contains cells with thickened walls and many storage bodies (Plate 3.20). A special hole--the hilum is formed in the lateral side of the seed and may form from the chalazal shifted in the raphal direction (Plate 3.27).

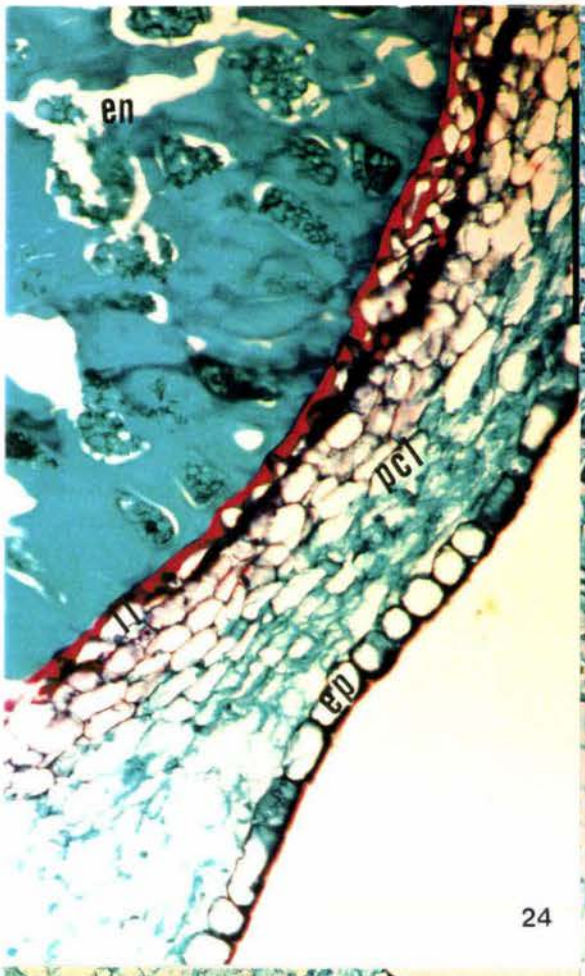
The tip of the radicle in the mature seed is situated at the micropylar end facing the seed coat and is separated from it only by a thin layer of the endosperm (containing

Plate 3.24 Seed coat at maturity stage (56 DAP, ×200). ep, epidermis; pcl, parenchyma cell layer; ll, lignified layer; en, endosperm.

Plate 3.25 Charazal part of a longitudinal section of a developing seed at 42 DAP (×100). en, endosperm; ts, transfer cells.

Plate 3.26 Micropylar part of longitudinal section of developing seed (42 DAP, ×160). em, embryo; en, endosperm; cp, cylindrical protuberance; s, strophiole.

Plate 3.27 Longitudinal section of a dry seed (×40). en, endosperm; h, hilum.



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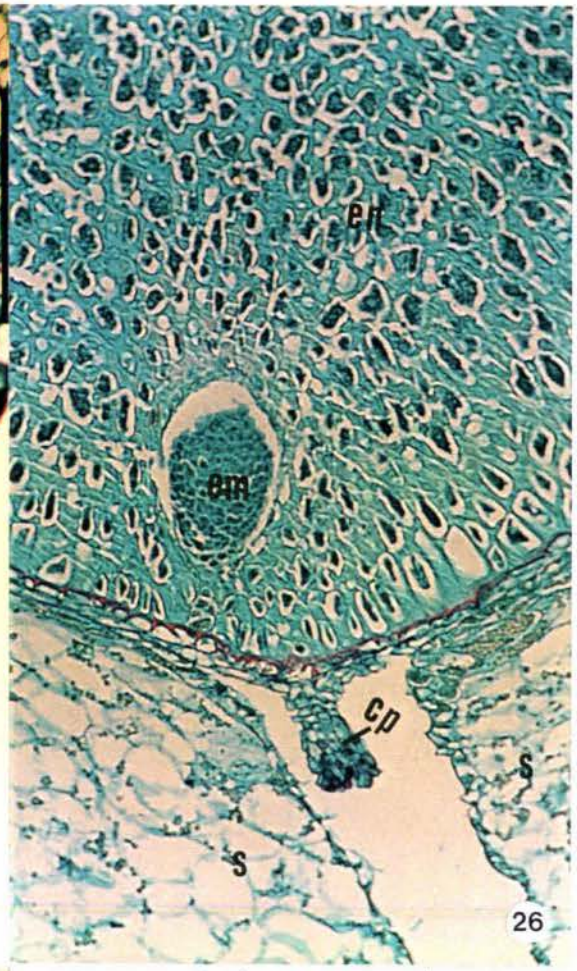

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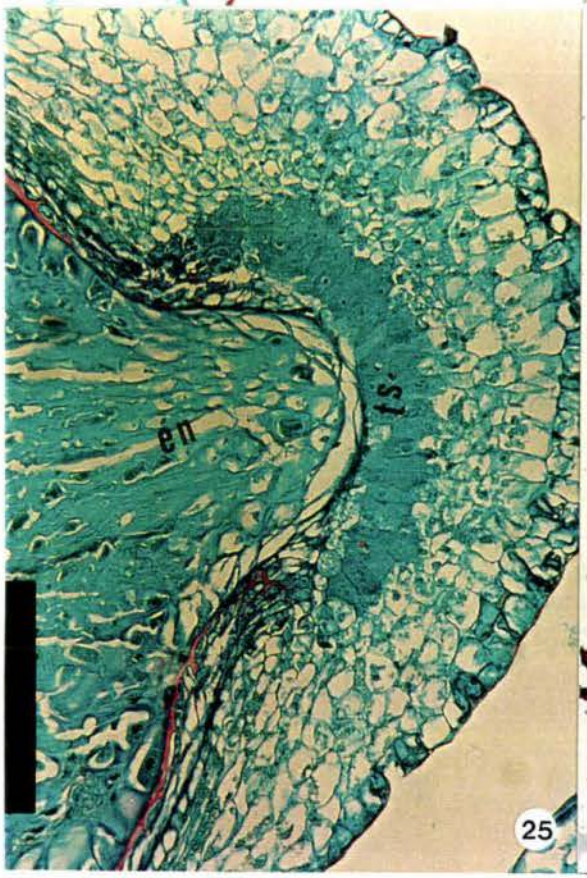

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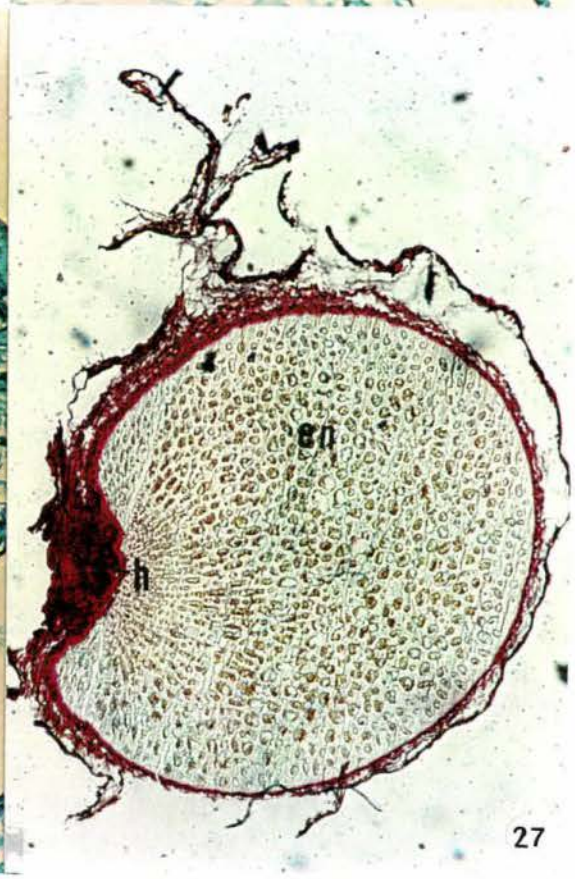

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1-2 layers of cells, Plate 3.22 I). The inner part of seed coat above the radicle tip is conical to cylindrical in shape (Plates 3.17, 3.19, 3.22 E and 3.26). This cylindrical protuberance of the inner integument at the micropylar side is formed from the inner integument at the micropylar part by closure of the micropylar tube during seed coat development.

As a result of observations in this study, seed development and associated histological events between pollination and seed maturity are summarized in Table 3.1.

Embryo and endosperm development in *Sandersonia* are not synchronous among ovules on an individual ovary. Specimens collected at any one time contain embryos of varying sizes and endosperm of varying stage. Specimens collected during any given week may contain embryos further developed than some taken the following week. Thus, the time shown in Table 3.1 is not very accurate. However, a general development scheme can be constructed by observing many randomly collected specimens.

Table 3.1 Correlation of seed development and histological stages

DAP	Diameter of ovules (mm)	Embryo	Endosperm	Seed coat
7-8	0.48 ± 0.06	Zygote at metaphase	Secondary nucleus	4-5 layers of cells in outer integument, 2 layers of cells in inner integument
8-9		Two-celled or three-celled proembryo	Free nuclear stage	
12				Cell horizontal elongation, more dense cytoplasm in inner integument
14	1.23 ± 0.19	Early globular stage	Cellularization of endosperm commenced	Cells enlarged and higher vacuolation in outer integument; cells crushed and initiate lignification in inner integument
21	2.02 ± 0.21	Late globular stage	Cellularization of endosperm completed	
28	2.37 ± 0.23	Cotyledonary primordia appeared (elongated spheroidal stage)		Four layers: epidermis, parenchyma layer, several layers of cells elongated horizontally, and the lignified layer
35-42	2.32 ± 0.17 2.58 ± 0.22	Growing point of shoot formed		
49-56	≈2.34 ± 0.27	Well developed embryo with radicle, small embryonic axis and a cotyledon	Well developed endosperm with thick-walled cells and many small bodied in the cells	Three layers: epidermis, subepidermal parenchyma, and the lignified layer

3.4 DISCUSSION

3.4.1 Ovary and Ovule

The present study confirms the results reported by Sterling (1975) that the ovary of *Sandersonia* has a tricarpellate form, a central hole or cavity in middle of ovary between the carpels. Each carpel contains numerous ovules which are anatropous, bitegmic and each ovule has an obturator on the concave side of the funiculus. However, the study also shows that there are open sutures which extend inward in the ovary along the septal boundaries of the carpels (this disagrees with Sterling's result), a well-developed, bowl-like hypostase below the embryo sac in the chalazal region and the ovule has a crassinucellate structure.

In a mature ovule of *Sandersonia* the exostome is an inverted Y-shaped, transverse slit with a middle notch (Plate 3.3). Both outer and inner integuments form a micropyle, due to the outer integument overgrowing the inner one (Plate 3.3). A special structure called the obturator is present on the concave side of the funiculus (Plate 3.3), which has a function in the guidance and growth of the pollen tube (Bouman 1984), and its surface opens to the micropyle. Tilton and Horner (1980) reported that a proliferation of tissue at the base of funiculus and at the tip of the carpel margins in *Ornithogalum caudatum* (Liliaceae) resulted in formation of a prominent obturator. They indicated that the first indication of obturator development was concomitant with prophase of megaspore meiosis. First, endothelial cells at the base of the funiculus divide anticlinally and then elongate slightly to form small swellings. Continued development in this region produces part of the obturator which lies at the opening of the micropylar exostome. The other part of the obturator is derived from growth of the tips of the carpel margins between the two rows of ovules in each locule. The dermal cells of the obturator are stained much more densely than those of the outer integument (Plate 3.3). Peterson *et al.* (1979) made a detailed ultrastructural study of this tissue in *Caltha*. On the basis of the presence of deeply staining cytoplasm, thick outer cell walls with electron-dense wall ingrowths, secreted material outside the cell walls, and numerous dictyosomes, they characterize the cells as transfer cells. The transfer cells are

supposedly involved in the short-distance transport of metabolites and/or the secretion of chemotropic substances for the growth of the pollen tube.

In addition to the obturator, the differentiation of the nucellar tissue at the chalazal region facing the antipodal end of the embryo sac is also present in *Sandersonia* ovule, which is referred as a "hypostase". At maturity, the hypostase is made up of a group nucellar cells which lose their cytoplasm and nuclei, and are thick-walled. These cells are surrounded by several layers of cells which are stained much more densely than those of the integument cells (Plates 3.3, 3.14 and 3.15) and could be referred as transfer cells. The hypostase is known in many families, e.g, Amaryllidaceae, Cyperaceae, Liliaceae, Zingiberaceae, Anacardiaceae, Bixaceae, Euphorbiaceae, Lauraceae, Sapindaceae, Theaceae (Bouman 1984). Many functions have been attributed to the hypostase (see section 2.1.1.2). It may be extant only in the mature ovule, and in certain stages of seed development, but may also persist in the mature seed (Bouman 1984). According to its structure, close to the end of the vascular bundle and surrounded by transfer cells, the hypostase in *Sandersonia* ovules may connect the vascular supply with the embryo sac and facilitate transport of nutritional material, but much detailed research is needed to elucidate its precise role in this species.

3.4.2 Ovule Growth

There is no obvious growth of the ovule before the zygote starts the first division, the mean diameters of ovule between day 0 and day 8 increasing from 0.39 mm to 0.48 mm (Fig. 3.1, Table 3.1). During this period, double fertilization occurs (not observed in this study), and then both the zygote and the primary endosperm nucleus are ready to start their divisions. As both the zygote and the primary endosperm nucleus begin the first division, a rapid enlargement of the embryo sac occurs, shown by rapid growth of the ovule (Fig. 3.1). Such enlargement is continuous and rapid until the embryo sac becomes fully cellular. After terminating cellularization of endosperm, there is a slight extension in diameter of ovule and the embryo becomes elongated spheroid due to the distal end elongating to form the cotyledonary initial (28 DAP). After 28 DAP, the endosperm cells gradually accumulate storage materials and become hardened (thickening of the cell walls), which probably restricts the extension of the ovule. When

the embryo develops into maturity at about 49-56 DAP, the ovule decreases in size (the mean diameter is about 2.34 mm) by seed desiccation. At this time, the embryo is linear in shape and has a radicle prominent, a small embryonic axis and a cotyledon. The endosperm tissue occupies much of the seed volume and contains many thick-walled cells in which many small storage bodies are present.

3.4.3 Development of Endosperm

Endosperm development in angiosperms is classified into three types: Nuclear, Cellular and Helobial (see section 2.1.2). Both Nuclear and Helobial types are characterized by the presence of a free-nuclear phase during the development of endosperm and are reported in Liliaceae (Davis 1966). Two main mechanisms of endosperm cellularization have been reported. In the first type, cellularization is accomplished through freely-growing walls between the endosperm nuclei, which arise from the embryo sac wall as projections. These walls fuse to form endosperm cells as reported in *Haemanthus katherinae* (Newcomb 1978), *Stellaria media* (Newcomb and Fowke 1973), *Helianthus annuus* (Newcomb 1973) *Iberis amara* (cited in Chitralekha and Bhandari 1992) and *Phaseolus vulgaris* (Yeung and Cavey 1988). In the second type, the freely-growing anticlinal walls arise between the nuclei in free-nuclear endosperm and later fuse to form open uninucleate compartments. Such compartments formed at the periphery of the embryo sac extend centripetally and divide periclinally to give rise to layers of endosperm cells as in wheat (Mares *et al.* 1977, Fineran *et al.* 1982), *Papaver somniferum* (Bhandari *et al.* 1986) and *Allium fistulosum* L. (Xiang-Yuan 1987).

Allium fistulosum L. a species in Alliaceae, exhibits embryo development in a very similar way to *Sandersonia* till the elongated spheroidal stage. The development of endosperm conforms to the Nuclear type (Xiang-Yuan 1987). Cell formation starts simultaneously at the micropylar and the chalazal ends of embryo sac when the embryo is in the late globular stage. The first anticlinal walls arise from the cell plates without phragmoplasts between the free interphase nuclei resulting in a layer of open cells. The first periclinal walls are initiated as a result of normal cytokinesis of the first formed open cells, and are associated with phragmoplasts and cell plates. The subsequent cell

divisions give rise to the endosperm cells centripetally until those from the opposite sides of the embryo sac meet (Xiang-Yuan 1987).

The present study shows the development of endosperm in *Sandersonia* also conforms to the Nuclear type. The cellularization of endosperm commences at the early globular stage and terminates at the late globular stage. That the differentiation of cellular endosperm began in the early globular stage of embryo development was also reported in *Helianthus annuus* (Newcomb 1973). Many researchers recorded that endosperm wall formation started at the micropylar end of the embryo sac and progressed toward the chalazal end, for example *Helianthus annuus* (Newcomb 1973), *Hamamelis virginiana* (Shoemaker 1905), *Haemanthus katherinae* (Newcomb 1978) and *Stellaria media* (Newcomb and Fowke 1973); occurred simultaneously in all regions as in *Asclepis cornuti* (Frye 1902); or in both micropylar and chalazal end, *Allium fistulosum* L. (Xiang-Yuan 1987). However, endosperm wall formation in *Sandersonia* starts at the chalazal end and proceeds micropylarly and centripetally.

The mechanism of cellularization of endosperm in *Sandersonia* is seen here to be different to that described for *Allium fistulosum* L. The first anticlinal walls in *Sandersonia* are associated with the embryo sac wall and project into the central vacuole. The open uninucleate compartments can not be found. According to these observations, the cellularization of endosperm in *Sandersonia* may conform to the first type (freely-growing walls between the endosperm nuclei arise from the embryo sac wall as projections), but further detailed investigations must be undertaken in this species.

It is widely accepted from *in situ* observations and *in vitro* studies that endosperm is an important source of metabolites for the developing embryo (Brink and Cooper 1947, Maheshwari 1950, Raghavan 1966). However, there are some reports that suggest the limited importance of endosperm during early embryogenesis. Schulz and Jensen (1969) have indicated that in *Capsella* at the heart stage of embryogenesis the endosperm is still developing and probably not contributing significantly to the nutrition of the embryo. In *Podelepis jaceoides* (Davis 1961) and *Minuria denticulata* (cited in Newcomb 1973) there is no indication of the digestion of endosperm during the early stages of embryogenesis such as occurs later. In sunflower, endosperm growth generally starts before that of the embryo (Newcomb 1973). After the elongated spheroidal stage

of embryogenesis of *Sandersonia* (about 28 DAP), a clear area appears adjacent to the embryo. This area is interpreted as representing the remains of digested endosperm (Newcomb 1973). Therefore, it seems reasonable to postulate that the embryo of *Sandersonia* is at least using the endosperm after the elongated spheroidal stage. The young embryo may be dependent on the suspensor as a source of metabolites since a proembryo with well-developed suspensor is present from the early globular stage.

The antipodal cells play an important role in endosperm development of *Papaver somniferum* (Bhandari *et al.* 1986) and *Ranunculus sceleratus* Linn. (Chitralekha and Bhandari 1991). High concentrations of DNA in the nuclei of antipodal cells, and in the endosperm nuclei around them in *Lilium* (cited in Bhandari *et al.* 1986), *Ranunculus sceleratus* (Vijayaraghavan and Bhat 1980) and *Papaver somniferum* (cited in Bhandari *et al.* 1986) point to a similar conclusion. Maze and Lin (1975) speculate that some cytological activity of the antipodal cells may involve the synthesis or transfer of growth controlling substances. Maze and Bohm (1974) suggested that the antipodal cells of *Agrostis interrupta* possibly accumulated and then released hormones for endosperm growth.

The antipodal cells in *Sandersonia* are prominent, persistent and enlarge after fertilization. They often degenerate before initiation of cellularization of endosperm. Similar results were also reported in other species, such as *Papaver somniferum* (Bhandari *et al.* 1986), *P. nudicaule* (Olson and Cass 1981), *Ranunculus sceleratus* Linn. (Chitralekha and Bhandari 1991) and wheat (cited in Bhandari *et al.* 1986). It is also found in developing ovules of *Sandersonia* that a well-developed, bowl-shaped hypostase, surrounding by one to two layers of transfer cells, below the antipodal cells and a vascular bundle terminates at the base of the hypostase (Plates 3.3 and 3.4). These results suggest that the antipodal cells assist in the nutrition of endosperm at least till the early cellularization period by transferring nutrients from the nucellar cells (hypostase) to the endosperm.

3.4.4 Development of Embryo

The nucleus of the egg cell in *Sandersonia* is located at the micropylar pole before

fertilization, and then is transferred to the chalazal pole (Plates 3.7, 3.8 and 3.9). A post-fertilization shift of nucleus and cytoplasmic contents towards the chalazal pole has also been observed in *Papaver nudicaule* (Olson and Cass 1981) and *Zea mays* (cited in Natesh and Rau 1984). In the unfertilized egg of *Papaver nudicaule* the nucleus is sited towards the micropylar pole with a large vacuole towards the chalazal pole. However, at fertilization there is a reversal of cytoplasmic polarity; the nucleus shifts to the chalazal pole and the micropylar pole is occupied by a vacuole (Olson and Cass 1981).

Veyret (1974) indicated different embryo types according to the early cell division pattern of the embryo. Six main types of embryogeny have been described by Schnarf as well as Johansen (see section 2.1.3) based on the transverse division of the zygote and the respective contributions of the two daughter cells to subsequent formation of the embryo and its suspensor. The specific initial events of cell division do not affect subsequent embryo development (Yeung and Law 1992). In *Sandersonia*, the first division of the zygote is transverse resulting in a smaller apical and a larger basal cell. The basal cell generally divides first, into two cells. These results are similar to those in *Lilium parryi* (Liliaceae) described by Johansen (1950). The following divisions could not determined in this study, and hence developing *Sandersonia* embryos can not be classified to any particular type of embryogeny. However, the Caryophyllad and Piperad types can be eliminated since the basal cell never divides again in the former and the first division of zygote is vertical in the latter (see Fig. 2.1).

Embryo development of *Sandersonia* passes through the following stages: the early globular (about 14 DAP), the late globular (about 21 DAP), the elongated spheroidal (about 28 DAP), and the linear embryo (after 35 DAP). The mature embryo has little differentiation, but a prominent radicle, a small embryonic axis, shoot apex and a cotyledon can be recognised (Plate 3.22 I).

The development of embryos in *Allium fistulosum* L. is of the Asterad type (Xiang-Yuan 1987) and its pattern of embryo development is very similar to those in *Sandersonia* till the elongated spheroidal stage. However, embryo development in *Allium fistulosum* L. is faster and results in a well-developed, curved embryo (see details in Table 3.2).

Table 3.2 Comparison of embryo development between *Allium fistulosum* L. and *Sandersonia aurantiaca*

DAA	*Allium fistulosum* L	DAP*	*Sandersonia aurantiaca*
4-5	Zygote at metaphase	7-8	Zygote at metaphase
5-6	2 to 4-celled proembryo	9	2 to 4-celled proembryo
7	8 to 16-celled embryo proper	14	Early globular stage
8-10	Globular stage	21	Late globular stage
11	Spheroidal stage (form cotyledonary initial)	28	Elongated spheroidal stage (form cotyledonary initial)
12-13	Growing point of the shoot at the lateral side of a embryo in the form of concavity	35-42	Growing point of the shoot at the lateral side of a linear embryo
13-15	Stick-shaped embryo	49	Linear embryo
>15	Curved mature embryo with a prominent radicle, shoot apex and a elongation cotyledon	56	Linear mature embryo with a prominent radicle, shoot apex and a cotyledon

* DAP for *Sandersonia* flowers = DAA+2 days

A well-developed suspensor was found in the *Sandersonia* embryo from about 14 DAP to 49 DAP. It contains a larger basal cell and a number of suspensor cells arranged in one or two tiers under the embryo proper. *Allium fistulosum* L. has a short suspensor comprised of two tiers of 4 cells each which remains to the spheroidal embryo stage (Xiang-Yuan 1987). Similar suspensor structures are also found in some species of Liliaceae. For example, a suspensor of five or more cells in *Calochortus* remains more or less intact when the embryo is mature (Johansen 1950); a well-developed filamentous suspensor is found in *Pushkinia scilloides* (Johansen 1950); and *Anthericum ramosum* possesses a suspensor in the form of several superposed flattened cells (Johansen 1950). In addition to pushing the growing embryo into the embryo sac, the suspensor in the following plants is believed to play an important role during early embryo development. It has been shown in *Phaseolus* that the suspensor is an important uptake site for the developing embryo (Yeung 1980) and is a major site for gibberellin synthesis (Picciarelli and Alpi 1986). In *Calypso*, the basal cell of the suspensor enlarges rapidly (Yeung and Law 1992). This rapid expansion presses the cell tightly against the integuments of the maternal tissues. It may be reasonable to suppose that the suspensor in *Sandersonia* embryo may play an important role at least during early embryo development, because: (1) the two synergids disappear before the first division of the zygote; (2) the suspensor remains a long time until the embryo is nearly into the mature stage; (3) the basal cell is tightly connected on the embryo sac wall at the micropylar end during the whole period of embryo development; and (4) small storage bodies which are present in mature

endosperm cells are also found both in the suspensor cells and the basal cell (plate 3.22 D, F).

3.4.5 Seed Coat Development and Seed Structure

Developing integuments show both anticlinal and periclinal divisions. The periclinal divisions are mainly responsible for the increase in the number of layers of the concerned integument, whereas anticlinal divisions mainly cause the growth in length (Boesewinkel and Bouman 1984). Integuments with mainly anticlinal divisions and no increase in the number of layers are called non-multiplicative integuments (Corner 1976), which generally have a simple structure and may be two- or three- or paucilayered. The evidence from this study suggests that none or hardly any cell divisions take place after fertilization, and the seed coat of *Sandersonia* is formed through the elongation and differentiation of integumentary cells. The seed coat in *Sandersonia* derives from the two integuments. The inner integument has two layers of cells and develops before the outer one does. At maturity, the cells of the inner integument are completely crushed, lignified, and finally forms a mechanical barrier surrounding the endosperm tissue. The outer integument develops 2 days later than the inner one. It is non-multiplicative and reduced in thickness and forms an outer epidermis (one layer of cells), subepidermal parenchyma layer (several layers of cells). The innermost layer of outer integument cells (Plate 3.23 C) may be degenerated or fused with inner integument cells to form the lignified layer.

A special structure – a conical or cylindrical protuberance is observed in the inner part of the seed coat, which derives from the inner integument at the micropylar part by closing the micropylar tube during seed coat development. A similar structure is also found in iris (Iridaceae) seed by Blumenthal *et al.* (1986). The anatomical differences between *Iris lorteti* and *I. atropurpurea* were described and the resistance of the seed coat were tested by measurement of the force required to rupture the seed coat at the micropylar region (see section 4.4.3 in Chapter 4). Blumenthal *et al.* (1986) indicated that the hardness of the seed coat appeared to be the main cause for seed dormancy in irises, and no germination of iris seeds can occur unless the structure of the seed coat in the micropylar region was loosened.

Mature seeds of *Sandersonia* are difficult to germinate. Their inability of germination can be attributed to a combination of seed coat impermeability and mechanical restriction with embryo dormancy. The detail investigations of mechanism of seed dormancy in this species are shown in Chapter 4 of this thesis.

In summary, seed development from pollination to maturity takes about 60 days. The embryo passes through the early globular, the late globular, the elongated spheroidal and the linear embryo stages. Endosperm development conforms to the Nuclear type. The cellularization of endosperm starts at about 14 DAP when the embryo reaches the early globular stage, and terminates at about 21 DAP when the embryo is at the late globular stage. The mature seed is globose with a strophiole around the micropyle. The embryo is linear and shows a prominent radicle, a small embryonic axis, shoot apex and a large cotyledon. The endosperm constitutes most of the seed volume and contains cells with thickened walls and many storage bodies. The seed coat of a mature seed differentiates into three layers: the epidermis, the subepidermal parenchyma (middle layer), and the lignified layer. Both epidermis and subepidermis come from the outer integument. The lignified layer is derived from both outer and inner or only inner integuments.

Chapter 4

GERMINATION OF *Sandersonia aurantiaca* (HOOK.) PROMOTED BY INTERACTION BETWEEN SCARIFICATION AND A PLANT GROWTH REGULATOR

4.1 INTRODUCTION

Sandersonia aurantiaca (Colchicaceae) is a species of interest for both horticultural (see Chapter 1) and medicinal purposes (Finnie and van Staden 1991). Seed dormancy is a feature of *Sandersonia*. The germination of seeds ripening in a particular year is spread over very long periods. Only about 10 % of seeds germinate during the first year when they are sown in soil; 50 % germinate during the second year (P. Guo, personal communication). The rest of the seeds fail to germinate after two years because they are susceptible to a host of fungi. Clark's work suggests that the *Sandersonia* seeds have a double dormancy mechanism and that chilling and leaching is required to overcome the dormancy (unpublished). However, no successful methods for propagating this species from seeds are reported in the scientific literature.

Generally, researchers recognize two types of seed dormancy: embryo dormancy, where the mechanism resides inside the embryo itself; and coat-imposed dormancy, where the mechanism is located within structures that enclose the embryo (Nikolaeva 1977, Bewley and Black 1994). Seed coats and surrounding structures may influence the ability of a seed to germinate through interference with water uptake, gas exchange, diffusion of endogenous inhibitors, or by mechanical restriction of embryo growth (Ikuma and Thimann 1963, Mayer and Shain 1974). Some researchers reported that the endosperm might mechanically restrict embryo expansion, thus preventing radicle emergence (see section 2.2.3.3). Weakening the endosperm tissue around the radicle tip can enhance seed germination in some species, such as pepper (Watkins and Cantliffe 1983), iris (Blumenthal *et al.* 1986), tomato (Groot and Karssen 1987), and muskmelon (Welbeum and Bradford. 1990).

Seeds with morphophysiological dormancy (MPD) have under-developed, dormant embryos, which means they have a combination of morphological and physiological dormancy (Baskin and Baskin 1991). The MPD often occurs in many primitive plant families which have either rudimentary or linear embryos (Atwater and Vivrette 1987). Before seeds with MPD can germinate, physiological dormancy must be broken, and embryos must grow to a certain critical length (Baskin and Baskin 1991, 1994). This kind of dormancy can be removed by such treatments as cold stratification, warm followed by cold stratification, growth stimulators (e.g., GA, kinetin, KNO_3), or two warm periods followed by cold stratification (Nikolaeva 1977).

Gibberellic acid (GA_3) is active in breaking dormancy. It is well established fact that application of GA to seeds breaks dormancy and accelerates germination of non-dormant seeds (Karssen *et al.* 1989). Since exogenous gibberellins were applied to promote the germination of lettuce seeds by Lona in 1956 (reviewed in Karssen *et al.* 1989) GA_3 has been often used as a promotory agent for breaking seed dormancy in a wide variety of species, such as *Avena fatua* (Hsiao 1979), *Cyclopia intermedia* (Whitehead and Sutcliffe 1995), *Gloriosa superba* L. (Suparna *et al.* 1993), *Lactuca serriola* L. (Small and Gutterman 1992), *Rhodanthe moschata* Wilson (Bunker 1994) and *Vernonia galamensis* (Teketay 1993). There is often an increased effect with an increase in the length of the after-ripening period (Green and Helgeson 1957). The presence of hull structures (lemma and palea) in grains reduces the effect of GA_3 and puncturing the seed coverings (Hsiao 1979) or scarification by immersion in sulfuric acid (Keogh and Bannister 1992) enhances GA_3 penetration which increases the availability of GA_3 for germination promotion. GAs which play a key role in seed germination of many species have several separate promotive actions (1) they control endosperm mobilization which seems to serve two purposes: (a) decrease of mechanical restraint imposed on embryo (b) mobilization of reserve food; and (2) they promote embryo growth (Karssen *et al.* 1989, Hilhorst and Karssen 1992). Hartmann *et al.* (1990) indicated that gibberellins appeared to play a role in two different stages of germination. One occurs at the initial enzyme induction in their transcription from the chromosomes. The second is at Stage III (seedling growth stage) in the activation of reserve food mobilizing systems.

The purpose of this study was to find methods to promote the seed germination of *Sandersonia* and to assess the seed dormancy mechanisms of *Sandersonia aurantiaca* (Hook.).

4.2 MATERIALS AND METHODS

4.2.1 Seed Source

Sandersonia aurantiaca seeds and plants were supplied by Crop & Food Research Ltd. at the Levin Research Centre. The seeds were collected from commercial plants in February 1995, 1994 and 1993, and from plants growing in a glasshouse at the Department of Biology and Biotechnology, Massey University in January 1996. The glasshouse temperature varied between 26-32°C during the day and 15-20°C at night, and a natural photoperiod was experienced by the plants. The plants were watered 4 times per day for approximately 3 min. Seeds collected in 1995 were used in all experiments. Other seed lots collected in 1996, 1994 and 1993 were used in germination experiment 4 and imbibition measurements. All of the seeds were stored in sealed plastic containers at room temperature until used in the experiments. The ovules cultured *in vitro* were collected from ovaries of plants in the glasshouse at the Department of biology and biotechnology.

4.2.2 Seed Viability

100 seeds were placed in petri dishes on moist filter paper for at least 48 h at 20°C. The moistened seeds were cut longitudinally so that the embryos were exposed before being stained with solution. Prepared seeds were immersed in a 1% solution of 2,3,5-triphenyl tetrazolium chloride (TTC) for 48 h in darkness at 20°C (Association of Official Seed Analysts 1970) and then observed under a light microscope. Seeds were classified as either non-viable (no staining) or viable (showing red color on the embryo).

4.2.3 Seed Germination

All experiments, except those on temperature effects, were carried out at 20°C. Except

when the light effect was studied, most experiments were carried out in nominal darkness, i.e., brief exposure to laboratory light for germination counting only. Seeds were placed on two layers of moistened filter paper (Whatman No. 1) in 9 cm petri dishes with 4 replicates of 40 seeds for each treatment. Petri dishes were sealed with a strip of Parafilm to maintain moisture and incubated in a controlled temperature incubator. Germination, defined as the protrusion of the radicle, was recorded every week up to about 150 days in experiment 1, about 60 days in experiment 2, and about 30 days in experiment 3 and 4.

4.2.3.1 Experiment 1: A Survey of Dormancy Breaking Treatments on *Sandersonia* Seeds

This experiment was conducted to test 85 treatments. Some of these are better classified as pre-treatments as the treatment was applied to the seeds before incubation began. However they are all referred to "treatments" in the following pages. Treatment methods were grouped into eight categories: (1) temperature, (2) light, (3) stratification, (4) mechanical, (5) heat, (6) chemicals, (7) hormone, and (8) combinations which consisted of two or more types of treatments (Table 4.1). 2.5 g/l thiram was included in the germination media to protect seeds from fungal contamination (Persson 1993). From this experiment the most promising methods were retained for the next experiment while the unsatisfactory methods were discarded. After 150 days, all treatments were divided into four groups: (1) replicate one was kept at 20°C, (2) replicate two was given heat shock (5°C for 5 days, 25°C for 5 days, 5°C for 5 days again, and then back to 20°C), (3) replicate three was dehydrated for three days (water content was reduced to about 10%), and (4) some of replicate four were scarified with #240 sandpaper and then cut with a scalpel.

4.2.3.2 Experiment 2: Optimization of Germination of *Sandersonia* Seeds

The most promising treatment selected from Experiment 1 was the treatment No. 85– seeds scarified with #240 sandpaper 1 min, and then nicked near the radicle end (see section 4.3 Results and Fig. 4.1). The germination percentage in this treatment however was only 10.6%. In order to try to obtain a higher germination percentage and reduce

Table 4.1 Eighty five treatment methods employed in Experiment 1

Treatment No	Treatment description	References[1]
Control		
1	20°C	
Temperature		
2, 3, 4, 5, 6, 7	5, 20, 30°C; or 5, 20, 30°C in a light impermeable black bag	Rudnicki & Kaukovirta 1991, Small & Gutterman 1992
Light		
8, 9, 10, 11	Red light 0 or 30 min, Far Red light 30 min, Blue light 30 min	Hou & Simpson 1993, Koller *et al.* 1964
Stratification		
12, 13, 14, 15	Wet seeds, 5°C, 2, 4, 8 or 12 weeks	
16, 17, 18, 19	Wet seeds, 5°C, 2, 4, 8 or 12 weeks in a light impermeable black bag	Paynter & Dixon 1991
Mechanical		
20, 21	Leaching in running tap water 2 days or 1 week	Plummer *et al.* 1995
22	# 240 sandpaper 1 min	Kondo 1993
23	Nicking seed	Paytner & Dixon 1991
Heat		
24, 25	Heat shock, 100°C/tap water 1/1 or 5/5 min	Teketay 1993
Chemicals		
26, 27	98% H_2SO_4 10 or 30 min + rinse 10 min	Kondo 1993
28, 29, 30, 31, 32	KNO_3 0, 10^{-4} or 10^{-2} M with light, or in a light impermeable black bag	McNeil & Duran 1992
33, 34	200 mM ethanol 2 or 24 h	Cohn 1989
35, 36	100 mM butanol 2 or 24 h	Cohn 1989
37, 38	10 mM $NaNO_2$ 2 or 24 h	Cohn & Hughes 1986
39, 40	0.5 mM NaN_3 2 or 24 h	Cohn & Hughes 1986
41, 42	10 mM NH_2OH-HCl 24 or 72 h	Cohn & Hughes 1986
43, 44, 45, 46	1000 or 4000 ppm thiourea 2 or 24 h	Suparna *et al.* 1993
47, 48, 49, 50, 51, 52	10, 25 or 50 mM glutathione 2 or 24 h	Fontaine *et al.* 1995
53, 54	10 mM salicylic acid 2 or 24 h	Cohn 1989
55, 56	20 mM benzoic acid 2 or 24 h	Cohn 1989
57, 58	20 mM propionic acid 2 or 24 h	Cohn *et al.* 1987
59, 60	28 mM isobutyric acid 28 mM 2 or 24 h	Cohn *et al.* 1987
61, 62	53 mM acetic acid 2 or 24 h	Cohn *et al.* 1987
63, 64	100 mM propanol 2 or 24 h	Cohn 1989
65, 66	100 mM ethyl acetate 2 or 24 h	Cohn 1989
67, 68	300 mM lactic acid 2 or 24 h	Cohn 1989
69, 70	500 mM succinic acid 2 or 24 h	Cohn 1989
71, 72	500 mM methanol 2 or 24 h	Cohn 1989
Hormone		
73, 74, 75, 76	10, 100, 300 or 1000 ppm GA_3	Laura *et al.* 1994
77, 78, 79, 80, 81	0, 1, 10, 100 or 1000 ppm C_2H_4 [2] 24 h	
82, 83	50 or 100 ppm 6-BA	Suparna *et al.* 1993
Combination		
84	5°C 8 weeks + 20°C 3 weeks + 5°C 8 weeks	Clark unpublished
85	# 240 sandpaper 1 min + nicking near radicle end	

[1] The methods used in this experiment referred to the references shown in this table, but there may be some change within the methods according to our proposal.

[2] C_2H_4 was obtained from a gas bottle by a syringe and then diluted to an appropriate concentration in a 600 ml air-tight container by removing an appropriate volume of air from the container and then injecting same volume of C_2H_4 into it through a rubber port.

seed dormancy, 31 new treatment methods were used in Experiment 2. Treatment No. 85 was substituted for a control in this test (Table 4.2). The effect of fungicide-thiram or surface sterilization on germination of *Sandersonia* seeds was also studied. 2.5 g/l thiram was included in the germination media for most of treatments except where noted otherwise.

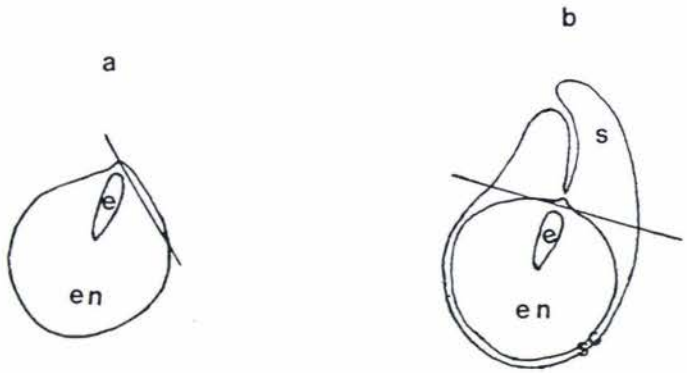

Figure 4.1 Position of seed nick from the radicle end of the seed. (a) Nick near the radicle end for seeds scarified by sandpaper. (b) Nick at the radicle end for intact seeds. e, embryo; en, endosperm; s, strophiole; sc, seed coat.

4.2.3.3 Experiment 3: Effect of Gibberellic Acid on *Sandersonia* Seed Germination

The aim in this experiment was to gain information as to the effect of concentration and contact time with gibberellic acid (GA_3). Firstly all 40×4 seeds per treatment were scarified with # 180 sandpaper by hand (1-1.5 min) and cut near the radicle end (Fig. 4.1). Then the seeds were placed on two layers of filter paper (Whatman No.1) and moistened with 6 ml of gibberellic acid (GA_3 10, 100, 200, 300, 500 and 1000 ppm; 6 ml distilled water as control) solutions for testing the effect of concentration. GA_3 at 300 ppm was applied for 1, 2, 4, 7 days and 14^+ days (seeds in GA_3 solution till the testing finished) to test contact time.

4.2.3.4 Experiment 4: Germination Optimization Treatments Applied to Other Seed Lots of *Sandersonia*

Two groups of four replicates of 40 seeds collected in 1993, 1994, 1995 and 1996 were used in this experiment. (1) The seeds were weighed, heated to 105° C for 16 h, cooled

Table 4.2 Thirty one treatment methods employed in Experiment 2

Treatment No	Treatment description
Control	
1	# 240 sandpaper 1 min + nicking near radicle end (no thiram) 20°C
Temperature and Light	
2	# 240 sandpaper 1 min + nicking near radicle end, 25°C, 16 h light / 8 h dark
3	# 240 sandpaper 1 min + nicking near radicle end, 30°C
4	# 240 sandpaper 1 min + nicking near radicle end, in a light impermeable black bag
Stratification	
5, 6	# 240 sandpaper 1 min + nicking near radicle end + 5°C, 1.25 g/l thiram, 4 weeks, or 25 % bleach* 20 min, 8 weeks,
Mechanical	
7	# 240 sandpaper 1 min (no thiram)
8	# 180 sandpaper 2 min + 25 % bleach* 20 min
9	# 120 sandpaper 4 min, no thiram
10	Soaking 4 days + pricked with needle
11	# 240 sandpaper 1 min + nicking not near radicle end
Leaching	
12	Sandpaper 1-1.5 min + nicking near radicle end + leaching in running water 1 week
13	Sandpaper 1-1.5 min + leaching in running water 1 week + nicking near radicle end
14	Leaching in running water 1 week + soaking 1 week + nicking near radicle end
Heat	
15	Whole seed + 85°C water bath 1 min
16	Sandpaper 1-1.5 min + 85°C water bath 1 min + nicking near radicle end
Chemicals	
17	Sandpaper 1-1.5 min + nicking near radicle end (dusted with thiram powder) + 10^{-2} M KNO_3, 25°C, 16 h light / 8 h dark
18	Sandpaper 1-1.5 min + nicking near radicle end + soaking in water 24 h + soaking in 4000 ppm thiourea 40 h + 1.25 g/l thiram
19	Sandpaper 1-1.5 min+nicking near radicle end+10 mM NH_2OH-HCl 72 h
20	Sandpaper 1-1.5 min + nicking near radicle end + 1 mM NaN_3 24 h + 1.25 g/l thiram
Hormone	
21	Whole seed + GA_3 300 ppm (no thiram)
22	Nicking near radicle end + GA_3 300 ppm (no thiram)
23	Sandpaper 1-1.5 min + GA_3 300 ppm (no thiram)
24	Sandpaper 1-1.5 min+nicking near radicle end+300 ppm GA_3.(no thiram)
25	Sandpaper 1-1.5 min+nicking near radicle end+50 ppm 6-BA (no thiram)
26	Sandpaper 1-1.5 min + nicking near radicle end + 300 ppm GA_3 + 50 ppm 6-BA (no thiram)
Effect of Thiram and Bleach	
27	Whole seeds (no thiram and bleach*)
28	Whole seeds +50 % bleach 20 min
29	Whole seeds + 2.5 g/l thiram
30	Sandpaper 1-1.5 min + nicking near radicle end + 25 % bleach* 20 min
31	Sandpaper 1-1.5 min + nicking near radicle end + 2.5 g/l thiram

* 35 g/ l NaOCl

in a desiccator, and reweighed to determine fresh weight, dry weight, and water content (WC expressed on dry weight basis). (2) The seeds were scarified with #180 sandpaper by hand (1-1.5 min), and cut near the radicle end (Fig. 4.1). Then the seeds were placed on two layers of filter paper (Whatman No.1) and moistened with 6 ml of 300 ppm GA_3 solution for germination testing at 20°C.

4.2.4 Imbibition Measurements

Intact seeds, de-coated seeds (testa removed by #240 sandpaper), nicked seeds, seeds pricked with a needle, and de-coated + nicked seeds (testa removed and then nicked by scalpel) were placed on two layers of moistened filter paper (Whatman No. 1) in 9 cm petri dishes with 4 replicates of 40 seeds for each treatment at 20°C. At 0, 1, 2, 4, 7, 24, 48, 72, 96, 120, 144, 168 and 192 h, each sample was lightly blotted with Hygenex paper towels to remove excess moisture, weighed, and returned to the petri dishes. The fresh weight of each sample was measured using a Mettler Toledo AG 204 balance. The percent water absorption was calculated for all samples based on the dry weight of the seeds (Welbaum and Bradford 1990). Intact seeds collected in different years or collected in 1995 but with different storage duration were also used in testing imbibition.

The diameters of intact seeds collected in different years were also measured in this experiment. 40 seeds were moistened on two layers of moist filter paper in a 9 cm petri dishes at 20°C and measured with a calibrated eyepiece micrometer under a light microscope at 0, 2, 24, 48, 96 and 192 h.

4.2.5 Allelopathic Effect on Lettuce Seeds

The possible presence of germination inhibitory substances in *Sandersonia* seeds was tested for by developing a germination inhibition assay using *Lactuca sativa* L. cv. 'Grand Rapids' seeds.

Extracts were prepared by soaking four replicates of 40 intact, de-coated, de-coated + rinsed (testa removed by #240 sandpaper and then rinsed in tap water for 1 min) or nicked seeds of *Sandersonia* in 5 cm petri dishes with two layers of moistened filter paper at 20°C for 1 week and then removing the seeds from the petri dishes. Four

replicates of 15 lettuce seeds were then placed in the petri dishes in which the *Sandersonia* seeds were soaked. The germination percentage of lettuce seeds and the radicle length of seedling was determined every 24 h up to 96 h.

Four replicates of 40 intact, de-coated, de-coated + nicked, or de-coated + leaching-nicked (nicking seeds after leaching for 1 week) seeds of *Sandersonia* were firstly leached for 1 week and then used for allelopathic tests as above.

The solution, in which the *Sandersonia* seeds were incubated for 5 months, of several treatments in germination experiment 1 was collected and then used for soaking four replicates of 15 lettuce seeds (3 ml of solution per petri dishes) for testing for possible allelopathic effects. The germination percentage of lettuce seeds and the radicle length of seedlings was determined at 72 h.

4.2.6 Ovule or Excised Embryo Growth in Vitro

The medium used in this experiment (supplied by Crop & Food Research Ltd. at the Levin Research Centre) comprised MS macronutrient salts (Murashige and Skoog 1962) at half strength, MS micronutrient salts and iron, LS vitamins (Linsmaier and Skoog 1965) and 30 g l^{-1} sucrose. The medium was solidified by the addition of 7.5 g l^{-1} agar. Medium pH was adjusted to 5.7 with either 0.1 NaOH or 0.1 N HCl prior to autoclaving at 121°C and 103 kPa for 15 min. Vessels used for culture were 9 cm disposable plastic petri dishes containing 25 ml of medium.

Seeds were allowed to imbibe water for 5 days at 20°C before being surface sterilized by dipping in 50 % ethanol 5 sec, sterilized water for 15 min, 50% bleach for 20 min, and finally 3 changes of sterile water. Embryos were then excised aseptically with a sterile scalpel and fine forceps by cutting the seed longitudinally and removing the embryo located in a groove in one half of the cut seed under a dissecting microscope. The embryos were placed on the culture medium and grown in a culture environment (Sanyo Growth Cabinet) of 25°C in a 16 h light/8 h dark cycle with a light intensity of 30 $\mu E\ m^{-2}\ s^{-1}$ and 70 % RH. After three days embryos were transferred to a new medium if any microbial contamination was present elsewhere in the plate.

Ovaries at 8, 14, 21, 28, 35 and 42 days after pollination were collected from plants grown in the glasshouse at the Department of Biology and Biotechnology and surface sterilized by dipping in 50 % bleach 20 min, 3 changes of sterile water. Ovules were then excised aseptically from the ovaries and placed on the culture medium for incubation in the same environment as that described above for embryo culture. Six petri dishes were used in each treatment with 5 ovules. Germination was defined as an increase in diameter of ovule by more than two times or production of a root.

4.2.7 Statistical Analysis

Experiments were of a replicated factorial design with randomized blocks (replicates). An Analysis of Variance (ANOVA) was conducted on percentage germination for the germination tests in experiment 2; and experiments 3 and 4 at each time interval. The data were transformed and arcsin values were used in performing the analysis, but the data were presented without transformation in tables and figures. Before transformation, zero values were increased by 0.05 %. The ANOVA was also used on water absorption rates for the imbibition measurements at each time interval. A Duncan's Multiple Range Test was used to test the significance of treatments in all experiments except the experiment of allelopathic effect on lettuce seeds (Little and Hills 1978). All treatments were considered statistically significant at $p<0.05$.

4.3 RESULTS

4.3.1 Seed Viability

100 seeds per seed lot (1995 and 1996 seed lots) were used to examine viability by the Tetrazolium test. The numbers of stained embryos and their topographical staining patterns are shown in Table 4.3. According to the germination percentage in germination experiments and the criteria for interpreting tetrazolium test results on wheat, Pensacoia, Bahiagrass, and Bermudagrass seeds (Association of Official Seed Analysts 1970), the topographical staining pattern of the *Sandersonia* embryo was

evaluated for each seed and six viability classes were determined (Table 4.3). The viability of 1993 and 1994 seed lots have not been tested due to the limited amount of seeds.

Table 4.3 The numbers of stained embryos and their topographical stain evaluation classes

	1995 lot	1996 lot	Description	Viability
1	23/100	7/100	Entire embryo stained bright red	Germinable
2	14/100	0/100	Extremities of scutellum or radicle unstained	Germinable
3	9/100	24/100	Entire embryo stained pink	Germinable
4	32/100	5/100	half of embryo unstained	Weak germinable
5	15/100	17/100	Embryo stained very light pink	Non-germinable
6	7/100	20/100	Embryo completely unstained	Non-germinable
7	0/100	27/100	Rotted seeds	Non-germinable

4.3.2 Seed Germination

4.3.2.1 Experiment 1: A Survey of Dormancy Breaking Treatments on *Sandersonia* Seeds

Absolutely no germination response was noted for 84 of 85 treatment tests at 150 days (Table 4.4). Only the treatment No. 85--seeds scarified first with sandpaper for 1 min then nicked near the radicle end showed increased germination from 0% to 10.6% by 30 days, at 20°C (Table 4.4). After 150 days, seeds in replicates 1, 2 and 3 were separately tested in a second set of three potentially dormancy breaking treatments. The treatments were: (1) retained at 20° C, (2) heat shock, (3) dehydration. There was no germination in any treatment (Table 4.5). The seeds (replicate four of some treatments) after incubation for 150 days, e.g., control, stratification for 2 or 4 weeks, leaching for 1 week, thiourea 1000 ppm for 2 h; 4000 ppm for 2 or 24 h, GA_3 100 or 300 ppm, and C_2H_4 1 or 1000 ppm, were scarified with #240 sandpaper and then cut with a scalpel near the radicle end. Table 4.6 shows there were 1-5 seeds germinated out of 40 in these treatments.

Table 4.4 The results of 85 seed treatments employed in Experiment 1

Treatment No.	Treatment description	Seed germinated / 40 [1]				Mean germination (%)
		1	2	3	4	
Control						
1	20°C	0	0	0	0	0
Temperature						
2, 3, 4, 5, 6, 7	5, 20, 30°C; or 5, 20, 30°C in a light impermeable black bag	0	0	0	0	0
Light						
8, 9, 10, 11	Red light 0 or 30 min, Far Red light 30 min, Blue light 30 min	0	0	0	0	0
Stratification						
12, 13, 14, 15	Wet seeds, 5°C, 2, 4, 8 or 12 weeks	0	0	0	0	0
16, 17, 18, 19	Wet seeds, 5°C, 2, 4, 8 or 12 weeks in a light impermeable black bag	0	0	0	0	0
Mechanical						
20, 21	Leaching in running tap water 2 days or 1 week	0	0	0	0	0
22	# 240 sandpaper 1 min	0	0	0	0	0
23	Nicking seed	0	0	0	0	0
Heat						
24, 25	Heat shock, 100°C/tap water 1/1 or 5/5 min	0	0	0	0	0
Chemicals						
26, 27	98% H_2SO_4 10 or 30 min + rinse 10 min	0	0	0	0	0
28, 29, 30, 31, 32	KNO_3 0, 10^{-4} or 10^{-2} M with light, or in a light impermeable black bag	0	0	0	0	0
33, 34	200 mM ethanol 2 or 24 hours	0	0	0	0	0
35, 36	100 mM butanol 2 or 24 hours	0	0	0	0	0
37, 38	10 mM $NaNO_2$ 2 or 24 hours	0	0	0	0	0
39, 40	0.5 mM NaN_3 2 or 24 hours	0	0	0	0	0
41, 42	10 mM NH_2OH-HCl 24 or 72 hours	0	0	0	0	0

Table 4.4 (Continued)

Treatment No	Treatment description	Seed germinated / 40[1]				Mean germination (%)
		1	2	3	4	
43, 44, 45, 46	1000 or 4000 ppm thiourea 2 or 24 hours	0	0	0	0	0
47, 48, 49, 50, 51, 52	10, 25 or 50 mM glutathione 2 or 24 hours	0	0	0	0	0
53, 54	10 mM salicylic acid 2 or 24 hours	0	0	0	0	0
55, 56	20 mM benzoic acid 2 or 24 hours	0	0	0	0	0
57, 58	20 mM propionic acid 2 or 24 hours	0	0	0	0	0
59, 60	28 mM isobutyric acid 28 mM 2 or 24 hours	0	0	0	0	0
61, 62	53 mM acetic acid 2 or 24 hours	0	0	0	0	0
63, 64	100 mM propanol 2 or 24 hours	0	0	0	0	0
65, 66	100 mM ethyl acetate 2 or 24 hours	0	0	0	0	0
67, 68	300 mM lactic acid 2 or 24 hours	0	0	0	0	0
69, 70	500 mM succinic acid 2 or 24 hours	0	0	0	0	0
71, 72	500 mM methanol 2 or 24 hours	0	0	0	0	0
Hormone						
73, 74, 75, 76	10, 100, 300 or 1000 ppm GA_3	0	0	0	0	0
77, 78, 79, 80, 81	0, 1, 10, 100 or 1000 ppm C_2H_4 24 hours	0	0	0	0	0
82, 83	50 or 100 ppm 6-BA	0	0	0	0	0
Combination						
84	5°C 8 weeks + 20°C 3 weeks + 5°C 8 weeks	0	0	0	0	0
85 [2]	# 240 sandpaper 1 min + nicking near the radicle end	2	9	4	2	10.6

[1] The result may represent the results of several treatments according to the treatment number.

[2] No. 85 Treatment was discovered by accident during imbibition measurements. It only took 30 days to get 10.6 % germination of *Sandersonia* seeds.

Table 4.5 Four secondary treatments employed during experiment 1 and their results (see details in text)

	Secondary treatment description	Results
Replicate one	Kept at 20°C	No germination out of 40 seeds in all treatments
Replicate two	Heat shock (5°C for 5 days, 25°C for 5 days, 5°C for 5 days again)	No germination out of 40 seeds in all treatments
Replicate three	Dehydration for 3 days	No germination out of 40 seeds in all treatments
Replicate four (some)	Scarified with #240 sandpaper + nicked near the radicle end	1-5 seeds germinated out of 40 seeds (see Table 4.6)

Table 4.6 The results of secondary treatments (scarified with #240 sandpaper + nicked near radicle end) of replicate four in experiment 1

No [1]	Treatment description [2]	Seed germinated / 40
1	20°C (control)	2
12	Wet seeds, 5°C for 2 weeks	2
13	Wet seeds, 5°C for 4 weeks	3
21	Leaching for 1 week	1
43	1000 ppm thiourea for 2 hours	5
45	4000 ppm thiourea for 2 hours	2
46	4000 ppm thiourea for 24 hours	4
74	100 ppm GA_3	2
75	300 ppm GA_3	5
78	1 ppm C_2H_4	2
81	1000 ppm C_2H_4	5

[1] The number of each treatment corresponds to the numbering system in Table 4.1 and Table 4.4.
[2] Treatment description corresponds to the treatment description in Table 4.1 and Table 4.4.

4.3.2.2 Experiment 2: Optimization of Germination of *Sandersonia* Seeds

Table 4.7 to 4.11 shows that seven of the 31 treatments produced encouraging germination responses. These were scarifying with sandpaper + nicking near the radicle end (16 %, Table 4.7), scarifying with sandpaper + nicking near the radicle end + 25 % bleach 20 min (21 %, Table 4.11) or 2.5 g/l thiram (16 %, Table 4.11), soaking 1 week + leaching 1 week + nicking near the radicle end (23 %, Table 4.8), scarifying with sandpaper + 300 ppm GA_3 (38 %, Table 4.10) and scarifying with sandpaper + nicking near the radicle end + 300 ppm GA_3 (69 %, Table 4.10). The two treatments, scarifying with sandpaper + 300 ppm GA_3, and scarifying with sandpaper + nicking near

Table 4.7 Effect of temperature and light on seed germination percentage, rate of germination and days taken for germination in *Sandersonia aurantiaca*

No[1]	Treatments	Seed germinated / 40				Mean germination (%)	Significance (P<0.05)	Days[2] for initiation of germination
		1	2	3	4			
1	Sandpaper+nick (control)	3	13	5	5	16 (61)[3]	ab[4]	10
2	Sandpaper+nick, 25°C, 16h light/8h dark	1	2	2	1	4 (64)	c	22
3	Sandpaper+nick, 30°C	1	1	0	0	1 (61)	c	22
4	Sandpaper+nick+in black bag	3	5	13	11	20 (60)	a	-
5	Sandpaper+nick+5°C 4 week+1.25 g/l thiram	3	2	2	2	6 (37)	c	10
6	Sandpaper+nick+5°C 8 week+25% bleach	3	0	1	5	6 (38)	c	17

[1] The number of each treatment corresponds to the numbering system in Table 4.2.

[2] Time taken in days for initiation of germination in any replicate. This was not determined for treatment 4.

[3] The figures in parenthesis denote the number of days for completing the germination experiments.

[4] Treatments with the same letter were not significantly different at P=0.05 according to Duncan's Multiple Range Test.

Table 4.8 Effect of mechanical treatments on seed germination percentage, rate of germination and days taken for germination in *Sandersonia aurantiaca*

No[1]	Treatments	Seed germinated / 40				Mean germination (%)	Significance (P<0.05)	Days[2] for initiation of germination
		1	2	3	4			
1	Sandpaper+nick (control)	3	13	5	5	16 (61)[3]	a[4]	10
7	#240 sandpaper 1 min	1	1	0	1	2 (61)	bcd	20
8	#180 sandpaper 2 min	1	3	3	2	6 (58)	bc	14
9	#120 sandpaper 4 min	1	3	3	1	5 (64)	bc	11
10	Soaking 4 days+needle	0	0	0	0	0 (59)	d	-
11	Sandpaper+nick (not near the radicle end)	1	0	1	2	3 (59)	bcd	24
12	Sandpaper+nick+leaching 1 week	2	0	1	1	3 (66)	bcd	17
13	Sandpaper+leaching 1 week+nick	1	0	0	2	2 (63)	cd	14
14	Leaching 1 week+soaking 1 week+nick	8	11	11	6	23 (63)	a	7
15	Whole seed + 85°C water bath 1 min	0	0	0	0	0 (49)	d	-
16	Sandpaper+85°C water bath 1 min+nick	3	1	1	6	7 (57)	b	10

[1] The number of each treatment corresponds to the numbering system in Table 4.2.

[2] Time taken in days for initiation of germination in any replicate.

[3] The figures in parenthesis denote the number of days for completing the germination experiments.

[4] Treatments with the same letter were not significantly different at P=0.05 according to Duncan's Multiple Range Test.

Table 4.9 Effect of chemicals on seed germination percentage, rate of germination and days taken for germination in *Sandersonia aurantiaca*

No[1]	Treatments	Seed germinated / 40				Mean germination (%)	Significance (P<0.05)	Days[2] for initiation of germination
		1	2	3	4			
1	Sandpaper+nick (control)	3	13	5	5	16 (61)[3]	a[4]	10
17	Sandpaper+nick+10^{-2} M KNO_3,25°C, 16h L/8h D	1	2	2	0	3 (57)	bc	16
18	Sandpaper+nick+soaking 24h+4000ppm thiourea 40h	1	0	1	2	3 (60)	bc	21
19	Sandpaper+nick+10 mM NH_2OH-HCl 72h	0	0	0	0	0 (53)	c	-
20	Sandpaper+nick+1 mM NaN_3 24 h	2	1	2	4	6 (63)	b	16

[1] The number of each treatment corresponds to the numbering system in Table 4.2.

[2] Time taken in days for initiation of germination in any replicate.

[3] The figures in parenthesis denote the number of days for completing the germination experiments.

[4] Treatments with the same letter were not significantly different at P=0.05 according to Duncan's Multiple Range Test.

Table 4.10 Effect of hormones on seed germination percentage, rate of germination and days taken for germination in *Sandersonia aurantiaca*

No[1]	Treatments	Seed germinated / 40				Mean germination (%)	Significance (P<0.05)	Days[2] for initiation of germination
		1	2	3	4			
1	Sandpaper+nick (control)	3	13	5	5	16 (61)[3]	c[4]	10
21	Whole seed + 300 ppm GA_3	0	0	0	0	0 (60)	d	-
22	Nick+300 ppm GA_3	3	1	8	5	11 (33)	c	12
23	Sandpaper+300 ppm GA_3	12	16	16	17	38 (35)	b	8
24	Sandpaper+nick+300 ppm GA_3	28	29	23	30	69 (31)	a	7
25	Sandpaper+nick+50 ppm 6-BA	1	0	1	2	3 (48)	d	30
26	Sandpaper+nick+300 ppm GA_3+50 ppm 6-BA	5	3	2	5	9 (48)	c	9

[1] The number of each treatment corresponds to the numbering system in Table 4.2.

[2] Time taken in days for initiation of germination in any replicate.

[3] The figures in parenthesis denote the number of days for completing the germination experiments.

[4] Treatments with the same letter were not significantly different at P=0.05 according to Duncan's Multiple Range Test.

Table 4.11 Effect of thiram and bleach on seed germination percentage, rate of germination and days taken for germination in *Sandersonia aurantiaca*

No[1]	Treatments	Seed germinated / 40				Mean germination (%)	Significance (P<0.05)	Days[2] for initiation of germination
		1	2	3	4			
1	Sandpaper+nick (control)	3	13	5	5	16 (61)[3]	a[4]	10
27	Whole seeds	0	0	0	0	0 (61)	b	-
28	Whole seeds+50% bleach 20 min	0	0	0	0	0 (59)	b	-
29	Whole seeds+2.5 g/l thiram	0	0	0	0	0 (60)	b	-
30	Sandpaper+nick+25% bleach 20 min	10	8	11	5	21 (62)	a	9
31	Sandpaper+nick+2.5 g/l thiram	10	3	6	7	16 (59)	a	11

[1] The number of each treatment corresponds to the numbering system in Table 4.2.

[2] Time taken in days for initiation of germination in any replicate.

[3] The figures in parenthesis denote the number of days for completing the germination experiments.

[4] Treatments with the same letter were not significantly different at P=0.05 according to Duncan's Multiple Range Test.

the radicle end +300 ppm GA_3, were significantly better than the remaining treatments (Table 4.10). However, the most satisfactory germination response was the latter, scarifying seeds with sandpaper and then nicking near the radicle end and soaking in 300 ppm GA_3 solution until seed germination. The mean germination of this treatment was 68.8 % and significantly higher than the control (16%) in experiment 2 and that (0%) in experiment 1 ($P<0.05$).

Plate 4.1 The effect of fungicide--thiram and surface sterilization on germination of *Sandersonia* seeds. (A) surface sterilization by 25 % bleach for 20 min, (B) 2.5 g/l thiram in germination medium.

Intact seeds without scarifying or nicking showed no response to any treatments (Tables 4.4, 4.8, 4.10 and 4.11), even when supplied GA_3 in the incubation solution (Table 4.10). There was no significant difference in the germination of scarified and nicked seeds exposed to light or in darkness (Table 4.7). Both high temperature (25°C or 30°C) and low temperature (chilling) decreased germination percentage from 16% to 1-6% ($P<0.05$) (Table 4.7). The seeds scarified with sandpaper or pricked with a needle had lower germination percentages (0-6%) than the seeds with both scarifying and nicking treatments (16 %, Table 4.8). The nick position in seed was important, and there

was significant difference ($P<0.05$) in germination percentage between seeds nicked near the radicle end or not (Table 4.8). Leaching seeds after scarifying and/or nicking also reduced germination percentage (Table 4.8), but leaching intact seeds did not affect final germination of nicked seeds (23%) compared with control (16%, Table 4.8). Chemical and heat treatments did not promote germination of *Sandersonia* seeds and decreased the germination percentage from 16% to 0-7 % (Tables 4.8 and 4.9). The response of *Sandersonia* seeds to 6-BA was negative (Table 4.10), even when applied in combination with GA_3 (Table 4.10).

Neither the thiram nor the 25% bleach affected germination percentage of *Sandersonia* seed (Table 4.11). However thiram inhibited radicle elongation in both of *Sandersonia* and lettuce seeds (Plate 4.1 and Fig. 4.10).

4.3.2.3 Experiment 3: Effect of Gibberellic Acid on *Sandersonia* Seed Germination

The effect of concentration of gibberellic acid (GA_3) on germination of *Sandersonia aurantiaca* seeds is shown in Fig. 4.2. GA_3 treatment had a positive effect on dormancy breakage of *Sandersonia* seeds when the seeds were mechanically scarified (Table 4.10, Figs. 4.2 and 4.3), but intact seeds showed no response to GA_3 treatment (Table 4.10). The optimum effect was obtained with a concentration of between 100 and 500 ppm GA_3. There was no significant difference between 100 and 500 ppm from 11 days after the treatment began, whatever the concentrations used (Fig. 4.2). At lower and greater concentrations, GA_3 exhibited a significantly lower effect on dormancy breakage (Fig. 4.2).

In another set of experiments, the effect of contact time of gibberellic acid (GA_3) on germination of *Sandersonia aurantiaca* seeds was studied (Fig. 4.3). Seeds were treated for 1, 2, 4, 7 and 14^+ days with 300 ppm GA_3 solutions, then transferred on to filter paper moistened with distilled water. The germination percentage of seeds increased with extension of contact time. No significant difference between the treatments was found before 11 days, whatever the soaking duration used (Fig. 4.3). An increase on seed germination was observed as early as 14 days after the treatment began. The increase was also seen of 21 and 28 days. There was no real difference on

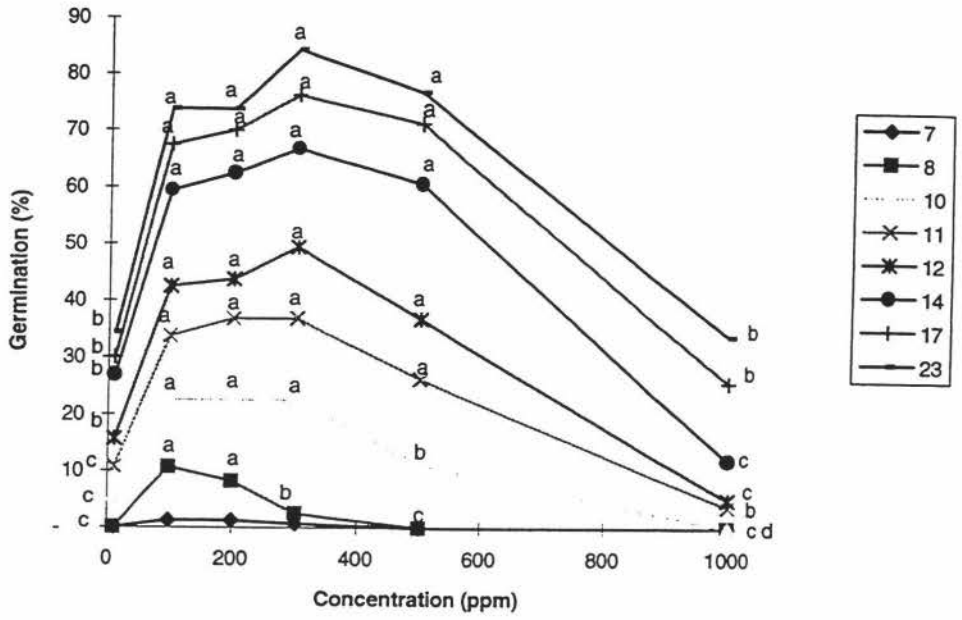

Figure 4.2 Effect of exogenous GA_3 concentration on germination percentages measured 7 days, 8 days, 10 days, 11 days, 12 days, 14 days, 17 days and 23 days after *Sandersonia* seeds were treated. Germination of treatments with the same letter was not significantly different (P = 0.05) within each curve according to Duncan's multiple range test.

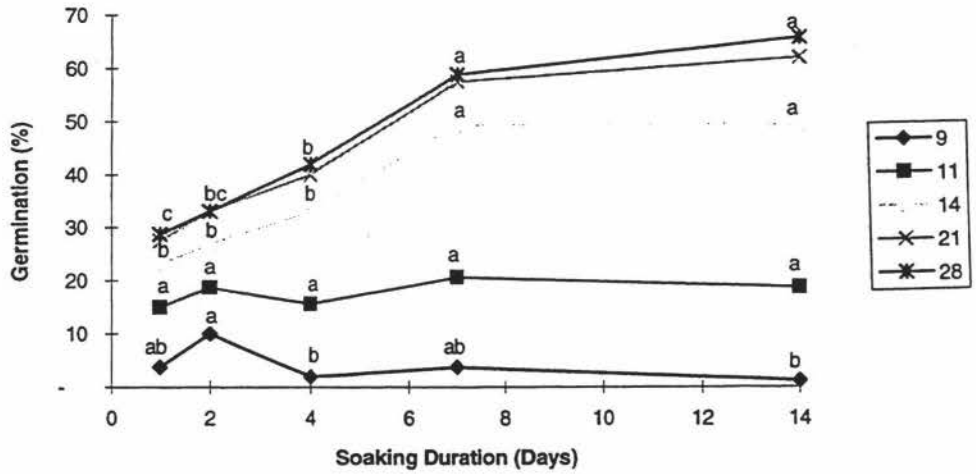

Figure 4.3 Effect on *Sandersonia* seed germination percentages of different contact time of 300 ppm GA_3. Results were measured at 9 days, 11 days, 14 days, 21 days and 28 days. Germination of treatments with the same letter was not significantly different (P = 0.05) within each curve according to Duncan's multiple range test.

germination percentage by contact of the *Sandersonia* seeds with GA_3 solution longer than 7 days (Fig. 4.3).

4.3.2.4 Experiment 4: Germination Optimization Treatments Applied to Other Seed Lots of *Sandersonia*

Information on seed characteristics of *Sandersonia aurantiaca* seeds from different seed lots is shown in Table 4.12. There was a significant difference between the different seed lots in fresh weight of 100 seeds, embryo size and germination test. The tetrazolium test indicated that the seeds in 1996 had a higher percentage of live seeds (36 %) than the number of seeds able to be germinated (19%). For the 1995 seed lot, tetrazolium results were similar to germination results.

Table 4.12 Fresh weight, dry weight per 100 seeds, viability, embryo size, and percentage germination of different lots of *Sandersonia* seeds. All treatments performed in nominal darkness at 20°C with 4 replicates (×2 times), 40 seeds per petri dishes.

	Fresh weight of 100 seeds (g)	Dry weight of 100 seeds (g)[1]	Viability (%)	Embryo size (mm) Length	width	Germination Test (%) Control	Treatment[2]
1993	0.6068 a[3]	0.5479	ND[4]	ND	ND	0	34 b
1994	0.5044 c	0.4545	ND	ND	ND	0	32 b
1995	0.5617 b	0.5079	78	0.80 a	0.25 a	0	75 a
1996	0.4409 d	0.3976	36	0.67 b	0.22 b	0	19 c

[1] Dry weight of 100 seeds = fresh weight of 100 seeds × percentage of dry matter.
[2] Seeds firstly were scarified with sandpaper and nicked by scalpel, and then incubated in 300 ppm GA_3 solution.
[3] Treatments with the same letter were not significantly different at P=0.05 according to Duncan's Multiple Range Test.
[4] ND = not done due to the limited amount of seeds.

4.3.3 Imbibition Measurements

The water content (WC) of intact seeds increased gradually (phase I) and reached a plateau (phase II) at 80% after approximately 144 h (Fig. 4.4). Seed WC then remained constant. Imbibition of de-coated seeds (testa removed by # 240 sandpaper before imbibition) lagged behind that of intact seeds, and the WC reached a plateau level at 50% after approximately 96 h (Fig 4.4). The imbibition pattern of nicked seeds showed an initial rapid water uptake followed by a relatively constant WC during phase II (Fig 4.4).

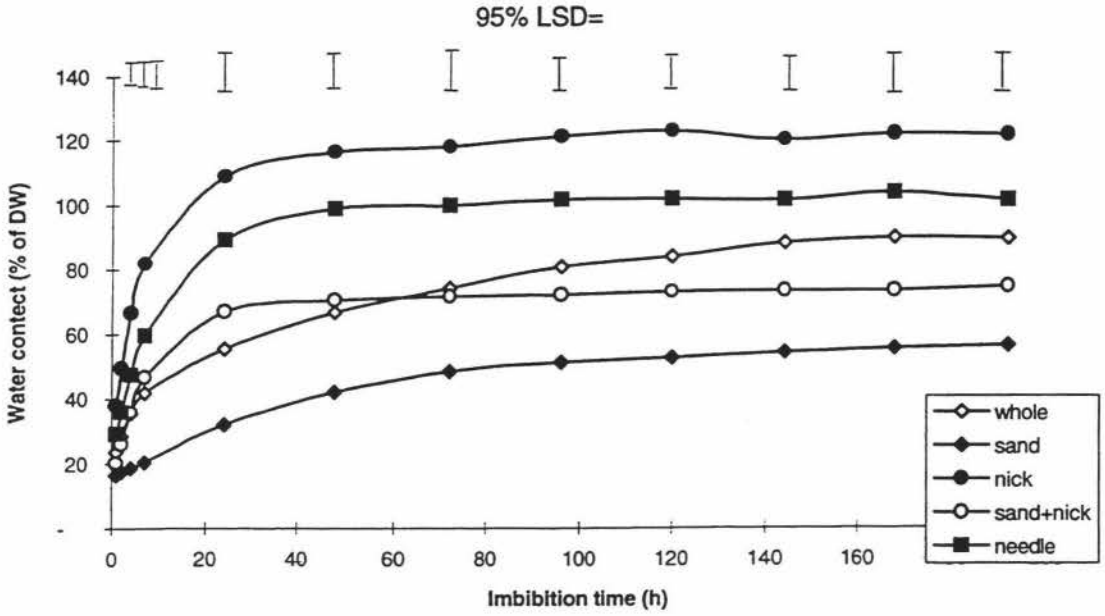

Figure 4.4 Water uptake patterns for intact seeds, de-coated seeds, nicked seeds, seeds pricked with a needle, and de-coated + nicked seeds during 192 hours of imbibition.

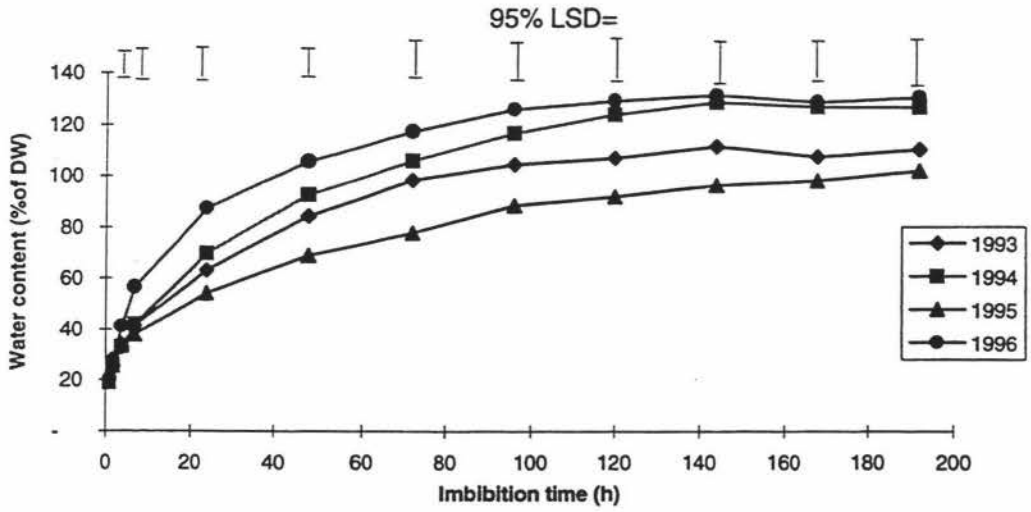

Figure 4.5 Water uptake patterns for seeds collected in different years during 192 hours of imbibition.

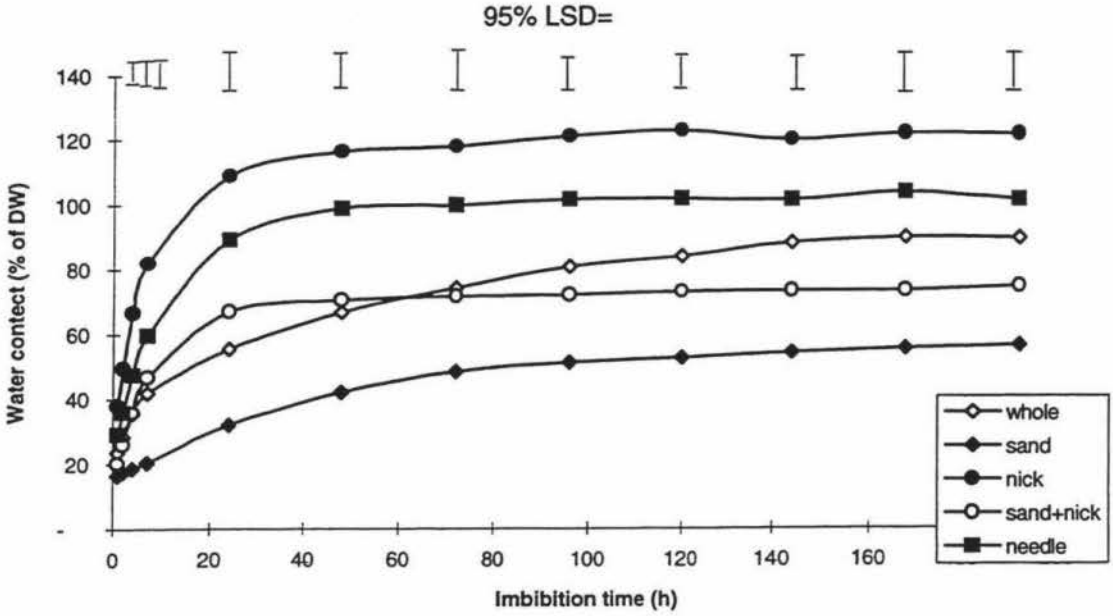

Figure 4.4 Water uptake patterns for intact seeds, de-coated seeds, nicked seeds, seeds pricked with a needle, and de-coated + nicked seeds during 192 hours of imbibition.

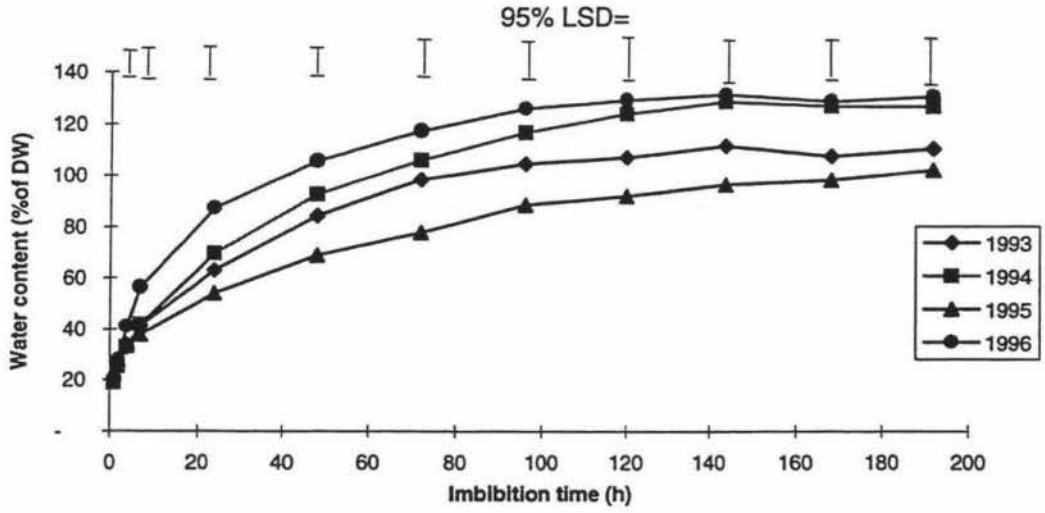

Figure 4.5 Water uptake patterns for seeds collected in different years during 192 hours of imbibition.

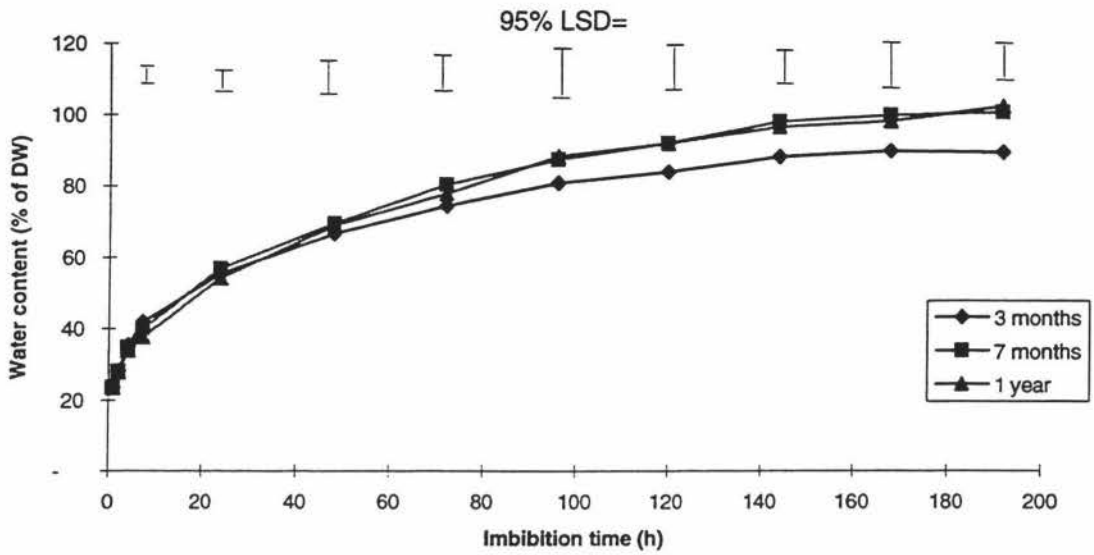

Figure 4.6 Water uptake patterns for seeds collected in 1995, but after different storage duration, during 192 hours of imbibition.

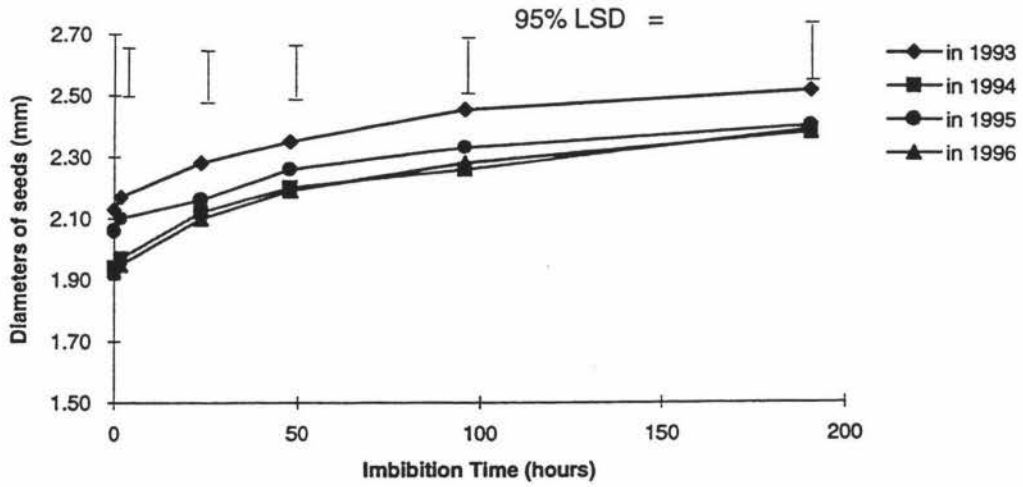

Figure 4.7 Differences in mean diameters of soaking seeds collected in different years.

The WC values of nicked seeds were higher(by 40%, $P<0.001$) than for intact seeds, and the plateau WC of 120% was attained within 48 h (Fig. 4.4). The seeds pricked with a needle had a similar imbibition pattern to nicked seeds, but the WC values were lower (by more than 10%, $P<0.001$) than that of nicked seeds (Fig. 4.4). De-coated + nicked seeds (testa removed and then nicked by scalpel before imbibition) also displayed a rapid increase in phase I water uptake (24 h) and a relatively constant phase II. The values of final WC were higher (by 20%, $P<0.001$) than for the de-coated seeds, but lower (by 30%, $P<0.001$ or by 50%, $P<0.001$) than for the pricked seeds or the nicked seeds (Fig. 4.4).

The imbibition patterns of intact seeds collected in different years or collected in 1995 but subjected to different storage duration are shown in Fig. 4.5 and Fig. 4.6, respectively. The former shows that the seeds collected in different years had different WC values, when they were imbibed. The seeds collected in 1996 had the highest values and the seeds from 1995 had the lowest ones (Fig. 4.5). Figure 4.6 shows that imbibition of seeds was increased by increasing the storage duration during the first several months of storage. The longer the storage time, the higher the water uptake. However, there was no real difference on the water uptake between seeds stored for 7 months and for one year.

The average diameters (during imbibition) of seeds collected in different years differed depending on the year and the weight of 100 seeds (Fig. 4.7). For example, the diameters of seeds from 1993 was changed from 2.13 (the mean diameter of 40 seeds) mm before imbibition to 2.52 mm after imbibition, but for the 1996 seeds, only from 1.93 mm to 2.38 mm.

4.3.4 Allelopathic Effect on Lettuce Seeds

Solutions, in which the different treatments of *Sandersonia* seeds (40 / petri dishes) were soaked for 1 week; or leached and soaked each for 1 week, showed an inhibitory effect on germination rate and radicle elongation of lettuce seeds, but did not affect the final germination percentage (Fig. 4.8 and Fig. 4.9). The inhibitory effect on germination of lettuce seeds was higher with solution from soaked only than that from leached plus

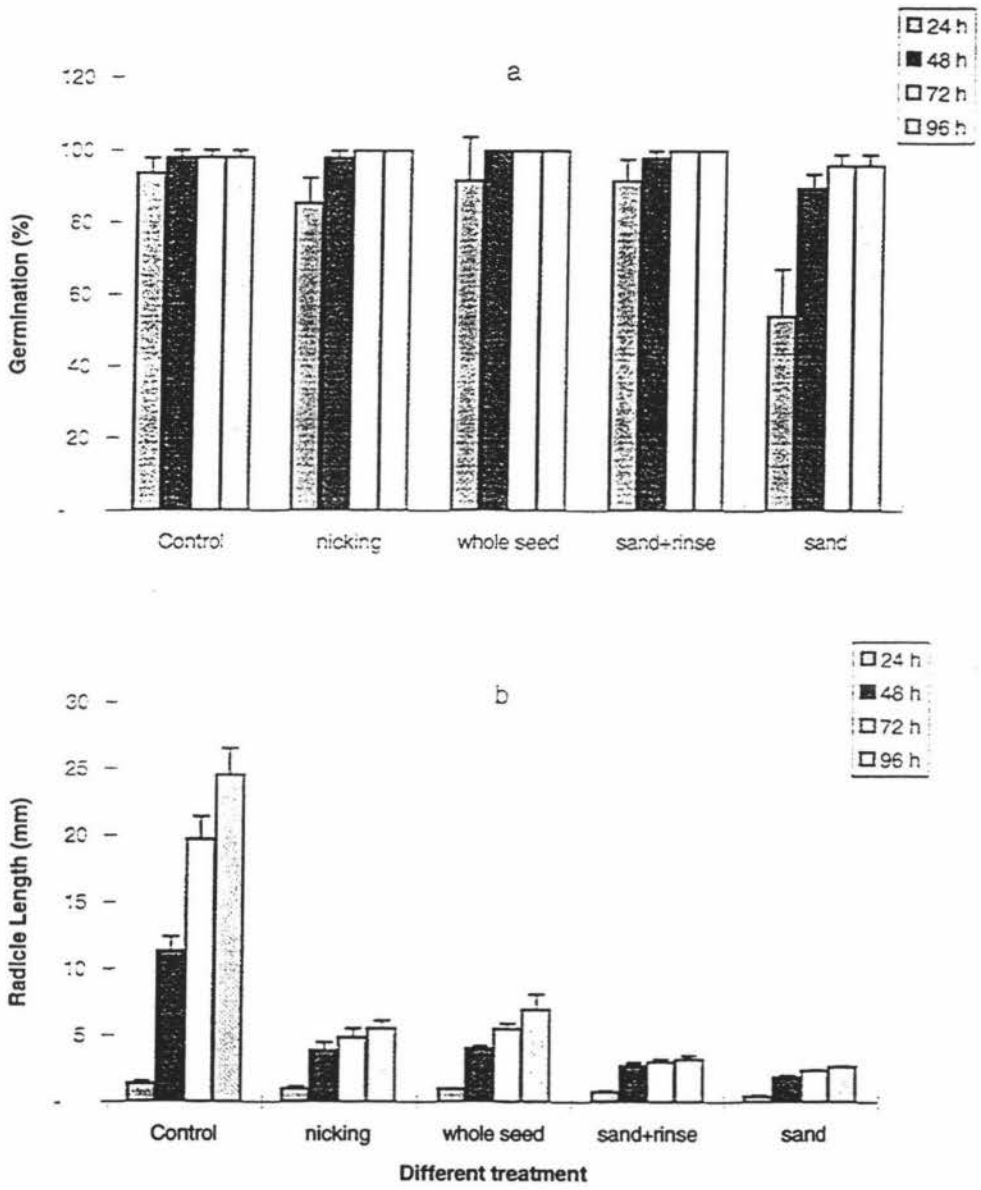

Figure 4.8 Effect on (a) lettuce seed germination and (b) radicle length of the solutions, in which the different treatment of *Sandersonia* seeds were soaked for 1 week. Results were measured after 24 h, 48 h, 72 h and 96 h. The treatments were: (1) control, (2) nicked seeds, (3) intact seeds, (4) de-coated seeds + rinse, and (5) de-coated seeds. The values are the mean of four replicates (15 seeds each) ± mean standard error.

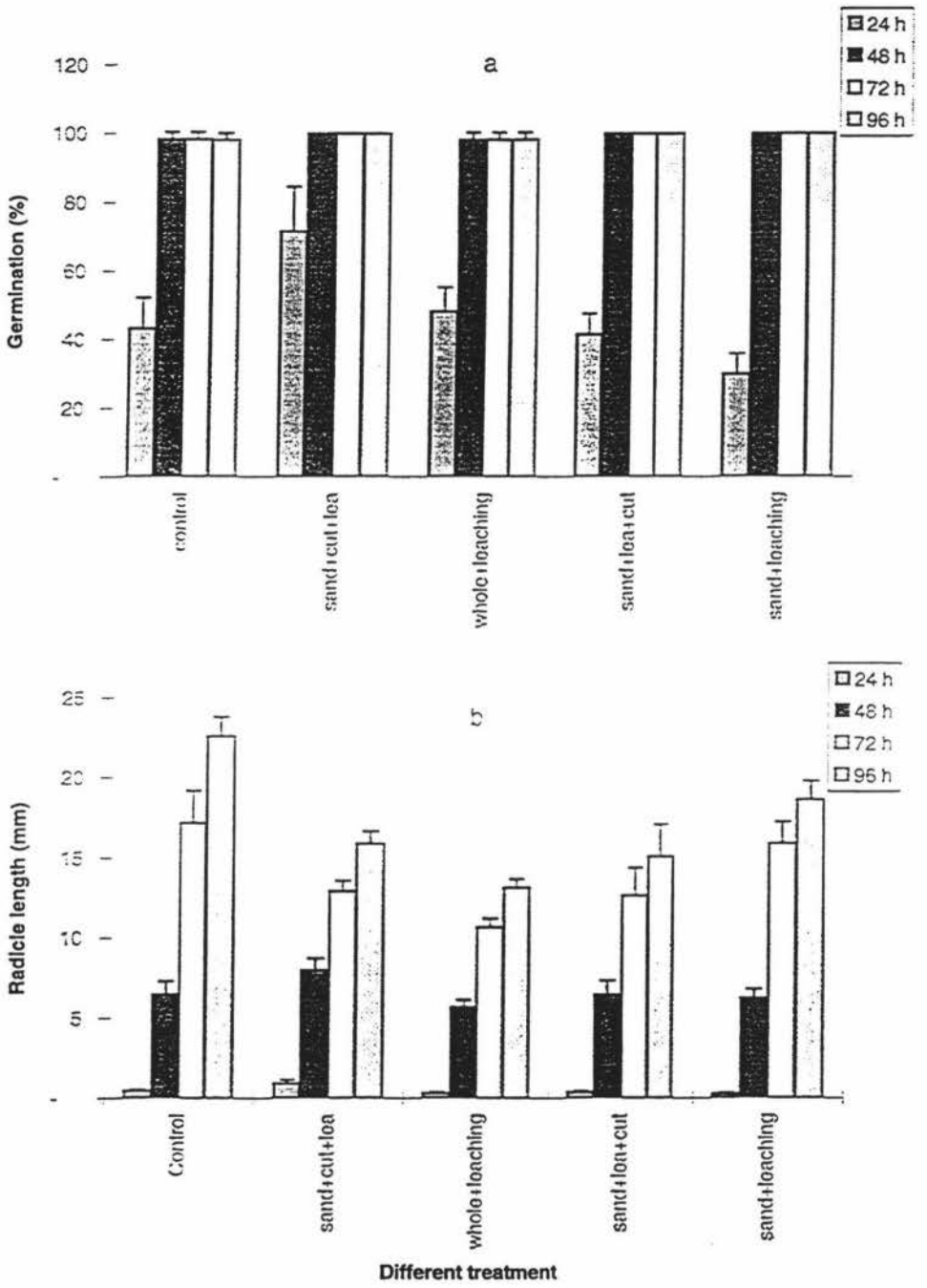

Figure 4.9 Effect on (a) lettuce seed germination and (b) radicle length of the solutions, in which the different treatment of leaching (1 week) *Sandersonia* seeds were soaked for 1 week. Results were measured after 24 h, 48 h, 72 h and 96 h. The treatments were: (1) control, (2) de-coated + nicked, (3) intact seeds, (4) de-coated + nicked (nicking the seeds after leaching for 1 week), and (5) de-coated. The values are the mean of four replicates (15 seeds each) ± mean standard error.

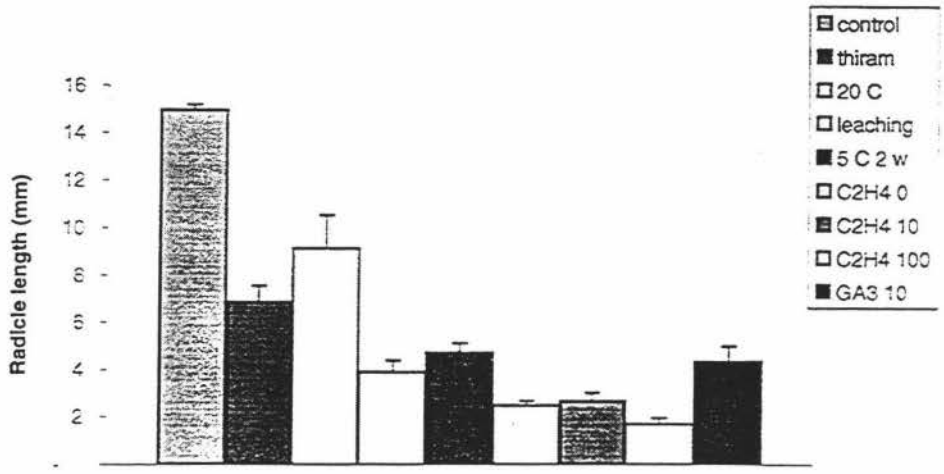

Figure 4.10 Effect on radicle length of lettuce seeds of the nine solutions, in which different treatment of *Sandersonia* seeds were soaked for 5 months, respectively. Results were measured 72 hours after incubation began. The values are the mean of four replicates (15 seeds each) ± mean standard error.

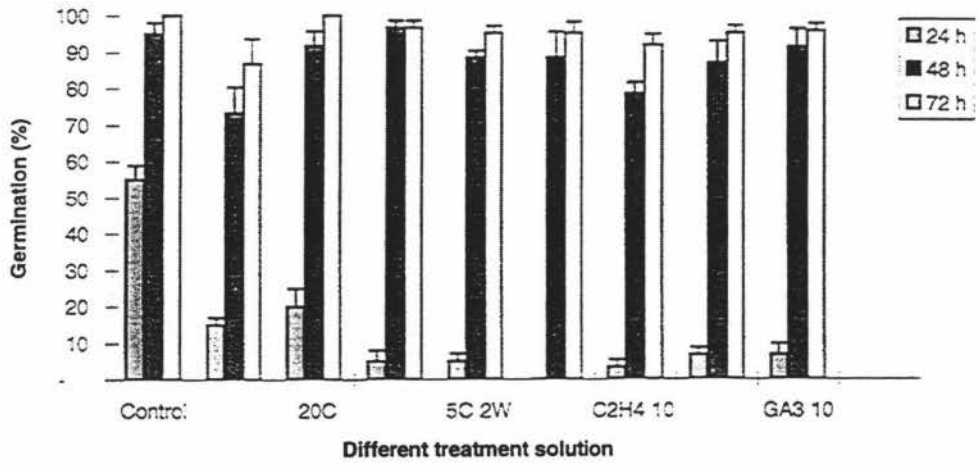

Figure 4.11 Effect on germination percentage of lettuce seeds of the nine solutions, in which different treatment of *Sandersonia* seeds were soaked for 5 months, respectively. Results were measured 24 h, 48 h, and 72 h after incubation began. The values are the mean of four replicates (15 seeds each) ± mean standard error.

soaked *Sandersonia* seeds.

The same inhibitory effect on lettuce seed germination was also obtained with the solution in which *Sandersonia* seeds were incubated for 150 days (Fig. 4.10 and Fig. 4.11).

4.3.5 Ovule or Excised Embryo Growth *in Vitro*

Dissection of the seed revealed that the embryo was linear with a cotyledon, a small embryonic axis, shoot apex and a prominent radicle, and measured approximately 0.80 mm (the mean length of 40 embryos) in length and 0.25 mm in width (Plate 3.22 I). Of 33 embryos excised and cultured only 9 embryos (27%) grew after incubation at 25°C, 16 h light / 8 h dark on a solid agar medium. The embryos enlarged by about three to four times to about 1.18-2.94 mm in length and 0.82-1.92 mm in width, and then stopped growth.

The results of ovule development and germination *in vitro* are shown in Table 4.13 and Fig. 4.12. Ovules harvested before 14 days after pollination could germinate, while only a few germinated beyond 14 days after pollination.

Table 4.13 The final results of ovule culture for embryo rescue

Ovule age (DAP)[1]	No. of ovule enlarged or rooting/5						Mean of germination (%)	Days[2] for initiation of germination	Morphology
	1	2	3	4	5	6			
8	3	2	2	1	2	4	47 (98)[3]	42	Callus, roots, 1 ovule produced a shoot (dead later)
14	0	0	1	1	1	1	13 (92)	43	Callus, roots
21	0	0	0	0	0	0	0 (137)	-	-
28	1	1	0	0	0	0	7 (131)	70	Root, callus
35	0	0	0	1	0	0	3 (111)	65	Root only
42	0	0	0	0	0	0	0 (107)	-	-

[1] DAP=Days after pollination.
[2] Time taken in days for initiation of germination in any replicate.
[3] The figures in parenthesis denote the number of days for completing incubation.

All ovules enlarged in the first week of incubation except the ovules harvested at 28 days after pollination (Figs. 4.12a and 4.12b). From the second week, the size of

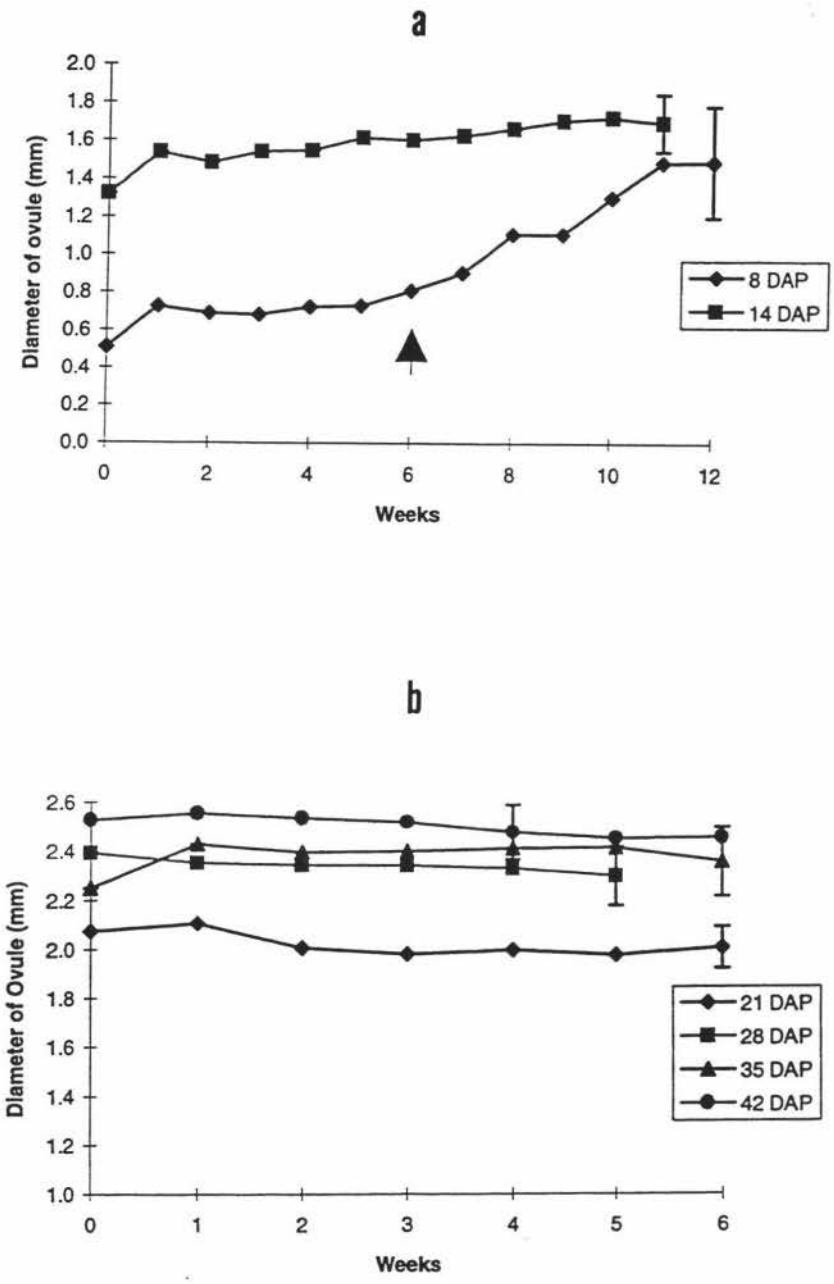

Figure 4.12 Growth rate of *Sandersonia* ovules *in vitro*. (a) the ovules were collected at younger stages (8 and 14 DAP), (b) collected at older stages (21-42 DAP). The ovules were placed on half strength MS medium (see section 4.2.6) and incubated in 16 h light /d at 25° C. Arrow marks the time of initiation of ovule germination in any replicate. Vertical bars represent 95 % LSD.

older ovules (21 DAP to 42 DAP) slightly decreased with increase of incubation duration (Fig. 4.12b). However, the difference was not significant ($P>0.05$). After incubation for 5 weeks, some ovules cultured at the younger stage (8 DAP) increased rapidly in size ($P<0.05$), and produced a root and/or callus (Fig. 4.12a). No real extension ($P<0.05$) of older ovules occurred whether a root was produced or not (Fig. 4.12b).

The results, which are presented in Table 4.13, also show that the ovules of *Sandersonia* grow very slowly *in vitro*. No seedlings were produced from ovules in this experiment.

4.4 DISCUSSION

Zero percentage germination of *Sandersonia* seeds in optimal temperatures (see control in Table 4.4) leads to the question whether such a poor germination rate is caused by seed dormancy or is the result of poor seed quality. In order to answer this question *Sandersonia* seeds were subjected to 85 treatments (see Table 4.1) that had been found to be promotory for seed germination in a wide range of other species.

The results, which are presented in Table 4.4, show that intact seeds of *Sandersonia* do not have any response to any treatments which are used in Table 4.1. Only the treatment in which seeds were scarified first with sandpaper for 1 min and then nicked near the radicle end showed increased germination from 0 to 10.6%. The nicked seeds took up more water (approx. 40%) than intact seeds. The nick position in seed was also important (Table 4.8), the seeds not nicked near the embryos only showed 3% germination which was significantly lower than that of the seeds nicked near the embryos. GA_3 treatment had a positive effect on improvement of germination of *Sandersonia* seeds treated with scarification (Table 4.10, Fig. 4.2 and Fig. 4.3). The excised embryos from the mature seeds could not grow and germinate very well when they were aseptically cultured *in vitro* (see section 4.3.5). Thus, our initial conclusion was that poor germination of *Sandersonia* seeds could be induced either by the structures surrounding the embryo, which influenced seed permeability to water, oxygen or mechanically restricted embryo growth, or by the embryo itself.

4.4.1 Seed Viability and Germination

4.4.1.1 Seed Viability

The tetrazolium test is a biochemical test for seed viability which is often used for a quick estimate of the viability of seed samples in general and those showing dormancy in particular (ISTA 1985). In this study, the tetrazolium test was used to examine the viability of *Sandersonia* seeds collected in 1995 and 1996 (newly harvested). Six staining patterns were observed with the tetrazolium test (see Table 4.3) and 78% and 36 % viability were determined in 1995 and 1996 seed lots, respectively. The tetrazolium test indicated that the seeds in 1996 had a higher percentage of live seeds (36%) than the number of seeds able to be germinated (19%). For the 1995 seed lot, tetrazolium results were similar to germination results. These suggest that the freshly harvested seeds exhibit a deeper dormancy than the after-ripened seeds and can partly lose their dormancy by the process of after-ripening. The results also indicated that the seeds collected in 1995 had higher quality than the seeds in 1996 did, because the former showed higher viability and no seeds rotted during testing.

The testing of *Sandersonia* seed viability using tetrazolium was not very satisfactory because the small and soft embryo was easily broken, and even lost from hard endosperm tissue when the seed was cut.

4.4.1.2 Optimization of Germination of *Sandersonia* seed

In the first part of this study, we selected 85 treatments, but the intact seeds of *Sandersonia* show no response to any treatment. This result implied that *Sandersonia* seeds had seed coat-imposed dormancy rather than embryo dormancy, and the hardness seed coat influenced the ability of seeds to absorb water and oxygen, perhaps even hindering a seed response to treatment agents e.g. by GA_3 penetration (Hsiao 1979, Keogh and Bannister 1992).

The treatment in which the seeds were scarified plus nicked partially removed dormancy of *Sandersonia* seed and increased the germination percentage from 0 to 10.6% (Table 4.4). The optimum temperature for germination appeared to be close to

20°C which was consistent with optimum temperature for tuber pre-sprouting for this species (20-23°C, Clark 1994). Higher temperatures (25-30°C) decreased germination (Table 4.7), and such an inhibitory effect might be related to a low oxygen solubility in water and a higher oxidation of phenolic compounds at higher temperature as has been suggested for *Cistus albidus* L. and C. *lanrifolius* L. seeds (Corral *et al.* 1990). The competition for oxygen could limit the provision of oxygen to the embryo.

The two treatments designed to elucidate chemical dormancy: leaching (to remove germination inhibitors) and stratification (to change the levels of germination promoters) (Paynter and Dixon 1991) did not increase germination percentage. Leaching decreased the potential for seeds to germinate when the seeds were scarified before leaching (Table 4.8). This result was similar to the observation of leaching depressing the germination percentage of nicked seeds of Yellow Bells (Paynter and Dixon 1991). Stratification did not stimulate germination, and even significantly decreased germination percentage compared with the control in experiment 2 (Table 4.7).

There was no significant difference between scarified plus nicked seeds germinated in darkness and in nominal darkness (control) at 20°C (Table 4.7). But the final germination was reduced by incubating them at 25°C with 16 h light/d (Table 4.7). According to these results, *Sandersonia* seed probably are not light-requiring seeds. Light may even inhibit their germination. Embryo dormancy involving light was present in some species although this was generally overcome after treatment with GA_3 (Bunker 1994). Karssen and Lacka (1986) proposed that environmental conditions such as light may affect germination by changing the sensitivity of seeds to GA. The seeds of some species, such as *Meconopsis* Vig., are light sensitive. GA_3 alone cannot break dormancy and the interaction of light and GA_3 results in breaking of dormancy (Sulaiman 1993). However, it appears that the *Sandersonia* seeds were sensitive to exogenous GA_3 even without exposure to light and the light was not required for the development of a sensitivity to GAs. Environmental factors such as light did not play a role in the response of seed to GA_3 was found by Derkx and Karssen (1993) in *Arabidopsis thaliana* and inhibited seed germination in *Arum maculatum* (Sowter 1949, Pritchard *et al.* 1993).

Seed germination for *Sandersonia* declined from 75 % to 32 or 34 % as age of the seed increased from 1 years old to 2 or 3 years old, respectively. This may indicate that *Sandersonia* seeds lose their germination capacity with increasing storage duration; or the difference of germination percentage of different seed lots was caused by different responses to the treatment or to different GA_3 concentration. That the newly harvested seeds (1996 lot) show lower germination percentage and lower seed viability than those of old seed lots may be due to variation in seed quality. The 1996 seeds have small seed weight, size and small embryos (Table 4.12).

Both sodium hypochlorite and thiram which protect seeds from fungal contamination (Paynter and Dixon 1991, Persson 1993, Cousens *et al.* 1994, Bunker 1994, Plummer *et al.* 1995) at low concentration were used in germination tests here. The results indicate that the former did not affect seed germination both in germination percentage and radicle elongation, but the latter obviously inhibited radicle elongation both in *Sandersonia* and lettuce seeds and may produce abnormal seedlings (Plate 4.1 and Fig. 4.10). Thiram, however, did not significantly affect germination *per se* and thus is considered to be a useful and non-interfering component of the germination assay system.

4.4.1.3 The Role of Chemicals

In order to overcome dormancy and improve germination of *Sandersonia* seeds, 19 chemicals agents (see Table 4.1) at appropriate concentrations were used to treat the intact seeds and 4 (see Table 4.2) used to treat the scarified plus nicked seeds in this study. Intact seeds showed no response to any chemicals and the presence of chemical agents for scarified plus nicked seeds decreased their germination percentage ($P<0.05$) compared with the control (Table 4.9).

Some nitrogenous compounds, such as nitrate, nitrite, and thiourea (Esashi *et al.* 1979a, Cohn and Hughes 1986, Suparna *et al.* 1993, Giba *et al.* 1994), and nitrogenous respiration inhibitors, such as hydroxylamine, azide and cyanide (Esashi *et al.* 1979a, Cohn and Hughes 1986, Naidu and Amritphale 1994) are known to stimulate the germination of many plant seeds. The mode of action of these compounds in

germination is not well known. Some hypotheses about the action mechanisms of these compounds have been suggested by several authors. These included the stimulation of the pentose phosphate pathway which was required for the initial step of germination process (Roberts 1973, Roberts and Smith 1977), the involvement of alternative respiration in the control seed dormancy (Yu *et al.* 1979, Esashi *et al.* 1979b), and the induction of membrane potential change (Hilhorst and Karssen 1989) or promotion of the rate of leaching of water-soluble inhibitors (Sukhvibul and Considine 1994, Suparna *et al.* 1993).

An appropriate concentration of chemicals is an important factor for inducing seed germination. For example, 3, 4 and 10 mM azide completely inhibited germination in the seeds of *Avena fatua* L. (ND lines) (Upadhyaya *et al.* 1982). Under these conditions, respiration apparently is inhibited and seeds cannot germinate even if dormancy has been broken. In addition to chemical concentration and seed exposure time, the dormancy-breaking response to chemicals has been found to be highly dependent upon incubation medium pH values (Cohn and Hughes 1986, Cohn 1989). In the current study, the finding that the chemicals (KNO_3, NaN_3, NH_2OH-HCl and thiourea) did not affect seed germination of *Sandersonia*, and even inhibited their germination might be due to unsuitable concentration and pH value in the medium.

It is known that some nitrogenous compounds and nitrogenous respiration inhibitors interact with other factors, such as temperature (Henson 1970, Karssen and Vries 1983), light (Hilton 1985, Hilhorst and Karssen 1988, Singh and Amritphale 1992) and hormones (Upadhyaya *et al.* 1982, Saini *et al.* 1985). Azide failed to break dormancy in *Avena fatua* Montana 73, and a combination of 0.8 or 1 mM with 0.14 or 0.28 mM GA_3 gave a strong synergistic interaction in inducing germination (Upadhyaya *et al.* 1982). Upadhyaya *et al.* (1982) indicated that the dormant state of *Avena fatua* seeds had been attributed to their inability to synthesize gibberellins, and stimulation of germination by azide depending on gibberellin biosynthesis. Some similar examples are also reported by some authors, such as the germination of *Chenopodium album* L. seeds which are synergistically promoted by a combination of ethylene or gibberellin and nitrate (Saini *et al.* 1985), and the germination of apple embryos stimulated by applying cyanide in light or in the presence of gibberellin (Dziewanowska and Lewak 1982). According to

results reported here for *Sandersonia* seeds showing a lack of response to the chemicals KNO_3, NaN_3, NH_2OH-HCl and thiourea, the suggestion is made that these chemical agents inhibit the ability of the embryo to synthesize gibberellin.

4.4.1.4 The Role of Plant Growth Regulators

Dormancy was partially removed when seeds were scarified plus nicked at 20°C ($P<0.05$, Table 4.4 and Table 4.7). Under this condition, dormancy was completely removed by application of certain plant growth regulators (Table 4.10). Of the two growth regulators (in experiment 2) used, gibberellic acid (GA_3) was most effective (Table 4.10), the optimum concentration was 100-500 ppm (Fig. 4.2) and treatment for one week at the beginning of imbibition was sufficient to stimulate maximal germination (Fig. 4.3). 6-Benzyladenine did not have any effect at the given concentrations when applied singly or combined with GA_3 (Table 4.10), and even decreased the germination percentage and offset the positive response to application of gibberellic acid (GA_3) (Table 4.10). In general, combined growth regulators do not promote germination more than when applied singly (Samimy 1994).

In some species where gibberellin is poorly effective on the intact seed, an effect can be induced by various treatments of the seed coat such as brief water immersion, NaOCl treatment, removal of hulls (Hsiao 1979) and acid scarification (Keogh and Bannister 1992, Sukhvibul and Considine 1994). Sequential application of acid scarification followed by imbibition with 10^{-4} M GA_3 is a technique widely used to germinate seeds with a hard seed coat and embryo dormancy (cited in Keogh and Bannister 1992). The results of this study confirmed that scarification of seeds is a first important step for seed germination in *Sandersonia*. This treatment might promote germination by various means. It may allow leaching out of germination inhibitors present in seeds. It may also increase water and oxygen uptake or reduce mechanical restriction. Here, the seeds treated with scarification still required exogenous GA for germination. GA_3 strongly stimulated germination when applied to seeds which were scarified and nicked, but not when applied to intact seeds. A lower response was seen for seeds which were only scarified. These indicated that seed coat and/or endosperm may restrict GA_3 penetration or GA_3 may not be able to counter their inhibitory

influences over germination. The scarifying plus nicking apparently increased GA_3 penetration and allowed GA_3 to directly stimulate embryo growth. This might have been by stimulation of cell expansion, cell division or by stimulating hydrolytic enzyme synthesis which released soluble sugars for embryo growth (Ouellette and Bewley 1986).

4.4.2 Embryo Dormancy

Embryo dormancy is defined as a viable, mature embryo of dormant seed failing to germinate, even after it has been separated from the rest of the seed (Bewley and Black 1982). In many families, the seed has a rudimentary or linear, and under-developed embryo (Martin 1946, Atwater and Vivrette 1987) with physiological dormancy (Nikolaeva 1977, Baskin and Baskin 1994). In such a case, the germination occurs only when environmental conditions are appropriate for both dormancy loss and embryo growth (Nikolaeva 1969). *Sandersonia* seed has very small (approx. 0.80×0.25 mm), linear embryo with a cotyledon, a small embryonic axis, shoot apex and a prominent radicle (Plate 3.22 I). Freshly harvested seeds of *Sandersonia* showed considerable dormancy when they were scarified plus nicked and germinated in GA_3 solution (Table 4.12). They germinated slower (data not shown) and attained a lower final germination than the after-ripened seeds. Also, excised embryos from mature seeds showed relatively slow growth and a poor germination suggestive of residual embryo dormancy. The treatment of scarifying plus nicking, which often removes barriers to water uptake and oxygen diffusion, was unable to elicit a full germination response and exogenous GA was still required. Therefore, dormancy in *Sandersonia* seeds can be partly attributed to the inability of the embryo to grow.

4.4.3 Imbibition and Seed Coat-imposed dormancy

Seed dormancy in many species is due to seed coat impermeability to water. The uptake of water by seeds is an essential, initial step toward germination (Bewley and Black 1994). The results of the imbibition measurement demonstrated that the testa of *Sandersonia* had a low degree of permeability but that it was not impermeable. Intact seeds show lower permeability ($P<0.001$) to water than those of nicked or pierced seeds (Fig. 4.4). The water uptake was improved when a small portion of seed tissue was

removed or the seeds were pierced by a sharp needle (Fig. 4.4). The nicked seeds showed an increase in weight to well over 108% of their dry weight after 24 hours imbibition and 122% at 192 hours. In contrast, intact seeds presented a lower weight increase, which ranged from 55% (at 24 hours) to 89% (at 192 hours). So, removal of a small portion of seed tissue almost doubled the capability of seeds to absorb water after 24 hours imbibition and the highest percentage of water uptake was reached at the 120 hours. The water uptake increased gradually in the intact seeds and the highest percentage was reached at 168 hours. The permeability of seeds with scarification by sandpaper only was not increased and significantly lower ($P<0.001$) than that of the seeds with the treatment by scarifying plus nicking (Fig. 4.4). From these results it could be suggested that a low degree of permeability to water is one of the limiting factors to germination of *Sandersonia* seeds. This may be induced not only by the testa but also by the tissues inside the testa. According to the structure of mature seeds of *Sandersonia* (see Chapter 3), both the lignified tegmen and the thick-walled endosperm may contribute to the seed impermeability towards water.

Seed coat-imposed dormancy is in many plant species due to mechanical constraints to embryo growth (Bewley and Black 1982). Brown and van Staden (1973) found that the dormant condition of *Protea compacta* was partially caused by mechanical restriction of embryo growth by the seed coat and that removal of a small portion of the seed coat from the radicle end improved germination. They also suggested that removal of a small portion of the testa between the radicle and style end did not improve germination, possibly because the mechanical forces exerted by the coat were not sufficiently altered to allow the embryo to fully develop. These phenomena appear to be very similar to those found in *Sandersonia* seeds. This could explain why the treatment with scarification by sandpaper had only limited success in inducing a germination response (Table 4.10).

The structure of *Sandersonia* seeds is very similar to structure of iris seeds (see Fig. 4.13 and section 3.3.7 in chapter 3). The tip of the radicle in both seeds is situated at the micropylar end facing the seed coat and is separated from it only by a thin layer of the endosperm. The inner part of the seed coat above the radicle tip is conical in shape in iris seed (Fig. 4.14), but cylindrical in *Sandersonia* seeds (Plate 3.26). This structure

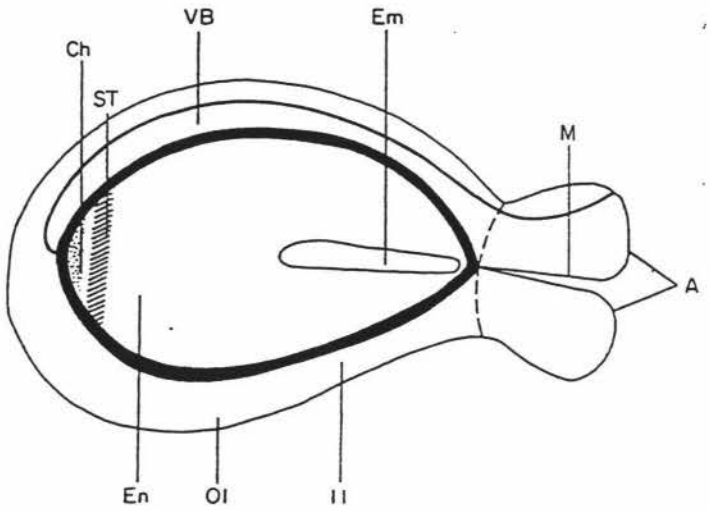

Figure 4.13 Schematic drawing of median longitudinal section of an iris seed from the Oncocyclus section. M, micropyle; A, aril; Em, embryo; VB, vascular bundle; Ch, chalaza; ST, suberized tissue; II, inner integument; OI, outer integument; En, endosperm. OI, II and A together form the seed coat (Source: Blumenthal *et al.* 1986).

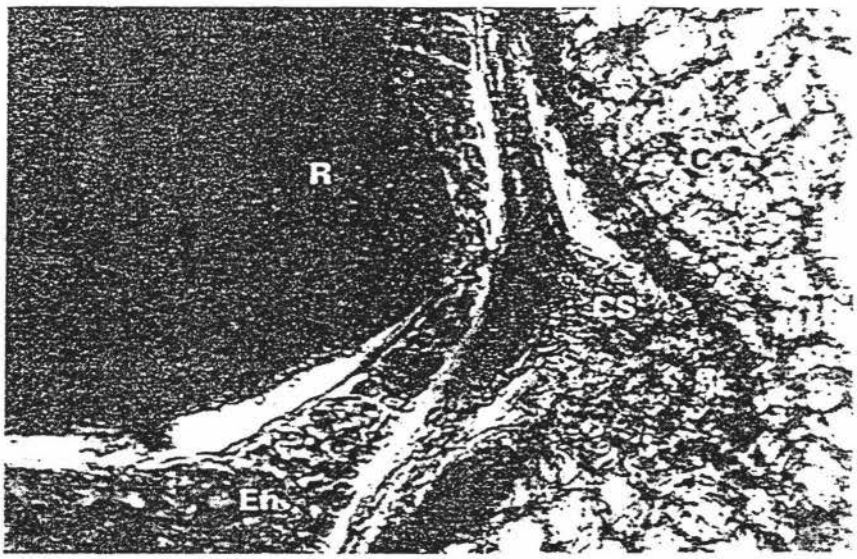

Figure 4.14 Radicle tip of *Iris atropurpurea* seeds close to inner micropylar end. ×300. C, seed coat; CS, conical protuberance; En, endosperm; R, radicle (Source: Blumenthal *et al.* 1986).

was formed by closure of the micropylar tube during ripening at the inner integument (Blumenthal *et al.* 1986). Blumenthal *et al.* (1986) tested experimentally the mechanical strength of the micropylar region of iris species and found that the seed coat of *I. lorteti* required approximately twice as much pressure as that of *I. atropurpurea* before it ruptured because the two species had an anatomical difference in conical protuberance of the inner integument at the micropylar side. The former showed a larger one which consisted of 8 to 9 cells long (16 to 18 cross walls) than the latter 4 to 5 cells long, (8 to 10 cross walls). The great similarity in structure between *Sandersonia* and *Iris* seeds implies that *Sandersonia* may have a similar mechanism in seed coat-imposed dormancy. This may also explain why scarifying plus nicking near the radicle end enabled the seeds to germinate.

4.4.4 Germination Inhibitors

Seed coats of many dormant seeds contain inhibitors (Bewley and Black 1982, Thapliyal and Nautiyal 1989). Frequently, the inhibitors that exist in seeds have been detected by their effect on germination of lettuce seeds, such as the inhibitors in papaya seeds (Chow and Lin 1991), and in fruits of *Muntingia calabura* L. (Laura *et al.* 1994). Figs. 4.8, 4.9, 4.10 and 4.11 show that the extracts of *Sandersonia* seeds have a significant inhibitory effect both on germination rate and radicle elongation of lettuce seeds. The inhibitory activity could be located either in the seed coat or endosperm because all solutions, in which intact, de-coated or nicked seeds of *Sandersonia* were soaked for one week, respectively, inhibited radicle elongation of lettuce seeds (Fig. 4.8b) and the inhibitory effect could be obviously decreased by leaching *Sandersonia* seeds for one week (Fig. 4.9b). Neither the extracts of the *Sandersonia* seeds for long period (150 days) nor for short period (1 week) incubations affected the final germination percentage of lettuce seeds (Figs. 4.8a, 4.9a and 4.11). This suggested the germination-preventing compound present in the surrounding tissues is unlikely to be the main cause of dormancy in seeds of *Sandersonia.*

Suparna *et al.* (1993) has suggested that seed dormancy of *Gloriosa superba* L. (related to *Sandersonia*) was due to the presence of certain growth inhibitors in the seeds. A rhizome extract of *Gloriosa superba* L. decreased the germination, total

number of roots, root length, vigour index and length of leaves of *Allium cepa* L. and these inhibitory effects could be induced by colchicine (Suganthi *et al.* 1993). It was reported both *Gloriosa superba* L. and *Sandersonia aurantiaca* contained colchcine by Subbaratnam (1952, 1954), Sarin *et al.* (1974), Thakur *et al.* (1975), Wildman and Pursey (1968) and Finnie and van Staden (1991). Colchcine levels in corms of *Gloriosa superba* L. and *Sandersonia aurantiaca* were 0.91% and 0.80% of dry matter (Finnie and van Staden 1991), respectively. The level in *Gloriosa* seeds was 0.61%, but no accurate amount was reported in *Sandersonia* seeds. The reduction of radicle length of lettuce seeds by inhibitors in *Sandersonia* seeds shown here, is perhaps the result of an inhibitory action on cell division by colchicine alternatively; colchicine may cause cell injury (Lilly 1965).

4.4.5 Ovule and Excised Embryo Growth *in Vitro*

Finnie and van Staden (1989) reported a marked difference in seed germination *in vitro* between *Sandersonia* and *Gloriosa*. The germination percentage in the former was 0% and in the latter was 95%, respectively. Experiments reported here show a similar result for *Sandersonia*. Embryos excised from mature seeds did not germinate very well *in vitro*. This indicates that the dormancy mechanism of *Sandersonia* seeds could well be located in the embryo. Exogenous hormones, such as IAA, cytokinin, especially GA_3, applied in growth medium may induce embryo germination and growth because GA_3 promoted the scarified and nicked seed germination. It was apparent that the difficulty in embryo culture of *Sandersonia* was not only in excising embryos from mature seeds but also in contamination of the medium by the embryo.

Ovule culture of *Sandersonia in vitro* shows more satisfactory results than embryo culture. 43% and 13% ovules germinated and produced callus and/or roots when they were cultured at 8 and 14 days after pollination, respectively. No seedlings however were produced in this study. From the results obtained we can suggest that (1) culture of ovules was more advantageous than that of embryo because they could be easily excised and sterilized, even at the zygote stage and the ovule provided a "maternal environment" for the developing embryo (Rangan 1984); (2) for the younger ovules (which were more successful) it may be an indication that the dormancy of *Sandersonia*

seeds initiated during seed development, may be started at the globular embryo stage. This hypothesis is consistent with the observations of seed development in chapter 3 where both inner integument lignification and endosperm cell formation initiated at 14 days after pollination. These developments may hinder embryo growth and induce embryo dormancy; (3) the observations of no seedling production and poor callus growth and differentiation may be due to the nutrient composition of the medium being unsuitable for ovule growth. Further study on these possibilities is required for the future.

In summary, I propose that the dormancy mechanism in *Sandersonia* seed is located in both the seed coat or embryo and that it consists of at least two steps that must be activated in sequence before germination can occur. The results in this study indicate that the first step can be activated prematurely by scarifying and nicking the seeds, thus allowing the seed coat to become permeable to water, oxygen or to reduce mechanical restriction. The second step can be activated directly by GA_3 which stimulates embryo growth.

The optimum condition for germination of *Sandersonia* seeds appears to be 20°C in nominal darkness. Higher temperatures (25-30°C) and 16 h light/d may inhibit their germination. *Sandersonia* seeds are shown to contain certain growth inhibitors which may be located in the surrounding tissues. These inhibitors however are not the primary cause of dormancy. Ovule culture may be an effective technique for both producing seedlings and investigating further dormancy mechanism.

Chapter 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

Sandersonia aurantiaca is a relatively new but nevertheless important cut flower crop in New Zealand. The information obtained in this thesis has both scientific and economic value since detailed works on seed development, dormancy characteristics and germination methods of *Sandersonia* have previously been limited.

From the results generated in this thesis the following conclusions can be drawn:

(1) The ovary is superior, tricarpellate, and trilocular with axile placentation. Each carpel contains numerous ovules, and each ovule is attached to the placentation by a funiculus.

(2) The ovules are anatropous, crassinucellate and bitegmic. Both integuments form the micropyle. Two special structures known as an obturator and a hypostase are present in the mature ovule. The former is located on the concave side of the funiculus and may be associated with pollen tube growth. The latter is present at the chalazal region just below the embryo sac and may play an important role in delivering nutrients from the maternal tissue to the embryo sac.

(3) The embryo sac is of the Polygonum type. The egg cell has a nucleus located at the micropylar pole before fertilization and shifted to the chalazal pole before the first zygote division. Two synergids disappear before embryo development. The antipodal cells are located at the chalazal end, enlarge after fertilization and remain till the initiation of cellularization of endosperm.

(4) The sequences of embryo and endosperm development are more or less similar to those in *Allium fistulosume*. Both embryo and endosperm develop almost simultaneously. The endosperm development conforms to the Nuclear type. The

cellularization starts at the early globular embryo stage and terminates at the late globular stage. The zygote begins to divide at about 8 DAP and progresses through the early globular, the late globular, the elongated spheroidal stages reaching a linear embryo at about 35 DAP. The embryo has a well-developed suspensor which remains till 49 DAP. In addition to pushing the embryo to the embryo sac, the suspensor may be a source of metabolites for embryo development.

(5) The embryo in a mature seed is linear, very small (about 0.80 × 0.25 mm) with a prominent radicle, a small embryonic axis, shoot apex and a cotyledon. When excised from the mature seeds, such small embryos show relatively slow growth and a poor germination suggestive of residual embryo dormancy.

(6) Both outer and inner integuments contribute to form the seed coat. The inner one develops faster than the outer one does. At maturity, the seed coat is simple, consisting of a number of layers: epidermis, subepidermis and lignified layer. The last one may act as a mechanical barrier to limit water and oxygen entry into seed, even restrict GA_3 penetration unless it is broken by some mechanical treatments. A special cylindrical structure is present in the inner seed coat at the micropylar part and also contributes to the hardness of the seed coat. These observations suggest that seed coat-imposed dormancy is another component of *Sandersonia* seed dormancy.

(7) From the results obtained in this study, we propose that the dormancy mechanism in *Sandersonia* seeds is located both in seed coat and embryo. Some germination-preventing compounds are shown to be present in the surrounding tissues, but are unlikely to be main cause of dormancy in seeds of *Sandersonia.*

(8) Dormancy is partially removed when seeds are scarified plus nicked near the radicle end. However, the most satisfactory method for breaking dormancy found in this study is when the seeds are scarified plus nicked near the radicle end, and then incubated in GA_3 solution (100-500 ppm) at least 1 week at 20°C with nominal darkness.

(9) According to results from ovule culture and the observations of seed development from pollination to maturity, it can be supposed that the dormancy of

Sandersonia seeds is initiated during seed development, and may start at the globular stage.

5.2 SUGGESTIONS FOR FUTURE RESEARCH

In this study, the traditional paraffin embedding technique has been used for preparing material for study under the light microscope. The results both for embryo and endosperm development are satisfactory at least after the globular stage. However, this method has been found to be unsatisfactory in the study of structures in early stages and mature seeds as the cytoplasm may be disrupted (Feder and O'Brien 1968), distorted and shrunk (Butler 1979) during fixation and embedding; in addition the endosperm tissue is very hard and the small, soft embryo is easily broken and lost in the mature seed. In order to obtain high-quality specimens giving good clarity, contrast, and resolution for light microscopic examination, plastic embedding methods are recommended for structural investigations because the hardness of the plastic blocks and thin sections (0.5-2 μm) can improve the resolution of specimen (Yeung 1984).

The safranin O-fast green FCF staining technique can be used for general histology (Setia and Richa 1989, Zavaleta-Mancera and Engleman 1994). Some histochemical staining techniques are often necessary for locating and identifying some special substances, such as callose by aniline blue (Eschrich and Currier 1964), lignin by phloroglucinol-HCl (Jensen 1962), lignin and some polyphenols by toluidine blue (Feder and Wolf 1965, O'Brien *et al.* 1965), carbohydrates by periodic acid-Schiff's reagent (PAS) (Jensen 1962), proteins by 1 % amido black in 7 % acetic acid (Schneider 1981), lipids by Sudan black B (cited in Setia and Richa 1989), starch by I_2KI (Jensen 1962) and nucleic acids by azure B (Jensen 1962). Therefore, the histochemical technique is also recommended to study structural development and may help to understand the mechanism of seed dormancy in *Sandersonia*.

Freshly matured seed of *Sandersonia* has a small embryo with a prominent radicle, a small embryonic axis, shoot apex and a cotyledon. According to this study, we could propose that the dormancy mechanism in *Sandersonia* seeds is located both in seed coat and embryo. What type of embryo dormancy the seeds have and whether embryos must grow to a certain critical size are still not very clear and also need to be studied.

One of most important findings in this study is that GA_3 strongly stimulated germination when applied to seeds which were scarified and nicked, but the reasons why GA_3 allows better germination of dormant *Sandersonia* seeds remain unknown. Thus there is a very interesting subject for further studies to understand the role of GA_3 on seed germination of this species. However, this technique should also facilitate further experimental studies on its reliability, stability and commercial application for producing large numbers of normal, healthy seedlings.

Many researchers are very interested in the study of dormancy mechanisms by combining study of seed coat structure, variations in seed coat structure during seed germination with germination behaviour (Poljakoff-Mayber *et al.* 1992, Angosto Trillo and Matilla Carro 1993, Todd-Bockarie 1993, Edelstein *et al.* 1995), in relation to factors such as water uptake, oxygen exchange and so on. The relationship between seed coat structure and oxygen availability of melon seeds at low temperature was studied by Edelstein *et al.* (1995). They indicated that the seed coat-imposed dormancy at low temperature in Noy Yizre'el was the combined result of more restricted oxygen diffusion through the seed coat and a greater embryo sensitivity to hypoxia, rather than to physical constraints of radicle break-through or impairment of imbibition. These studies also provide us with more suggestions for future studies in *Sandersonia* seeds.

That the ovules can germinate at an early stage *in vitro* suggests another topic for future study. The most important areas of ovule culture, even embryo culture are both in finding suitable media and appropriate technologies, which will be very useful for commercially producing a large number of seedlings by propagating *in vitro*, for breeding research and for further investigation of the mechanism of seed dormancy in *Sandersonia*.

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