PHYSIOLOGICAL STUDIES ON STERILITY INDUCED IN WHEAT BY HEAT AND WATER DEFICIT

by

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There are four things that make this world go round: love, energy, materials and information. We see about us a critical shortage of the first commodity, a near-critical shortage of the second, increasing shortage of the third, but an absolute glut of the fourth.

We in science, of necessity, must contribute to the glut ...

- Robert A. Day

To my parents whose inspiration has made this moment possible for me.

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3. RESULTS AND DISCUSSION

Research papers based on the results of the present investigation, published or accepted for publication before the completion of this thesis.

1. SAINI, H.S. and ASPINALL, D. (1981). Effect of water deficit on sporogenesis in wheat (*Triticum aestivum L.*) *Ann. Bot.* 48: 623-633.

2. SAINI, H.S. and ASPINALL, D. (1982). Abnormal sporogenesis in wheat (*Triticum aestivum* L.) induced by short periods of high temperature. *Ann. Bot.* 49: 835-846.

3. SAINI, H.S. and ASPINALL, D. (1982). Sterility in wheat (*Triticum aestivum* L.) induced by water deficit or high temperature : Possible mediation by abscisic acid. *Aust. J. Plant Physiol.* 9: in press.

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LIST OF ABBREVIATIONS

ABA	-	Abscisic acid
b.p.	-	Boiling point
C	-	Amount of DNA in a chromatid
CIG	-	Commonwealth Industrial Gases
Cm	-	Centimetre
Cv	-	Cultivar
DNPH	-	Dinitrophenyl hydrazine
EDTA	-	Ethylenediamine tetra-acetic acid
g	-	Gram
GLC	-	Gas-liquid chromatograph(y)
GMA	-	Glycol methacrylate
h	-	hour
HMDS	-	hexamethyldisilazane
IAA	-	Indol-3-acetic acid
l	-	Litre
lsd	-	least significant difference
M	-	Molar
m	-	Metre
µE	-	Micro Einstein
mg	-	Milligram
µg	-	Microgram
min	-	Minute
ml	-	Millilitre
µl	-	Microlitre
mm	-	Millimetre
µm	-	Micrometre
MMC	-	Megaspore mother cell
m.wt	-	Molecular weight
N	-	Normal
ng	-	Nanogram
nl	-	Nanolitre
nm	-	Nanometre
Ψ	-	Water potential
PAS	-	Periodic acid - Schiff's reagent
%	-	Percent

PMC	-	Pollen mother cell
ppm	-	Parts per million
PVC	-	Polyvinyl chloride
Rf	-	Distance migrated relative to the solvent front.
R.S.	-	Recycled soil
RWC	-	Relative water content
s	-	Second
SCOT	-	Support coated open tubular
S.E.	-	Standard error of difference of means
TBO	-	Toluidine blue O
TONY	-	p-tolylsulphonylmethylnitroso-amide
TTC	-	2,3,5-triphenyl tetrazalium chloride
u.c.	-	University of California - type soil mix
UV	-	Ultraviolet
u/v	-	Volume/volume (concentration)
W	-	Watt
w/v	-	Weight/volume (concentration)

SUMMARY

1. Wheat plants were subjected to water deficit by withholding water or to heat stress by exposure to 30°C for 3 days during various stages of development between the onset of meiosis and completion of anthesis. Both treatments when applied during meiosis in the pollen mother cells and tetrad break up (sensitive stage) caused significant reductions in grain set and yield.

2. High temperatures of 30°C for 1 day or 30°C day/20°C night for 3 days during the sensitive stage also caused significant reduction in grain set.

3. Decrease in grain set in response to these stresses was followed by a compensatory increase in the weight of the surviving grains, this increase being greater when the grain set was affected by water deficit than by heat.

4. Water stress during the sensitive stage caused a substantial drop in the relative water content and water potential of the leaf but did not affect spike water potential. Heat stress during the same period had no effect on any of these attributes. The results suggest that water stress affected grain set without causing desiccation of the spike tissue and that the effects of heat stress were not because of water loss from the plant as a result of elevated temperature.

5. Both water- and heat-stress caused reduction in grain set by inducing male sterility. However, only heat-stress affected female fertility.

6. Heat-stress induced structural abnormalities in the embryo sacs and nucellus tissue and adversely affected the growth of pollen tubes when heat-stressed pistils were pollinated with fertile pollen. It is proposed that the effects of heat on pollen tube growth were due either to the disruption of nutrition of pollen tubes by the stigma/style or to defective attraction signals from abnormal embryo sacs.

7. In about half of the abortive anthers on water-stressed plants, the pollen grains lost contact with the tapetum at about the time of first pollen grain mitosis and showed suppressed intine development and starch accumulation. These abnormalities were associated with the degeneration of the filament and vascular bundle. The remainder of the abnormal anthers showed similar disruption of pollen development but had apparently normal filaments and vascular bundles.

8. About half of the heat-affected anthers showed degeneration of microspores during meiosis coupled with premature tapetal degeneration. The rest of the anthers aborted in a similar way to those affected by water stress, but did not show abnormalities in the filament tissue. It is suggested that there may be qualitative differences between the effects of water stress and heat on sporogenesis in wheat.

9. Abnormal anthers induced by water- or heat-stress were morphologically similar. These anthers were relatively small, frequently shrivelled and generally failed to extrude or dehisce at anthesis. They contained pollen grains that did not stain with 2,3,5-triphenyl tetrazolium chloride, indicating a lack of viability. Pollen viability was reduced by stress also in anthers that looked apparently normal.

10 Water stress resulted in an accumulation of abscisic acid in the spikelets. Application of abscisic acid solution at the stress-sensitive stage of development augmented the endogenous concentration of the hormone, reduced grain set and induced male-sterility, but not female sterility. Application of the hormone after meiosis had no such effects. Although the abnormal anthers and pollen grains on abscisic acid-treated plants appeared similar to those affected by water stress, their pattern of development was different. Approximately 75% of the anthers that aborted in response to the application of abscisic acid showed the beginning of abnormal microspore and tapetal development at meiosis. Water-stress affected anthers aborted after the first pollen grain mitosis and, at least half had degenerate filaments. It is therefore, suggested that despite the apparent similarities in the responses to water stress and applied abscisic acid, the two may

affect pollen fertility through different mechanisms.

11. Compared with untreated plants, heat-stress (30-40°C for 1-3 days) did not cause an increase in the endogenous concentration of abscisic acid in spikelets or flag leaf, indicating that the mechanism of induction of sterility by heat is unlikely to involve abscisic acid.

12. Spraying plants with ethephon or exposing them to ethylene-supplemented air during the sensitive stage caused a reduction in grain set via an induction of male sterility. Application of either of the treatments after meiosis had no effect on grain set. Anthers and pollen grains affected by the application of ethephon or ethylene had similar structure to those sterilized by water stress or heat. In the light of the published information on the increase in ethylene emanation from the stressed tissues, it is postulated that ethylene may be involved in the mechanism of induction of sterility by stress.

STATEMENT

I hereby declare that the thesis here presented is my own work, that it contains no material previously published, except where due reference is made in the text, and that no part of it has been submitted for any other degree.

(Hargurdeep Singh Saini)

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The first stone in the foundations of this project was laid, perhaps, at my brief meeting with Dr. Don Aspinall during his visit to India in 1975 when the thought of working in his laboratory first entered my mind. It was not until nearly three years later that I arrived in this department and began what has been a stimulating episode. Dr. Aspinall has been an excellent supervisor, an inspiring coworker and a pleasant and enlightening companion from whom I have learnt much, not only about how to conduct research but also about the philosophy of science and of life in general. I am grateful to him for more things than I can mention.

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1. INTRODUCTION

There are two primary components of grain yield in cereals: grain number and grain weight. Grain number per unit ground area is further composed of the number of inflorescences, the number of spikelets per inflorescence, the number of florets per spikelet and the proportion of florets actually setting grains. Grain weight is determined by the extent of production and enlargement of endosperm cells and the accumulation of storage materials in them.

The life cycle of a wheat plant is distinctly phasic, being broadly delineated into the vegetative, the reproductive and the grain development phases. Each of these developmental phases may contribute towards the determination of one or more components of yield (Evans and Wardlaw, 1976). The number of inflorescences is controlled by the production of tillers during the vegetative phase and the death of excess tillers during the reproductive period, the number of spikelets per spike and florets per spikelet by floral development during the earlier part of the reproductive phase, and the proportion of florets that set grains by sporogenesis and fertilization later during the reproductive phase. Although the main event during the grain development is the determination of grain weight this phase may also influence grain number since some of the fertilized embryo sacs may fail to grow further.

This thesis deals with the effects of water deficit and high temperature on the factors controlling grain set in wheat and the physiological processes underlying these effects. The following review, therefore, will be restricted to a discussion of inflorescence development, sporogenesis and fertilization and their relationship to yield, the effects of water deficit and heat on the reproductive development, including the physiological role of abscisic acid and ethylene under these conditions.

1.1. FLORAL INDUCTION AND INFLORESCENCE DEVELOPMENT

Comprehensive accounts of the morphological and histological events during the transition of the vegetative apex of wheat to the reproductive phase, and the further development of the flowering apex are given by Barnard (1955), Bonnett (1966), and Kirby and Appleyard (1981).

Some time prior to the appearance of floral primordia, the shoot apex starts elongating. The reproductive stage is ushered in by the appearance of double ridges on the apex. The upper one of each pair of ridges then differentiates to form a spikelet while the lower one fails to develop further. Spikelet differentiation commences in the middle of the spike and proceeds in either direction. This sequence of development is maintained for each of the spikelet parts. Within a spikelet, however, the gradient of development is acropetal, whereas, within a floret the lemma appears first followed by the stamens, palea and the pistil in that order. In the stamens, the anthers are the first to be initiated and the filaments are formed later beneath the anthers. The ovary is the first part of the pistil to differentiate, the styles next, and the stigmas last.

The wheat inflorescence is of the determinate type (Bonnett, 1966) and once the terminal spikelet has been formed the process of spikelet formation is completed and no more are added. The apical spikelet is at right angles to the plane of the rest of the spikelets. Once the terminal spikelet has differentiated, rapid enlargement of the floral organs ensues coupled with the elongation of stem internodes. The floral development and stem elongation may compete with each other for assimilate supply (Evans and Wardlaw, 1976).

Usually, the floret primordia initiated are far in excess of the number that develop to maturity. Quite often only two to four basal primordia survive to form potentially fertile florets and the remainder die prematurely (Kirby, 1974).

Meiosis in the male and female sporogenous tissue takes place during the later part of the reproductive phase, followed by the development of male (pollen) and female (embryo sac) spores. Rapid growth of the stem pushes the developing spike through the surrounding leaf sheaths.

Anthesis occurs one to two weeks after the emergence of the spike. The terminal primordia grow faster than those in the middle of the spike, which tends to synchronize later events such as meiosis and anthesis (Kirby, 1974).

The timing of the reproductive cycle in wheat has an important influence on yield. The timing of floral induction is crucial as induction, too early, may increase the risk of frost injury while delayed induction may increase the risk of drought or heat damage. Vernalization and day length play an important role in the control of floral induction. In high latitudes, the spring wheats are generally responsive to long days whereas the winter wheats show a strong requirement for vernalization. Wheats in low latitudes, however, have a much lower requirement for either long days or vernalization (Evans *et al.*, 1975).

The duration of the reproductive cycle in wheat may vary from two weeks to several months, depending on the cultivar and the environment (Holmes, 1973; Evans *et al.*, 1975; Langer and Dougherty, 1976). Floral induction is hastened by high light intensity (Friend *et al.*, 1963; Rahman *et al.*, 1977), high temperature (Rawson, 1970; Halse and Weir, 1974; Rahman and Wilson, 1978), and long photoperiods (Riddell *et al.*, 1958; Allison and Daynard, 1976; Rahman and Wilson, 1977b). Post induction inflorescence development is also faster under high light intensity (Friend, 1965,66), long days (Williams and Williams, 1968; Rawson, 1970; Wall and Cartwright, 1974), and high temperature (Friend, 1965; Halse and Weir, 1970; Rawson, 1970).

The number of spikelets per spike can significantly influence grain yield (Rawson, 1970; Fischer, 1975). Generally, high light intensity increases the number of spikelets by prolonging the spikelet formation phase (Friend, 1965, Fischer, 1975; Rahman *et al.*, 1977). Willey and Holliday (1971), however, found that shading did not reduce spikelet number although it lowered grain set. Similarly, there is some uncertainty about the effect of crop density on spikelet number; some workers reporting a reduction in the number of spikelets per spike at higher plant densities (Puckridge, 1968) whereas others found no such effect (Darwinkel, 1980). A greater number of spikelets is formed under high levels of nitrogen (Beveridge *et al.*, 1965; Holmes, 1973; Langer

and Liew, 1973) and phosphorus (Rahman and Wilson, 1977a). Conditions, such as long days and high temperature, that accelerate floral induction usually reduce spikelet number by hastening the formation of the terminal spikelet (Rawson, 1970; Allison and Daynard, 1976; Rahman and Wilson, 1977b,78; Kolderup, 1979). The differentiation of spikelet primordia in both wheat and barley is severely retarded by water stress resulting in a lower final spikelet number (Hussain and Aspinall, 1970; Barlow *et al.*, 1977).

Once the terminal spikelet has been formed, environmental conditions have no further influence on spikelet number, although the number of florets within a spikelet may still be affected by environment. Floret development is hastened by longer days (Langer and Hanif, 1973). High nitrogen level delays floral development, consequently increasing the number of grains per spikelet (Single, 1964; Langer and Liew, 1973).

1.2. DEVELOPMENT OF SEX ORGANS AND FERTILIZATION

Following the initiation of stamens and pistils, their subsequent development to maturity takes about 3 weeks (Batygina, 1962). The wheat plant bears perfect, hypogynous flowers, each with a tristaminate androecium and a monocarpellary gynaecium with a unilocular ovary bearing a single ovule (Percival, 1921). The timing of development of microspores into pollen grains, and the male gametes therein, parallels that of the megaspore that differentiates into an embryo sac within which an egg or ovum is contained.

1.2.1. MALE REPRODUCTIVE DEVELOPMENT:

1.2.1.1. PREMEIOTIC ANTHER DEVELOPMENT:

The early development of the anther has been described by Percival (1921). Following their formation, the stamen primordia elongate rapidly. A single archesporial mother cell differentiates very early in each potential locule of the anther. Each of these cells undergoes a periclinal division into an outer 'primary perietal cell' and an inner 'primary sporogenous cell'. The outer divides twice radially to form four cells which join with the cells towards the inside of the archesporium to encircle the sporogenous cell(s). This encircling layer of cells, after two periclinal divisions, gives rise to the four layers of the anther wall: the innermost 'tapetum', the second 'middle layer', the third 'endothecium' and the outermost 'epidermis'. Anticlinal divisions in all these cells produce columnar layers of cells surrounding the sporogenous tissue. While these divisions of the primary parietal cell are in progress, each primary sporogenous cell also undergoes divisions in the radial and the longitudinal planes to form six columns of archesporial cells, each of which is in contact with the tapetum. The anther is supplied by a single longitudinal vascular bundle.

At 20°C, mitotic divisions in the archesporial columns continue until the anthers are 0.5 to 0.6mm long and contain between 90 and 140 cells, now termed pollen mother cells (PMCs). Until this stage the archesporium has been dividing asynchronously for about 5-6 days, with mitoses taking longer as the development proceeds. The final mitosis takes place about 48h before the synchronous onset of meiotic prophase in the PMCs. (Bennett *et al.*, 1973b)

1.2.1.2. MEIOSIS :

Detailed investigations into the cellular development during meiosis and microsporogenesis in 'Chinese Spring' and 'Holdfast' cultivars of wheat grown at 20°C and under continuous light have been conducted at the Plant Breeding Institute, Cambridge (Bennett *et al.*, 1971,73b; Bennett and Smith, 1972). Following the final pre-meiotic mitosis, PMC nuclei increase in size and assume a diffuse, reticulate appearance. At this stage the DNA content of PMCs is 2C while that of tapetal cells ranges from 2C to 4C. About 18h later, the PMC nuclei contract and stain more densely. The tapetal cells cease dividing and their DNA content, as well as that of PMCs is now 2C. The callose wall starts encircling each PMC, starting at the centre of the archesporial column. DNA synthesis takes place about 15h later in both the PMCs and the tapetal cells increasing their DNA content from 2C to 4C. In *Triticum durum* Desf. the DNA synthesis appears to occur at about the same time (Rudramuniyappa and Panchaksharappa, 1980). The nuclear size in the cells increases again and the nucleoli move closer to the surface of the nuclei. The deposition of callose in the PMC wall is completed before the commencement of meiosis. Throughout meiosis, each PMC is enclosed within its own callose envelope.

Meiosis in wheat takes place when the spike is still surrounded by two leaf sheaths belonging to the penultimate and the flag leaves. It takes about 24h at 20°C, proceeding at one of the quickest rates known in higher plants (Bennett *et al.*, 1973b). Batygina (1962), however, observed meiotic durations of several days under field conditions in Russia. Prophase, the longest stage of meiosis, takes about 17.0h, whereas metaphase-I, anaphase-I, telophase-I and dyad stages take 1.6, 0.5, 0.5 and 2.0h respectively. Meiosis-II is very quick and lasts about 2.4h. Most of the time during meiosis is taken up by prophase, perhaps because it is during this period that the major concentration of chromosome length and such complex molecular changes as chromosomal synapsis, assembly of the synaptonemal complex and the molecular rearrangements involved in recombination occur.

In addition to the major episode of DNA synthesis during the pre-meiotic interphase in wheat, some DNA synthesis also occurs during most stages of meiosis up to the second telophase (Riley and Bennett,

1971). Delayed synthesis of DNA, which has also been observed in other species (Sauter, 1971), is thought to be due either to delayed replication in chromosomal regions where synthesis was not completed in the previous interphase or to the continual repair of defects or breaches including those caused by crossing over.

Meiosis is synchronous within an anther with the exception of the cells towards the tip which are slightly more advanced in their development than those towards the base. This asynchrony between the PMCs within an anther does not exceed development that would take 1-2h to complete. Moreover, the separate anthers within the same floret are similarly synchronous. Within a spikelet the development of the primary floret is ahead of that of the secondary floret, by about 12±2h, and the development of the secondary floret is ahead of that of the tertiary floret by about 26±6h (Bennett *et al.*, 1973b).

Meiosis in each PMC produces an isobilateral tetrad of haploid microspores, each with 1C nuclear DNA content (Bennett *et al.*, 1973b). At this stage, each tetrad is enclosed within a callose wall. The tetrad stage lasts about 10h at 20°C during which period the microspore nuclei become rounded. then follows rapid loosening and eventual disappearance of the callose wall. Each tetrad eventually breaks up to release four irregularly-shaped, aporate and thin-walled microspores.

1.2.1.3. POLLEN DEVELOPMENT :

Development of microspores into mature pollen grains, following their release from the tetrad, has been described by Batygina (1962), Goss (1968) and Bennett *et al.* (1973b). At the time of tetrad bread-up, the mean volume of a microspore is approximately $1.14 \times 10^4 \mu m^3$, and this increases steadily throughout subsequent development. Soon after their release microspores round off to assume an almost spherical shape with nuclei occupying about half their volume. Germ pore rudiments start appearing on the microspore walls about four hours after tetrad break-up, and within about ten hours pore and wall formation is well underway. Initially there are several small vacuoles in the microspore cytoplasm which, about 12h after the bread up of the tetrads, coalesce to form a single vacuole near the pore. Thereafter, the vacuole increases rapidly

in size to occupy the greater part of the microspore. Because the vacuole is situated near the pore, Batygina (1962) has suggested that materials enter the growing pollen grain through the pore. during this period of vacuole development the nucleus undergoes considerable condensation and generally becomes located just beneath the germ pore. By about 48h after microspore release the vacuole grows large enough to occupy most of the pollen grain volume and the cytoplasm constitutes a thin layer around the periphery, extending into the lumen of the microspore only in the area around the nucleus.

About 36h following tetrad break-up DNA synthesis takes place, increasing the nuclear DNA content to the 2C level. Nuclear volume also increases in association with this synthetic activity. The first pollen grain mitosis starts almost synchronously within an anther, about 48h after the microspore release and lasts about 18-24h (Bennett and Smith, 1972; Bennett *et al.*, 1973b).

The first pollen grain mitosis produces a vegetative and a generative nucleus. The former rapidly increases in size, becomes diffuse and stains poorly, whereas the latter undergoes condensation and stains darkly. The vegetative nucleus is situated near the germ pore while the generative nucleus is at the opposite pole. The latter soon organizes its own cytoplasm thus dividing the cell roughly in a 1:9 ratio, into two - the generative cell and the vegetative cell.

Starch accumulation starts about 12-24h after the first pollen grain mitosis and by the time of the second pollen grain mitosis, the pollen grain is packed with starch (Watanabe, 1961; Goss, 1968; Bennett *et al.*, 1973b). The second mitosis in the pollen grain takes place about 60h after the first. During this division only the generative nucleus divides to give rise to two sperm nuclei. This division possibly takes a similar period to the first, but definite information is lacking. The second pollen grain mitosis, like the first, is fairly synchronous (Bennett and Smith, 1972; Bennett *et al.*, 1973b). The two sperm nuclei, which are initially round, soon move apart and elongate to constitute two sperms with pointed ends. One end of the sperm becomes blunt before dehiscence (Batygina, 1962). Anthesis occurs about 60h after the second pollen grain mitosis, or about 7.5 days after the tetrad stage (Bennett *et al.*,

1973b). By this time, the volume of the pollen grain has increased considerably to about $11 \times 10^4 \mu m^3$. Just before dehiscence starch granules disappear from the pole containing the generative cell and become gathered around the vegetative nucleus (Watanabe, 1962; Bennett *et al.*, 1973b).

The mature pollen grain of wheat is an ovoid structure, 48-57µm in diameter and contains three cells; the largest being the vegetative cell with a large, spherical nucleus about 15µm in diameter and two sperm cells with elongated nuclei, each about 35µm long and 3µm wide.

The pollen grain has a single pore, about 6.3 to 9µm in diameter, covered with a lid or operculum (Percival, 1921; Goss, 1968; Bennett *et al.*, 1973b). The pollen wall consists of an inner, thicker hyaline layer, the intine, and an outer thinner, exine. The rim of the pore and the operculum consist of two layers of exine. In the pore region, the intine is thickened (Batygina, 1962; Goss, 1968). The texture of the exine, and hence the pollen grain surface is granular (Goss, 1968).

Information on the chemical constitution of wheat pollen is very limited. Large quantities of starch are present (Goss, 1968; Joppa *et al.*, 1968; Bennett *et al.*, 1973b) and sugars such as glucose and fructose have been detected (Goss, 1968). The free amino acid complement includes cystine, lysine, histidine, arginine, asparagine, aspartic acid, glutamine, glycine, threonine, α-alanine, proline, tyrosine, α-amino-butyric acid, tryptophan, phenylalanine, leucine, isoleucine and glutathione (Goss, 1968). Serine is absent. Unlike most angiosperm pollen (Stanley and Linskens, 1974) only a relatively low concentration of proline is present (Goss, 1968).

At maturity the four pollen sacs or thecae are filled with loose, powdery pollen of a yellowish colour. On the average a wheat anther produces about 1000 pollen grains, with variations between cultivars and possibly between different environments (Goss, 1968; Joppa *et al.*, 1968; Beri and Anand, 1971).

1.2.1.4. DEVELOPMENT OF THE TAPETUM DURING SPOROGENESIS :

The tapetum, the innermost layer of the anther wall, plays an essential nutritive role in the formation of the pollen grains since all the metabolites entering the sporogenous calls must either pass through or be metabolized by the tapetum (Echlin, 1971). There are two kinds of tapetum, the plasmodial and the secretory types (Vasil, 1967). In a plasmodial tapetum there is a rapid disruption of the inner walls of the tapetum soon after meiosis and the protoplasts of the tapetal cells extend in an amoeboid fashion among the developing microspores. The cells of the secretory tapetum, on the other hand, retain their peripheral position and contribute to the anther sap by secretion from their inner faces until the layer breaks down completely at maturity.

The single layered tapetum of wheat is of the secretory type (Bennett *et al.*, 1973b). Tapetal cells continue to divide until about 30h before the onset of meiosis in the PMCs, about 18h after the cessation of premeiotic mitoses in the archesporium. At this stage the tapetal cells are considerably smaller than the PMCs but outnumber them. DNA synthesis in the tapetal cells takes place at the same time as in the PMCs and is followed by nuclear division, either just prior to the onset of meiosis or during early prophase, depending on the cultivar (Bennett and Smith, 1972; Bennett *et al.*, 1973b). No wall formation accompanies the final nuclear division, resulting in binucleate tapetal cells which remain in that state until anther dehiscence.

The tapetum starts degenerating soon after meiosis in the PMCs is completed (Percival, 1921; Joppa *et al.*, 1966). Initially, following meiosis, the starch content of the tapetum increases but, soon after the first pollen grain mitosis, the starch grains rapidly disappear from the tapetal cells. The degeneration of the tapetum is continuous and at maturity all that remains of the tapetum is a thin layer of residue lining the anther cavity.

Wheat has not been extensively utilized in studies on the role of the secretory tapetum in the development of pollen grains, hence most of the information available on the subject has come from work on other genera including *Beta, Dichanthium, Cannabis, Helleborus and Lilium* (Vasil,

1967; Echlin, 1971).

During the pre-meiotic period tapetal cells are connected to one another and to the peripheral archesporial cells through plasmodesmata. The peripheral archesporial cells are similarly linked with the cells at the core of the column (Heslop-Harrison, 1964). Following callose deposition around the PMCs, their protoplasmic connections with the tapetum are severed and reappear after prophase at different positions (Vasil, 1967). These connections and those between the radial walls of the tapetal cells, allow free movement of cytoplasmic organelles such as plastids, mitochondria, pinocytotic vesicles, components of endoplasmic reticulum and sometimes even whole nuclei. (Vasil, 1967). Once meiosis is complete, the PMCs lose all plasmodesmatal connections with the tapetum and with each other.

During the early stages of meiotic prophase, a large number of vesicles, sometimes termed 'pinocytotic blebs' are cut off from the plasmalemma of the PMCs into their cytoplasm. Once released inside the PMC, these vesicles dissolve, releasing their contents. It is surmised that these contents are acquired from outside the cell (Heslop-Harrison, 1964).

At meiosis the tapetal cells have large nuclei, prominent endoplasmic reticulum and dictyosomes (Hoefert, 1969; Echlin and Godwin, 1968; Echlin, 1971). Tapetal cells reach their peak maturity at late meiosis and by the end of tetrad stage they begin to show signs of autolysis. To begin with, the tapetal cell membrane becomes convoluted and vacuolation increases followed by a gradual disappearance of the cell wall to leave only a thin layer. As wall formation in the pollen grains progresses, the tapetum becomes progressively more disorganized. Mitochondria and plastids disappear first and other organelles follow. Vesiculation within the cytoplasm increases. In the final stages of degradation, the RNA content of the cells increases and invaginations, thought to be secretory structures, appear in the cell membrane. The last to be disrupted are the cell membrane, nucleus and the endoplasmic reticulum when whatever little remains of tapetal cell contents is discharged into the anther fluid (Knox, 1962; Echlin, 1971). The general trend in tapetal development, therefore, consists of an initial synthetic phase followed by a process of

degeneration. The stage of maximum pollen development coincides with the stage of tapetal degradation (Echlin, 1971).

An interesting feature of the development of secretory tapetum is the occurrence of a large number of spherical structures on the inner walls of the tapetal cells. These structures have been termed "Ubisch bodies", "orbicules" or "plaques" (Heslop-Harrison, 1968; Echlin, 1971). The walls of the Ubisch bodies consist of sporopollenin, the main constituent of mature pollen grain exine. Because of this homology between the coating of mature pollen grain and that of the Ubisch bodies, it is reasonable to suspect that the latter may play a role in the formation of pollen exine. However, there is little evidence to substantiate this notion (Heslop-Harrison and Dickinson, 1969; Echlin, 1971). Since the deposition of sporopollenin occurs almost simultaneously on both Ubisch bodies and pollen grains it is possible that the former may just be a by-product of metabolism, or that they have a function other than exine fromation (Echlin, 1971).

Despite a common belief that the tapetum contributes materials to the developing pollen grains (Echlin, 1971; Stanley and Linskens, 1974; Heslop-Harrison, 1979), most of the evidence supporting such a role is circumstantial. Definite proof of the transfer of materials and the mechanism of such transfer is still awaited.

1.2.2. FEMALE REPRODUCTIVE DEVELOPMENT :

Knowledge concerning the development and structure of the female gametophyte of wheat is even more sparse than that of the male counterpart. The primary reason for this seems to be the difficulties associated with the location and handling of the female generative tissue, which consists of a single cell or, at later stages, only a few cells burried deep in a large volume of somatic tissue.

1.2.2.1. PRE-MEIOTIC ARCHESPORIAL DEVELOPMENT :

The mature wheat ovary is covered by unicellular hairs and the ovary wall consists of 10-16 layers of loosely packed parenchyma cells, bounded by an outer and an inner epidermis (Percival, 1921). The ovary is

supplied by three vascular bundles. Within the unilocular ovary, there is a single, sessile ovule which is longitudinally symmetrical until meiosis but thereafter becomes anatropous. It seems that the ovule is derived from the morphological apex of the floral axis, its apparent lateral position being due to the rapid growth of one side of the axis before the carpel closes (Percival, 1921; Bennett *et al.*, 1973a). The ovule, which is attached to a basal placenta on the inner ventral surface of the ovary, is bitegmic, each integument consisting of a double layer of cells. There is a micropyle directly opposite the chalaza. Integuments are differentiated at about the time when the tapetum develops in the anther (Percival, 1921). At meiosis the nucellar dome is completely enclosed by the integuments (Bennett *et al.*, 1973a). The nucellus is parenchymatous with a broad chalaza and an epidermis of closely fitting cells (Percival, 1921).

The ovule, in its early stages, consists of a hemispherical mass of meristem within the carpellary cavity (Percival, 1921). When the nucellus is about 6-7 cells across, a cell immediately beneath the epidermis of the nucellar dome differentiates into a single celled magaspore mother cell (MMC) or archesporium (Percival, 1921; Bennett *et al.*, 1973a). The MMC is conspicuous among the nucellar cells by its large volume and pear-shape, being greatly elongated and having its micropylar end much wider than the chalazal end (Bennett *et al.*, 1973a,b).

1.2.2.2. FEMALE MEIOSIS :

Meiosis in the MMCs of wheat is very similar to that in the PMCs. The DNA content of the MMC nucleus is 4C during prophase-1 and decreases to 2C during anaphase-1 and to 1C during telophase-2. Before the onset of meiosis, the MMC develops a callose wall around it which becomes thicker during the later stages of division (Bennett *et al.*, 1973b).

Unlike the duration of male meiosis, which has been measured directly (Bennett et al., 1971,73b; Bennett and Smith, 1972), the duration of female meiosis has been estimated indirectly by monitoring male meiosis and relating it to the development of the MMC within the same floret (Bennett *et al.*, 1973a,b). Despite the possibility of minor errors, the results obtained by this method appear to be reliable. Male and female

meioses occur almost simultaneously and both have similar durations at 20°C (Bennett *et al.*, 1973a,b). The greatest asynchrony observed between male and female meiosis within a single floret is about 11h. This is less than half the known duration of male meiosis (24h). The durations of the various stages of female meiosis appear to be approximately identical to those of male meiosis.

According to Percival (1921), both the meiotic divisions in the MMC occur at right angles to the long axis of the nucellus, thus providing a linear tetrad of megaspores. Bennett *et al* (1973a,b), however, have claimed that although meiosis-1 is in the same plane as reported by Percival, the plane of meiosis-2 differs between the two cells of the dyad. In the micropylar cell it is at right angles to the plane of the first division, whereas, in the chalazal cell it is in the same plane as the first meiotic division. Thus the end product of meiosis is a T-shaped tetrad. This difference between the two observations may be attributed either to genotypic variability or to different environmental conditions.

1.2.2.3. EMBRYO SAC DEVELOPMENT :

Following meiosis, the outer three cells of the tetrad degenerate rapidly (Percival, 1921; Bennett *et al.*, 1973b). The surviving megaspore enlarges greatly, pushing apart the cells on the sides of the narrow channel left by the degenerated sister cells, and ultimately comes into contact with the epidermis. It is this megaspore that develops into the embryo sac. In most angiosperms only one of the four megaspores survives to produce an embryo sac, perhaps either because it inherits a large complement of cytoplasmic organelles and food materials or because it is situated at a position within the ovule which is nutritionally more favourable (Russell, 1979; Kapil and Bhatnagar, 1981). The callose wall may also play a role in determining which megaspore is to give rise to the embryo sac by selectively cutting off the nutrient supply to some megaspores and diverting it to others (Kapil and Bhatnagar, 1981).

Bennett *et al* (1973b) have given a detailed account of embryo sac development in wheat which is of the *Polygonum* type (Maheshwari, 1950; Davis, 1966). The first mitotic division of the functional megaspore produces two nuclei: the primary micropylar and the primary chalazal.

The second division gives rise to a pair of nuclei at either end of the megaspore and the third results in two quartets of haploid nuclei lying at opposite poles of the elongating embryo sac. Three cells of the micropylar quartet differentiate into the egg apparatus consisting of an egg cell flanked by two synergids, whereas the fourth cell develops as the upper polar nucleus. The chalazal quartet differentiates into the lower polar nucleus and three antipodal cells. The antipodal cells undergo repeated divisions to produce 20-30 cells in the mature embryo sac (Bennett *et al.*, 1973b). There appears to be variation in the number of antipodal cells, 6-10 being reported by Percival (1921), 8-20 by Morrison (1950), 12-17 by Hoshikawa and Higuchi (1960) and about 16 by Wojciechowska and Lange (1977).

The timing of embryo sac development at 20°C corresponds with pollen grain development (Bennett *et al.*, 1973,b). The callose wall around the functional megaspore is broken down before the latter starts dividing. The volume of the newly formed functional megaspore is about $8.5 \times 10^3 \mu m^3$ and increases steadily throughout embryo sac development. The first mitotic division in the megaspore occurs a few hours before the first mitosis in the pollen grains and the subsequent two follow in rapid succession. The second and third mitoses occur simultaneously in all the embryo sac nuclei. The duration of the first cell cycle in the developing megaspore is between 48 and 72h whereas the next two cell cycles last about 12h each. Hence by the time the first pollen grain mitosis is completed in all the microspores, the embryo sac contains eight nuclei. Soon after the first mitosis, a vacuole appears in the centre of the developing embryo sac. Cell walls are laid down only after the eight embryo sac nuclei have been formed. Following the first pollen grain mitosis, the antipodal cells in the embryo sac start proliferating and reach their maximum number before the completion of second mitosis in the pollen mother cells. Within 24-36h of the first mitosis in the pollen grains, the polar nuclei move together and take their position at the centre of the embryo sac. During the period from the second pollen grain mitosis to anther dehiscence, differentiation of the egg and synergid nuclei occurs in the embryo sac. Although the antipodal nuclei do not divide further after the second pollen grain mitosis, DNA synthesis within them continues until maturity. Because of these endomitoses, the average DNA content of the antipodal cells at anthesis is close to 52C. Hoshikawa

and Higuchi (1960) have reported a somewhat different course of development of the antipodal cells, where about 40 cells are produced by 3-4 days before fertilization. Part of these cells form a short lived haustorium but others on the micropylar side form a complex of 12-17 large mature cells by the time of flowering. They have suggested that the variation in the number of antipodal cells with age indicates that they contribute to the nutrition of the embryo sac.

At maturity the pyriform embryo sac measuring about 270x160µm, lies embedded in the nucellus with its narrow end in direct contact with the epidermis opposite the micropyle (Percival, 1921). The egg apparatus is situated at the micropylar end of the embryo sac and consists of one large centrally located egg cell or ovum about 50x32-35µm in size and two synergids measuring about 20x16µm (Percival, 1921; Bennett *et al.*, 1973b; Wojciechowska and Lange, 1977). The egg cell contains a large, spherical, faintly-staining nucleus usually with a single nucleolus. The cytoplasm of the egg cell is distinct from that of the surrounding cells in that it contains numerous small granules which are perhaps fatty in their constitution (Morrison, 1955; Bennett *et al.*, 1973b). Neither the synergids nor the central cell contains any such globules. The cytoplasm of the egg is rich in starch and, at earlier stages, in RNA the level of which declines to some extent at maturity (Kapil and Bhatnagar, 1981). Synergid nuclei are smaller than the egg nucleus and are somewhat elongated (Bennett *et al.*, 1973b). The polar nuclei lie close together in a band of cytoplasm between the egg cell and the nearest antipodal. Each of the polar nuclei is larger than the egg nucleus and has one large prominent nucleolus and several smaller ones (Bennett *et al.*, 1973b; Wojciechowska and Lange, 1977). The polar nuclei fuse before fertilization to give the 'polar nuclear body' (Percival, 1921; Bennett *et al.*, 1973b). Hoshikawa and Higuchi (1960) and Batygina (1978) however, have claimed that no such fusion of the polar nuclei occurs before fertilization. At the chalazal end lies a variable number of antipodal cells, each about 32-60µm in diameter, containing vacuolated cytoplasm and large nuclei, upto 40µm in diameter and having high DNA content through endo-reduplication (Percival, 1921; Bennett *et al.*, 1973b).

Kapil and Bhatnagar (1981) have reviewed ultrastructural aspects of

the development and constitution of embryo sac in flowering plants. Despite the diversity in fine structure, the embryo sacs of various genera among higher plants conform to a general trend. The structural features of the egg indicate a state of metabolic inertia while the stock of food reserves suggest its preparedness for future events. The synergids and antipodal cells, on the other hand, have characteristics of transfer cells suggesting a function in obtaining nutrients for the embryo sac.

1.2.3. FLOWERING AND GRAIN SET :

When the androecium and gynaecium reach their peak maturity, the wheat spikelet goes through a series of coordinated events constituting the process of flowering, which results in fertilization and the start of the next generation in the newly set seed.

1.2.3.1. ANTHESIS, DEHISCENCE AND POLLINATION :

Anthesis starts in a spikelet situated about two thirds of the way up from the base of the ear and proceeds in either direction along the spike. Among the florets within a spikelet the order of flowering is acropetal. A spike usually requires about 3-5 days to complete flowering (Peterson 1965; De Vries, 1971).

At the time of flowering, the lodicules rapidly draw water from the ovary and swell pushing the lemma and palea apart within about 5 minutes (Peterson, 1965; Goss, 1968; De Vries, 1971). Flowers usually remain open for 8-30 minutes (Evans *et al.*, 1975). At the same time the filaments elongate rapidly, reaching 2-3 times their original length within about 2-4 minutes, thus pushing the anthers out of the floret. Simultaneously the stigmas quickly separate from each other and appear more feathery. While the filament is elongating, the anthers dehisce, opening first at the tip and then splitting along both sides. The dehiscence of the anthers releases pollen, some of which falls on the stigma of the same floret but most outside the floret. The actual amount of pollen that is released outside the floret varies from cultivar to cultivar (Beri and Anand, 1971). A high proportion of fertile pollen within an anther and warm weather favour anther dehiscence (De Vries, 1971). As a general rule wheat is a self-pollinated crop, although a small amount of outcrossing also occurs (Leighty and Taylor, 1927; Peterson, 1965).

Soon after pollination is effected, the lodicules lose their turgidity and collapse, lemma and palea return to their original position and the floret is once again closed (De Vries, 1971). The extent to which the flowers open and anthers extrude is subject to genetic and environmental influences (Joppa *et al.*, 1968; De Vries, 1971). Florets containing sterile anthers remain open much longer than those with fertile anthers, possibly because of a delay in pollination. Apparently, the physiological mechanism controlling the collapse of the lodicules is activated soon after pollination (De Vries, 1971). If pollination fails at the time of flowering, the lemma and palea may again be pushed apart by expansion of the ovary about 3 days later. This re-opening of flowers facilitates cross-pollination in some of the unfertilized florets, though not necessarily in all since the pistils of some florets may lose their receptivity by this time (Hoshikawa, 1961).

In general, binucleate pollen grains have a much longer life span than trinucleate ones (Hoekstra and Bruinsma, 1975; Johri and Shivanna, 1977) and as graminaceous pollen belongs to the latter class it is generally of short viability (Davis, 1966). Pollen grains of wheat lose their viability within a very short period after release from the anther (Goss, 1968; De Vries, 1971). Although it is possible to prolong the longevity of wheat pollen to several hours or even a few days by careful storage, under natural conditions the pollen survives for no more than a few minutes following its release from the anther (De Vries, 1971). Indeed, viability may be lost completely within five minutes in some instances (Athwal and Kimber, 1970). Environmental conditions at the time of dehiscence may also affect the longevity of pollen to a certain extent (Goss, 1968; De Vries, 1971).

In a plant such as wheat, where the viability of the pollen grain is short, prolonged receptivity of the pistil is important for successful seed set by cross-pollination where self-pollination has failed. Such an advantage is available to wheat since stigma receptivity and ovule viability, and hence the capability to set grains is retained for about 7-8 days after anthesis (Imrie, 1966; De Vries, 1971; Evans *et al.*, 1972) but peak activity lasts only 3-5 days (De Vries, 1971). The duration of pistil receptivity is adversely affected by high temperature and low humidity during flowering (Imrie, 1966; De Vries, 1971).

1.2.3.2. POLLEN GERMINATION, POLLEN-TUBE GROWTH AND FERTILIZATION:

Even though the development of the sex-organs may have proceeded quite normally, grain setting can still be hindered by the failure of any single process among pollen germination, tube growth, fertilization and post-fertilization cell division in the primary endosperm nucleus and zygote. This problem is illustrated by the fact that not all florets reaching anthesis actually set grains (Evans *et al.*, 1972).

Depending on the nature of their receptive surfaces, angiosperm stigmas can be classified either as wet or dry (Heslop-Harrison and Shivanna, 1977). Stigma of wheat, as of most other grasses, belong to the latter category where there is little fluid secretion from the stigmatal surface at maturity and the receptive cells are dispersed in multiseriate branches. Once in contact with the feathery stigma, pollen grains usually germinate within a few minutes (Hoshikawa, 1959; Chandra and Bhatnagar, 1974; Batygina, 1978), although considerably longer germination times have been occasionally reported (Peterson, 1965). The percentage pollen germination is increased by a higher concentration of pollen on the stigma (Chandra and Bhatnagar, 1974; Ter-Avanesian, 1978). The optimum temperature for pollen germination is around 20°C (Hoshikawa, 1960).

The pollen tube emerges by dislodging the operculum and pushing its way out through the germ pore, which need not be in contact with the stigma (Chandra and Bhatnagar, 1974). Depending on whether or not the germ pore is in contact with a stigmatic hair, it takes a pollen tube about 2-15 minutes to penetrate the hair. Two generative nuclei, followed by the vegetative nucleus, leave the pollen grain and enter the pollen tube, moving forward as the tube grows at the tip. The subsequent growth of the pollen tube through the stylar tissue is rapid. Pollen tubes enter the ovarian locule, usually within 1h, though some may take several hours (Hoshikawa, 1959; Chandra and Bhatnagar, 1974; Wojciechowska and Lange, 1977; Batygina, 1978). Once within the locule, a pollen tube passes between the inner epidermis of the ovary and the outer integument of the ovule and travels to the tip of the micropyle where it enters the ovule (Chandra and Bhatnagar, 1974; Batygina, 1978). A few tubes may reach the micropyle but only one penetrates the embryo sac (Wojciechowska and Lange, 1977). It has been suggested that pollen tubes that do not penetrate the

embryo sac provide nutrients to the developing embryo (Goss, 1966).

Although initial wall synthesis in pollen tube is supported by the stored reserves in the pollen grain, the tissue of the angiospermic pistil soon assumes an important role in the nutrition of the growing tube. A large proportion of materials, notably carbohydrates utilized by the pollen tube, are obtained from the pistil tissue (Loewus and Labarca, 1973). The embryo sac also appears to play a significant role in directing the pollen tube growth towards the ovary (Rosen, 1964; Jensen, 1965; Sedgley, 1976). Pollen tubes are thought to be attracted chemotropically towards the embryo sac, possibly by cations such as Ca^{++} and K^{++} released from one of the synergids (Rosen, 1964; Jensen, 1965; Glenk *et al.*, 1971; Jensen and Ashton, 1981).

Upon coming in contact with the embryo sac, the pollen tube penetrates one of the synergids and discharges the two sperm nuclei which then travel to the cytoplasmic band between the egg cell and the polar nuclei (Hoshikawa, 1959; Wojciechowska and Lange, 1977; Batygina, 1978). The two sperms separate after this and travel in the opposite direction. One of them enters the egg by piercing through the cell membrane and fuses with its nucleus to from the zygote. Some controversy surrounds the exact manner in which the other sperm nucleus fuses with the polar nuclei. According to Hoshikawa (1959), this sperm nucleus first fuses with one polar nucleus and then, after a few hours, this combined nucleus fuses with the other polar nucleus to form the primary endosperm nucleus. The existence of two separate polar nuclei before fusion with the sperm nucleus has also been acknowledged by Wojciechowska and Lange (1977) and Batygina (1978). However, Bennett *et al.*, (1973b) have reported that the two polar nuclei fuse before fertilization to produce a 'primary nuclear body' which on fertilization by the sperm nucleus gives rise to the primary endosperm nucleus. This latter sequence has been reported in other species (Jensen, 1972).

Following the arrival of sperm nuclei, the fusion with the embryo sac nuclei is slow in comparison with the rapid travel of the pollen tube down to the ovule. For several hours after pollination the contents of the fusing nuclei can be seen in separate groups, closely appressed together, though it is not always possible to observe individual chromosomes

(Hoshikawa, 1959; Bennett *et al.*, 1973b; Wojciechowska and Lange, 1977; Batygina, 1978). The actual integration of chromosomes in the primary endosperm nucleus, in fact, is delayed until the commencement of its division about 5h after pollination. In the zygote, on the other hand, the fusion of chromatin materials is not achieved until 18-20h after pollination (Bennett *et al.*, 1973b; Batygina, 1978).

The zygote goes through an initial resting period of short duration (Wojciechowska and Lange, 1977), and cell division begins about 22h after pollination. At this time the endosperm, which started dividing 5h after pollination, already consists of about 16 nuclei (Hoshikawa, 1961; Bennett *et al.*, 1973b). About 3-5 days after fertilization the antipodal cells start degenerating, and within approximately nine days all traces of their existence are lost (Morrison, 1955; Bennett *et al.*, 1973b). The degenerating antipodal cells are replaced by the developing endosperm cells, an observation which has often led to speculation that the antipodal cells provide nutrition for the growing endosperm (Percival, 1921; Hoshikawa, 1961). Five days after fertilization, the embryo and the endosperm have approximately 96 and 5000 cells respectively (Bennett *et al.*, 1973b) and the young grains can be seen in florets.

1.3. ENVIRONMENTAL STRESS AND REPRODUCTION

Grain number per spike, one of the most important determinents of yield in wheat (Evans, 1978) is influenced by the number of spikelets per spike, the number of florets per spikelet and the proportion of florets that actually set grains (Evans and Wardlaw, 1976). That the number of spikelets and florets is influenced by environmental conditions prevailing during the period of spikelet- and floret- initiation has already been discussed in section 1.1. Environmental conditions later in the reproductive phase may affect grain set by affecting any of a number of processes such as the development of sex organs, meiotic divisions in the spore mother cells, sporogenesis, gametogenesis and fertilization. Effects of water deficit, heat and other environmental perturbations on the reproductive development after the initiation of sex organs in wheat are discussed here.

1.3.1. WATER DEFICIT:

Udol'skaja (1936) was probably the first to address the question of whether there was a specific stage of sensitivity to drought during the reproductive phase; she reported that the most severe reduction in yield of wheat occurs when water deficit coincides with the period of reduction division in the sex organs. Subsequent studies by many other Russian scientists which added to the initial observation of Udol'skaja were summarised by Skazkin (1961) who pointed out that cereals are highly susceptible to drought during the period from the appearance of "staminate tubercles" up to fertilization. Indeed it has been confirmed repeatedly that water deficit during this period of reproductive development causes serious reduction in grain set and hence yield of wheat (Salter and Goode, 1967; Dubetz and Bole, 1973; Fischer, 1973), barley (Skazkin and Fontalina, 1951; Aspinall *et al.*, 1964; Wells and Dubetz, 1966) maize (Denmead and Shaw, 1960) rice (Matsushima, 1962; O'Toole and Moya, 1981), oats (Van der Paauw, 1949; Novikov, 1952; Skazkin and Lerman, 1952) and other grasses (Salter and Goode, 1967).

Russian scientists, notably Skazkin and coworkers, were among the first to explore the reasons for reduced grain set under water stress conditions. Water stress during meiosis and tetrad formation was found to

induce extensive pollen sterility in wheat, barley and oats (Novikov, 1952; Skazkin, and Zavadskaya, 1957; Zavadskaya and Skazkin, 1960; Skazkin, 1961). Skazkin and Zavadskaya (1957) reported abnormalities in chromosomal pairing and separation during meiosis in barley PMCs, which probably led to an uneven distribution of chromosomes in daughter nuclei and hence to pollen sterility. Bingham (1966) reported that in wheat the maximum sensitivity to drought occurs during or a few days before meiosis and that the decline in grain set was due exclusively to the induction of male sterility since hand pollination of the stressed plants showed that female fertility was unaffected. The loss of grain set in the basal florets of a spikelet was partially compensated by additional grain set in the more distal florets that would normally have been barren. Compensatory grain set such as this, was also observed by Evans *et al.*, (1972) after manual sterilization of florets. Water deficit can also cause female sterility in oats additional to the male sterility (Skazkin and Lukomskaya, 1962). Female sterility, however, is induced only by "severe and prolonged" water stress. Under these conditions, the antipodal cells are deformed and gradually disappear; the lumen of the embryo sac becomes filled up with nucellar cells and the embryo sac degenerates to a withered strand lacking functional elements. Application of boron (Skazkin and Rozkova, 1956) or nitrogen (Skazkin and Zavadskaya, 1957) just before the critical period partially ameliorates the effects of water deficit on pollen fertility.

In most of these reports, the conclusions about the critical stage of development and the nature of stress-induced injury are not substantiated by convincing evidence. The use of confusing, unexplained and sometimes conflicting terminology by some authors renders critical evaluation of these investigations difficult. For instance the water deficit in the soil or the plants was rarely estimated reliably, making it impossible to compare the results of different studies or to assess their significance.

Grain set in wheat is not affected until the xylem water potential falls below -12 bar, but when the water potential approaches -24 bar, grain set is virtually nil (Fischer, 1973). Recently Morgan (1980) found no change in grain set until the leaf reached approximately zero turgor. Furthermore, despite a fall in the leaf turgor and grain set, the relative water content and turgor potential of the spikelets remained unchanged,

indicating that it was unlikely that the effect of water deficit on pollen fertility was due to the desiccation of the sporogenous tissue.

Water deficit during or immediately after anthesis can also cause some reduction in grain set in wheat, although this effect is less than that during meiosis and early pollen development (Wardlaw, 1971; Brocklehurst *et al.*, 1978). Whether this effect of water deficit during anthesis is due to the failure of fertilization, or to the abortion of grains, remains unclear.

1.3.2. HEAT :

The number of grains per spike of wheat is reduced when the plants are grown at temperatures above about 20°C during the reproductive phase (Fischer and Maurer, 1976; Warrington *et al.*, 1977; Kolderup, 1979; Rawson and Bagga, 1979). Apart from the reduction in grain number due to the reduction in spike size caused by high temperature during the period of spikelet initiation and development (Section 1.1.), high temperature during the development of sex organs also appears to affect grain set. Exposure to a hot wind for a short period at the 'boot stage' (Smika and Shawcroft, 1980) and brief periods of extremely high (up to 40.5°C) night temperature at anthesis (Langer and Olugbemi, 1970) can drastically reduce grain set in wheat. Failure of daughter cells to separate after the first meiotic division in the PMCs of rye plants grown at 25°C resulted in bilocular, sterile pollen (Bennett et al., 1972).

The duration of meiosis in PMCs of wheat and rye is temperature dependent, the division being faster at higher temperatures (Bennett *et al.*, 1972).

Wheat plants grown at 27°C Day and 22°C night temperature immediately following anthesis had lower grain set than those grown at 15°C day and 10°C night temperature (Wardlaw, 1970). High temperature during anthesis can have adverse effects on pollen viability and stigma receptivity in wheat (Imrie, 1966; Goss, 1968). Optimum temperature for best pollen tube growth within wheat styles appears to be about 20°C (Hoshikawa, 1960). Although pollen tube growth is faster at higher temperatures, the chances of successful fertilization are diminished at about 30°C Hoshikawa, 1959b, 66).

Effects of heat stress in the field are hard to separate from those of water stress, particularly in semi-arid environments, because these stresses usually occur together (Hall *et al.*, 1979). High temperatures such as those employed in several of the above mentioned studies may have caused tissue desiccation, making it impossible to differentiate between the effects of water deficit and those of heat *per se*. Hence, most of the abnormalities attributed to heat could have been caused by water deficit induced by excessive evapotranspiration at the high temperatures used. This controversy needs to be resolved by studying the direct effects of temperature for example by using high atmospheric humidity to prevent heat-induced water deficit.

1.3.3. OTHER ENVIRONMENTAL FACTORS :

Sporogenesis, particularly microsporogenesis, in Poaceae is sensitive to a wide variety of environmental perturbations. Exposure to temperature between 0-3°C during the first pollen grain mitosis can cause male sterility in wheat (Toda, 1962). Cooling rice plants to 12°C for four days during PMC meiosis causes substantial decrease in grain set (Hayase *et al.*, 1969). Low temperature affects grain set by inducing male sterility while not affecting female fertility (Hayase *et al.*, 1969; Ito *et al* 1970). The greatest sensitivity to low temperature in rice seems to occur not during meiosis but immediately thereafter, during the young microspore stage (Satake and Hayase, 1970,74). Abnormalities in anther development become apparent 4-6 days after meiosis when the respiratory activity and protein content of the anthers start declining. (Nishiyama, 1970). Abnormal anthers stop developing early and fail to extrude or dehisce at anthesis (Ito *et al.*, 1970). Low night temperatures just before PMC meiosis in *Sorghum* can also cause male sterility but not female sterility (Brooking, 1976).

Flooding of rice plants during reduction division in PMCs causes severe decline in grain set (Matsushima, 1962). Effects of flooding on fertility are possibly due to poor aeration, as has indeed been found by Campbell *et al* (1969) with wheat and barley, where lack of soil aeration leads to pollen sterility. Microsporogenesis and hence grain set in these two crops is also affected by deficiency of nitrogen (Skazkin and Zavadskaya, 1957; Zavadskaya and Skazkin, 1960; Campbell and Davidson,

1979; Campbell and Leyshon, 1980) and copper (Graham, 1975). Photoperiodic disturbances can cause male sterility in barley and induce floral abnormalities in maize (Galinat and Naylor, 1951) and wheat (Fisher, 1972). Grain set in wheat can be reduced by high plant density (Darwinkel, 1980). Since low light intensity during the reproductive development can also reduce grain set in wheat (Wardlaw, 1970; Willey and Holliday, 1971) and maize (Prine, 1971), the effects of high plant density on grain set may, at least in part, be due to increased shading.

1.4. HORMONAL CONTROL OF STRESS - INDUCED STERILITY

Plant growth and development is well known to be controlled by the interplay of various growth substances or hormones (Thimann, 1977). Stressful environmental conditions bring about changes in the concentration of hormones which may in turn modify various physiological processes and finally the developmental pattern of the plant (Hsiao, 1973; Vaadia, 1976; Aspinall, 1980). In keeping with the scope of the present investigation, the discussion here will be restricted to the effects of water stress and heat on abscisic acid and ethylene, and the role of these hormones in regulating floral fertility.

1.4.1. ABSCISIC ACID :

The most dramatic hormonal response to water stress on record is the rapid and substantial accumulation of abscisic acid (ABA). An increase in the inhibitor-ß content of excised wheat leaves was first reported by Wright (1969) and the inhibitor was identified, using optical rotary dispersion analysis, as (+)-abscisic acid (Wright and Hiron, 1969). These initial findings were followed by numerous other similar reports of water stress induced increase in ABA content of various organs of a wide array of plant species (Milborrow, 1974). Furthermore, in some species ABA also accumulates in response to high temperature (Hellali and Kester, 1979; Daie *et al.*, 1981; Daie and Campbell, 1981) and a number of other conditions of environmental stress such as low temperature, nutrient deficiency, radiation, salinity and water logging (Mizrahi *et al.*, 1971; Mizrahi and Richmond, 1972; Goldbach *et al.*, 1975; Shaybany and Martin, 1977; Degani and Itai, 1978; Rakhimbaev *et al.*, 1978; Daie *et al.*, 1979). Changes in ABA levels under these conditions, however, are not as marked as those under water stress.

There are few aspects of plant growth and development that are not affected by ABA. The current state of knowledge about the physiological effects of ABA, the mechanism of induction of ABA accumulation in response to stress, the biosynthesis and metabolism of the hormone, its localisation within the cell, its mode of action and the role it plays under stress conditions have been comprehensively reviewed in recent years (Milborrow, 1974; Aspinall, 1980; Walton, 1980, 80/81). This discussion, therefore, will be limited to the effects of ABA on floral fertility.

Only a limited amount of information is available on the effects of ABA on plant sexuality. Treatment with ABA promotes femaleness and reduces the positive effect of gibberellin on maleness in cucumber (Rudich and Halevy, 1974). In sweet corn, application of ABA during early tassel development mimics the effects of water stress which inhibits the growth of the terminal male inflorescence and promotes the growth of lower axillary inflorescences, consequently causing functional male sterility (Damptey and Aspinall, 1976; Damptey *et al.*, 1976,78). ABA may be involved in the inhibition of flowering in *Lolium* by water deficit (King and Evans, 1977).

Following immersion of flag leaves of wheat plants for 2 min in an ABA solution, Morgan (1980) observed a reduction in grain set. Application of ABA after the second pollen grain mitosis had no significant effect on grain set. The reduction in grain set was due to the induction of pollen sterility by ABA application. Since these effects were identical to those of water deficit, it was concluded that ABA may have a role in reducing grain set and male fertility in water-stressed wheat plants. Endogenous concentration of ABA in response to the exogenously supplied hormone was, however, not estimated. It is, therefore, difficult to assess whether the sterility caused by water deficit or application of exogenous ABA was also associated with equivalent endogenous concentrations of the hormone in the spikelets in response to the two treatments. Radley (1980) reported a reduction in grain set when ABA was applied at, or just before, anthesis but the hormone was applied at high concentrations and it is possible that the response was due to an excessive hormone concentration within the tissue.

1.4.2. ETHYLENE :

Ethylene is the only plant hormone which is a gas under physiological conditions of temperature and pressure. Considerable interest has been shown during the last two decades in the biosynthesis, metabolism and physiological role of ethylene in plants. Ethylene exerts a major regulatory influence on most, if not all, aspects of plant growth, development and senescence. Detailed reviews of the work in this field are abundant (Burg, 1962; Pratt and Goeschl, 1969; Abeles, 1972; Lieberman, 1979; Yang, 1980). Hence, only the work concerning ethylene

production in response to water deficit and heat and the effects of the hormone on floral fertility will be discussed here.

Ethylene production by a number of plant tissues is augmented by a variety of environmental stresses (Lieberman, 1979; Aspinall, 1980). Water stress enhances ethylene production in wheat (Wright, 1977) cotton (McMichael *et al.*, 1972), Avocado (Adato and Gazit, 1974), *Citrus* (Ben-Yehoshua and Aloni, 1974) and *Vicia faba* (El-Beltagy and Hall, 1974; Hall *et al.*, 1977). Leaves of well-watered wheat plants produce very little ethylene but, following the imposition of water stress, ethylene emanation increases more than 30-fold within 4h and subsequently declines to pre-stress levels within 24h (Apelbaum and Yang, 1980). These authors suggest that water stress induces *de novo* synthesis of 1-aminocyclopropanecarboxylic acid synthase in wheat, which is the rate-controlling enzyme in the pathway of ethylene biosynthesis. Water stress-induced ethylene evolution is thought to exert a controlling influence on leaf and fruit abscission (Jordan *et al.*, 1972; Lipe and Morgan, 1973).

Evolution of ethylene from leaf tissue of *Phaseolus* increases with increase in temperature up to about 37.5°C beyond which there is a rapid decline, and no detectable ethylene is produced above 42.5°C (Field, 1981a,b). Inhibition of ethylene evolution has also been reported to occur in cotton at 45°C (Vieira and Cothren, 1980) and apple and *Phaseolus* at 35-40°C (Yu *et al.*, 1980).

Leyshon and Sheard (1978) observed a decrease in grain yield of barley upon flooding and attributed this effect to oxygen deficiency and increased ethylene emanation from the flooded soil. Ethephon (2-Chloroethylphosphonic acid) is known to break down and release ethylene at pHs higher than 4.0, generally encountered in the living cells (Cooke and Randal, 1968; Edgerton and Blanpied, 1968). When sprayed on wheat and barley plants during the reproductive phase, in concentrations around 2000ppm, ethephon can induce complete or near complete male sterility without affecting female fertility (Rowell and Miller, 1971; Bennett and Hughes, 1972; Stoskopf and Law, 1972; Law and Stoskopf, 1973; Fairey and Stoskopf, 1975; Verma and Kumar, 1978). According to Bennett and Hughes (1972), ethephon affects male fertility in wheat by inducing additional mitosis in pollen grains. The treatment is effective only if applied

before the commencement of meiosis in the PMCs. Rowell and Miller (1971) were, however, able to induce complete sterility in wheat by applying ethephon even a few days after the completion of meiosis. Since water deficit, heat and many other environmental hazards may enhance the evolution of ethylene (Lieberman, 1979; Aspinall, 1980; Field, 1981b), the loss of grain set and male fertility under these conditions (Section 1.3) may well be mediated through this hormone.

1.4.3. INTERACTION BETWEEN ABSCISIC ACID AND ETHYLENE :

The nature of the interaction between ABA and ethylene is far from understood and it seems to vary with the type of tissue. Both ABA and ethylene inhibit the growth of etiolated pea seedlings (Lieberman and Kunishi, 1971) and ABA-inhibited seedlings show depressed ethylene production (Gertman and Fuchs, 1972). ABA and ethylene, however, appear to inhibit the growth of these seedlings by different mechanisms (Lieberman, 1979). Inhibition of ethylene production by ABA was also observed in IAA-stimulated etiolated mung bean hypocotyls (Kondo *et al.*, 1975). On the other hand, addition of ABA caused a sharp rise in ethylene production in senescent tobacco leaf disks (Lieberman and Fuchs, 1972). By using carbon dioxide to inhibit ethylene action, Mayak and Dilley (1975) showed that ABA hastens senescence of carnation flowers by advancing the onset of autocatalytic ethylene production. Conversely, application of ethylene to harvested *Citrus* fruit resulted in a large accumulation of ABA in the peel within 24h, similar to the accumulation of ABA when the fruits were allowed to senesce naturally on trees (Goldschmidt *et al.*, 1973). A reciprocal relationship between ABA and ethylene in causing senescence is apparent from these results.

Application of ABA can promote ethylene evolution from *Calamondin* leaves (Cooper *et al.*, 1968), *Citrus* bud cultures (Goren *et al.*, 1979) and *Citrus* leaf explants (Sagee *et al.*, 1980). Using inhibitors of ethylene biosynthesis, Goren *et al.*, (1979) and Sagee *et al.*, (1980) demonstrated that ABA-induced callus formation in *Citrus* bud cultures and abscission in *Citrus* leaf explants were mediated by ethylene.

Water stress induces accumulation of ABA as well as evolution of ethylene in detached wheat leaves (Wright, 1977). However, application of

ABA inhibits the production of ethylene induced by water stress in excised wheat leaves (Wright, 1980). Similarly, application of ABA to excised leaves of *Vicia faba* is without effect, or possibly has a depressive influence on ethylene emanation (Hall *et al.*, 1977). It is unlikely that the water-stress-induced evolution of ethylene has any stimulatory effect on the accumulation of ABA in excised wheat leaves since, upon imposition of stress, ABA increases earlier and at a faster rate than does ethylene (Wright, 1977). What effect endogenously synthesized ABA may have on ethylene evolution from stressed tissue, however, remains unknown. since there is usually a parallel increase in ABA accumulation and ethylene evolution from most of the stressed tissues, it is likely that the relationship of exogenously supplied ABA to ethylene is different from that of the endogenously produced hormone.

1.5. THE PRESENT INVESTIGATION :

Despite several past reports, the knowledge about the effects of water stress and heat on male and female sporogenesis in wheat is limited. This project was undertaken to further the knowledge about stress-induced abnormal sporogenesis by conducting systematic investigations into the effects of carefully monitored and measured water deficit, imposed during several specific stages of sexual development, on grain set. The investigation has also sought to distinguish between the effects of water deficit and heat by examining the response of grain set to elevated temperature in plants grown under high atmospheric relative humidity thus avoiding heat-induced water-loss. The nature of injury inflicted to the male and female spores by the two types of stress was studied through extensive microscopic examination of the developmental pattern and the structure of mature micro- and megaspores. Also studied was the germination of fertile pollen and pollen tube growth on heat-affected pistils. Finally an attempt was made to explore the physiological basis of stress-induced abnormal sporogenesis by investigating the possibility that the effects of water stress and heat on sporogenesis may be mediated by ABA and/or ethylene.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. PLANT MATERIAL:

The seed of *Triticum aestivum* L. Cv. Gabo was kindly supplied by Dr. A.J. Rathjan of the Department of Agronomy, Waite Agricultural Research Institute. This seed was multiplied in the glasshouse of the Department of Plant Physiology and was used to raise wheat plants throughout this project.

2.1.2. CHEMICALS AND REAGENTS:

All chemicals and reagents used for purification and gas liquid chromatography were of analytical grade and the remainder were of at least laboratory reagent grade. The source of important chemicals are given where they are first mentioned.

2. 1. 3. SOLVENTS:

All solvents used for the extraction of abscisic acid were distilled in a glass fractionating column before use.

Petroleum spirit (b.p. 60°-80°C) was dehydrated by distilling and storing over calcium chloride for two weeks after which it was stored over freshly extruded sodium wire in a tightly capped bottle.

2.2. METHODS

2.2.1. PLANT CULTURE :

In the earlier stages of this project, plants were grown in steam-sterilized recycled soil (R.S.) but later it was decided to switch over to the University of California Mix (U.C.) (Matkin and Chandler, 1972) since the latter gave better growth of plants. Only one type of soil was used in any one experiment. No extra nutrients were supplied to plants grown in R.S., whereas the pots of U.C. were watered with adequate liquid fertilizer to provide some run-through, when the plants were 4 weeks old and at intervals of two weeks thereafter. The fertilizer solution was prepared by dissolving 5g each of mono-ammonium phosphate, ammonium nitrate and potassium chloride in 10 litres of water. Plants were grown either in 5 litre pots, in which case they were thinned to 6 per pot after four weeks of growth, or in 1.4 litre pots where only one plant was retained per pot.

In experiments including water-stress treatments the water regime was controlled from daily measurements of pot weight. Except when water-stress was imposed, a high water regime was maintained by watering to field capacity whenever the water in the pots was depleted to 75 per cent of field capacity. In all experiments where plants were not subjected to water deficit, the pots were watered frequently to ensure a small volume of run-off.

Plants were raised in a cooled glasshouse for 4 weeks following germination and were then transferred to either one of two identical controlled-environment cabinets maintained at 20±1°C constant temperature, 16hr photoperiod and average canopy level irradiance of 350 $\mu E.m^{-2}S^{-1}$ (400-800nm) of fluorescent and incandescent illumination (40X85W cool white fluorescent tubes, and 8X60W incandescent bulbs). All treatments were applied in the controlled environment cabinets and the plants were returned to the glasshouse at the end of all treatments or the commencement of anthesis, whichever occurred later.

Usually no tillers were removed from the plants except when they were to be exposed to an ethylene-containing atmosphere, when either the main

shoots or primary tillers alone were retained. Whenever it was necessary to prevent cross-pollination, spikes were covered just before anthesis with glassine bags which were removed about two weeks later.

2.2.2. IMPOSITION OF STRESS TREATMENTS:

Water stress was imposed at various stages of development by withholding water and monitoring the soil water content from daily measurements of pot weight. Pots were rewatered to field capacity when their soil water content had been depleted to 20 per cent of field capacity and normal watering was continued thereafter. Control plants were watered normally throughout an experiment. Details of timing of water stress treatments in relation to the developmental stages are given along with the relevant experiments.

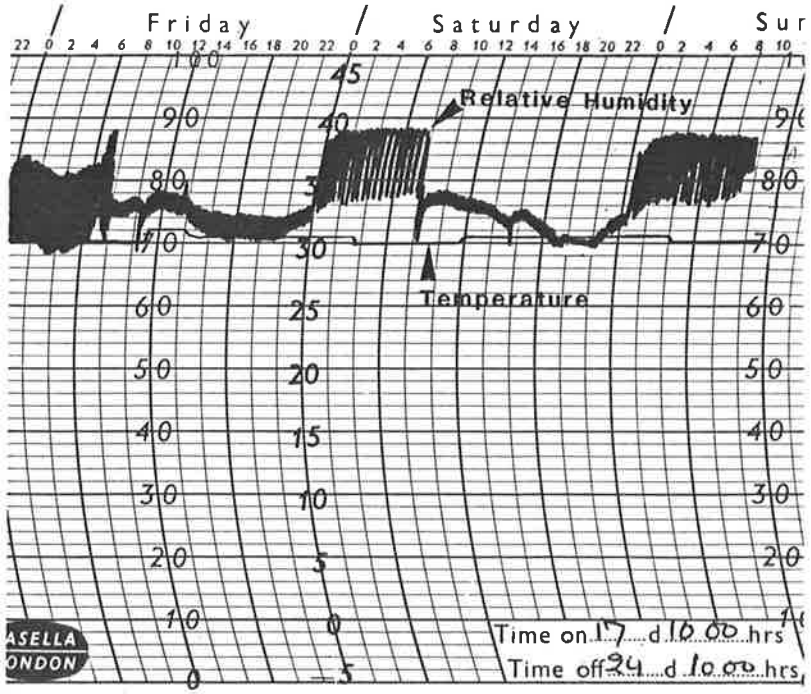


Fig. 2.2.1. A typical thermohydrograph recording during a heat treatment (30°C)

Plants were exposed to a high temperature at various developmental stages, by transferring them for the desired length of time to a controlled environment cabinet maintained at the required temperature. The atmospheric relative humidity was kept above 70 per cent during the high temperature treatments in order to prevent excessive evapotranspiration (Fig 2.2.1.). Control plants were always retained at 20°C.

The relationship between the various high temperature treatments and the stages of plant development is detailed in the sections describing the specific experiments.

2.2.3. APPLICATION OF ABSCISIC ACID AND TISSUE SAMPLING:

Solutions of abscisic acid (ABA) in water were applied through a wick in the manner described by Damptey *et al.* (1978). The base of a 575µl stoppered plastic centrifuge tube was cut off and an unwaxed cotton thread was passed through the cut end and out through the stopper such that about 2cm of cotton protruded from the base and about 15cm from the stopper. About 2mm of the base was then briefly immersed in molten wax and quickly cooled, sealing the base and anchoring the cotton firmly in the tube. The capacity of the tubes so prepared was just over 500µl.

Each tube was filled with 500µl of ABA (Sigma Chemical Co., U.S.A) solution and stoppered. The free end of the cotton thread was then passed, with a fine needle, through the culm immediately below the penultimate leaf node. The needle was then disengaged from the thread, leaving the latter embedded in the culm with the tube hanging from the side of the plant (Fig 2.2.2a). The top of the tube was positioned as close as possible to the point where the thread entered the culm. The thread thus acted as a wick through which 373±97 µl of ABA solution was absorbed into the plant within a 24 hr period, following which the wick was removed. Control plants were similarly supplied with deionised distilled water. When a comparison between plants supplied with ABA and those subjected to a water deficit was required, the latter were pierced with a dry wick which also remained in place for 24h.

Tissue samples for estimation of ABA concentration were collected just

Fig. 2.2.2. Application of ABA solution and sampling of spikelets.

(a) A wheat culm with the vial containing ABA solution, and the wick in position

(b) Removal of spikelets through the slit in the leaf sheaths

(c) The slit closed with a black tape after the operation

(d) A sample spike following emergence from the enfolding leaf sheaths.

c - culm, v - vial, w - wick, b - bandage, s - spikelet, ss - sampled spike.

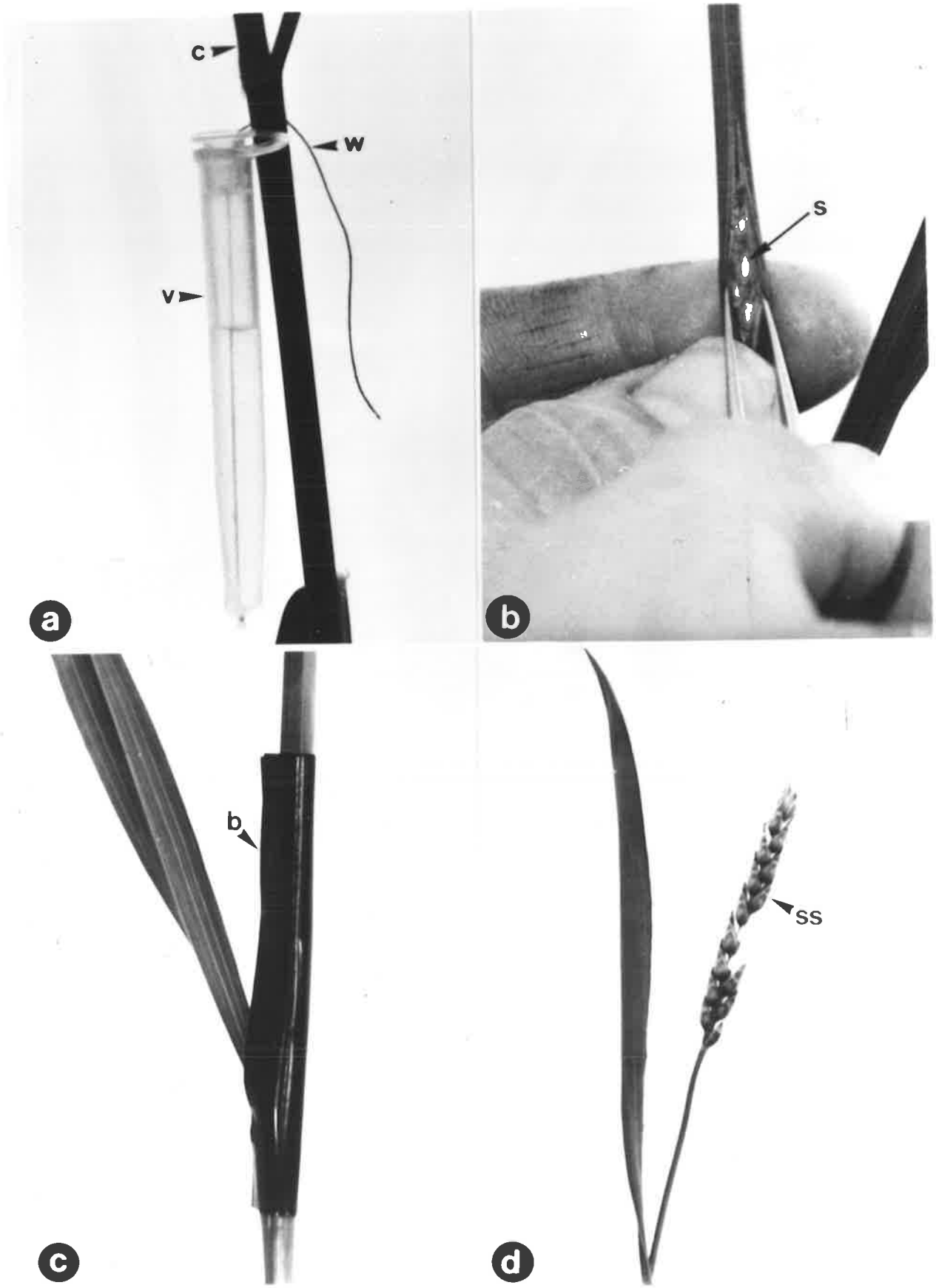


Fig. 2.2.2.

before terminating the stress treatments or removing the wicks unless stated otherwise in the description of the experiments. When grain set and endogenous ABA concentration were determined on the same spike, one or two spikelets were removed carefully with fine forsceps from the central region of the spike through a vertical incision in the two enfolding leaf sheaths (Fig 2.2.2.b). The incision was subsequently closed with black tape to protect the developing spike from desiccation and exposure to light (Fig 2.2.2c). The sampled spike emerged normally following this operation without any measurable effect of wounding on floret fertility (Fig 2.2.2d). In experiments where fertility was not recorded, ABA was determined on flag leaf or pooled samples of spikelets. Sampled tissue was immediately frozen in liquid N_2 and stored at -20°C until analysed.

2.2.4. EXPOSURE TO ETHYLENE GAS:

Whole shoots were exposed to various concentrations of ethylene gas through the continuous flow system shown in Fig 2.2.3. Air was drawn in with an air pump from the open atmosphere outside the room in which controlled-environment cabinet was situated. The outflow from the pump was divided into two streams through PVC tubes. The flow rate in each channel was adjusted to 10 litre min^{-1} with the help of a calibrated glass flow meter having a metallic float and a 3-way valve through which the excess air was bled out. Ethylene gas from a pressurised bottle (C.I.G) was injected into one of the air streams at various flow rates with the help of a calibrated glass flowmeter having a bead float. After directing the stream of ethylene-supplemented air through two successive 30 litre PVC drums to ensure mixing the air-stream was passed through a clear perspex chamber containing 1 or 2 plants. The other stream from the air pump was directed through another perspex chamber in a similar way but no ethylene was injected into this stream which provided the control treatment. Both perspex chambers were situated within a controlled-environment cabinet while the remainder of the apparatus was located outside the cabinet. The tubes carrying the outflow from both chambers were led out of the cabinet and joined together before the combined exhaust was released into the atmosphere outside the room and well away from the cabinet.

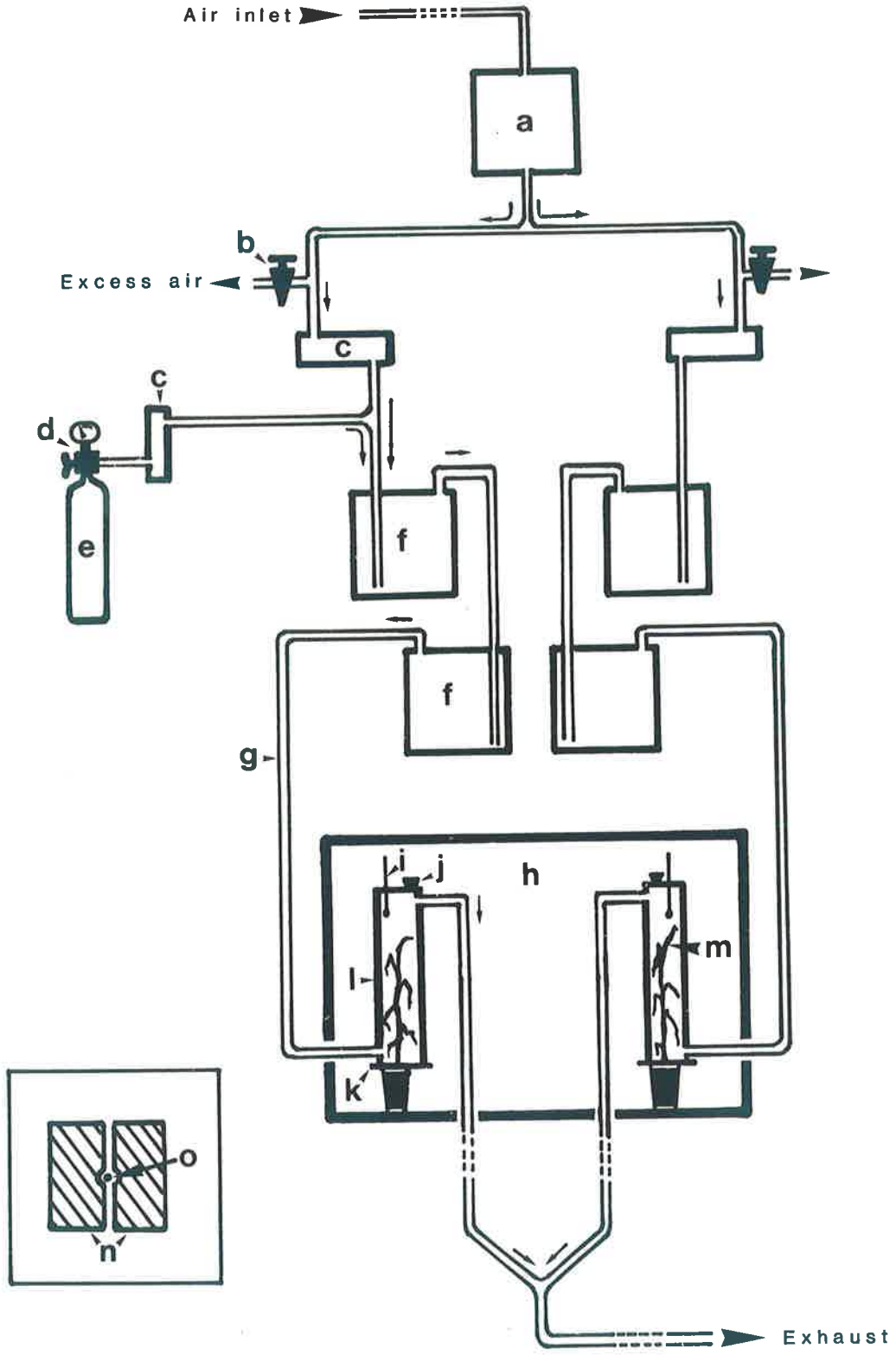


Fig. 2.2.3. Apparatus for exposing plants to a continuous flow of ethylene-supplemented air. Inset - Platform for supporting the plant-containing chamber over the pot.
a - air pump, b - valve, c - flow meter (glass), d - pressure and flow regulator, e - ethylene-gas bottle, f - mixing chamber (PVC), g - connecting tube (PVC), h - controlled-environment cabinet, i - thermometer, j - Subaseal septum, k - platform (clear perspex), l - plant-chamber (clear perspex), m - plant, n - platform segments, o - culm.

Each replicate comprised one or two plants, pruned to a single shoot, enclosed in each perspex chamber. The chamber rested on a perspex platform located on top of the pot containing the plant, the stem of the plant passing through a hole in the platfrom (Fig 2.2.3. - inset). All joints were sealed with Blutack (Emhart Australia Pty. Ltd. The temperature inside each chamber was monitored with a thermometer inserted at the top, and did not at any time differ significantly from the cabinet temperature. The concentration of ethylene in both chambers was monitored by periodically sampling the air with a syringe through a 'Suba Seal' septum inserted near the outlet and measuring the amount of ethylene with a gas-liquid chromatograph.

2.2.5. APPLICATION OF ETHEPHON:

Ethephon (2-chloroethylphosphonic acid; CIBA-GEIGY Australia Ltd.) was applied in various concentrations in water by spraying to run-off on plants during several stages of development. Control plants were sprayed with distilled water.

2.2.6 DETERMINATION OF STAGES OF DEVELOPMENT

Meioses in PMCs and MMC take place approximately at the same time at 20°C within a single floret ((Bennett et al, 1973a,b) and pollen development can be correlated with embryo sac development (Bennett et al., 1973b). Thus floral development was determined from the male tissue alone and treatments were timed to correspond with specific developmental stages. Representative plants from each treatment were sampled periodically and anthers from the first three florets of spikelets taken from the lower, middle and upper regions of the spike were stained according to the Feulgen method (Darlington and La Cour, 1960) and developmental stages were determined following microscopic examination. Whenever necessary, the stages of female development concurrent with the observed male development were determined by referring to the work of Bennett et al. (1973a,b)

Feulgen stain was prepared by pouring 250ml of boiling distilled water over 1.5g basic fuchsin (George T. Gurr Ltd., England). The mixture was agitated for 2h, cooled to 50°C and filtered. 30ml of 1N HCl and 3g of

potassium metabisulphite were added to the filterate and it was left in a tightly stoppered bottle in the dark for 24h before 1.0g of decolorizing carbon (freshly activated charcoal) was added with shaking for 5 min. The mixture was then filtered rapidly through Whatman No 1 paper. The stain was stored in an air tight dark bottle at 4°C.

The anthers were fixed for at least 24h at 4°C in a freshly prepared mixture of ethanol and glacial acetic acid (3:1 v/v) and were stored at 4°C for no more than a week before staining. Anthers to be stained were hydrolysed in 1N HCl at 60°C for 12 min, washed twice with distilled water and kept in Feulgen stain in the dark for 2h. Each anther was then squashed with a dissecting needle in a drop of 45% acetic acid on a microscope slide, covered with a cover-slip and observed under an Olympus BHA-HL-413 research microscope.

2.2.7. MEASUREMENT OF WATER STATUS:

Pilot experiments were carried out to establish a relationship between pot weight and soil water content. Pots were filled with a known amount of oven dried soil and weighed. These were then watered copiously, covered with polyethylene sheets, allowed to drain for 24h and weighed again. The difference between the pot weights on the two occasions represented the soil water content at field capacity. An estimate of the plant fresh weight was obtained periodically and this was used to correct the pot weights at various levels of soil water content at different stages of plant growth. Soil water content was the weight of water in the soil expressed as a percentage of that at field capacity.

Relative water content (RWC) of the leaf is defined as the water content of fresh tissue expressed as a percentage of water content at full turgor. RWC of the uppermost fully-expanded leaf of one representative plant from each pot was measured on each occasion by the modified method of Barrs and Weatherley (1962). The leaf was cut into 1cm segments with a sharp blade and transferred into a pre-weighed air-tight glass vial and leaf fresh weight was obtained. The segments were then allowed to take up water by floating them on distilled water in a Petri plate in the dark. They were removed from water 4h later and dried by placing them between six layers of Whatman No. 1 filter paper and applying a 500g weight at the

top for 30 seconds. Surface dried leaf segments were again transferred to airtight vials and their turgid weight taken. Segments were subsequently oven-dried for 48h at 80°C and their dry weight recorded. RWC was calculated from the following formula;

$$RWC = \frac{\text{Fresh Weight} - \text{Dry Weight}}{\text{Turgid Weight} - \text{Dry Weight}} \times 100$$

The water potential (ψ) of the leaf and of the spike were determined by a pressure bomb (Soilmoisture Equipment Corp., U.S.A.) following the method of Scholander *et al.* (1964). Whenever the ψ and the RWC of the same plant were determined, the leaf ψ was first measured taking care to pressurise the leaf for the shortest possible duration and the same leaf was then used to measure RWC. The spike from the same plant was dissected out and the ψ of this unemerged spike was measured using the pressure bomb, taking care not to enclose any cut surface within the chamber.

2.2.8. OBSERVATION OF MORPHOLOGICAL ATTRIBUTES:

Most observations were made either on the main shoot or the primary tillers, with the exception of plant height which was measured from the tallest shoot and the total weight which comprised the whole aerial part of the plant. Grain set was the number of florets that developed grains as a percentage of those that were judged to be potentially fertile. The florets containing a feathery stigma and 3 anthers at anthesis were considered potentially fertile.

2.2.9. ESTIMATION OF ABSCISIC ACID:

ABA was determined by electron capture detection on a gas liquid chromatograph.

2.2.9.1. PREPARATION FOR ASSAY:

2.2.9.1.1. PREPARATION OF CHROMATOGRAPHY PAPERS:

All Whatman No 3MM papers used for chromatography were washed by sequential elution for 24h with each of 0.1M EDTA, distilled deionised

water, 2N acetic acid, distilled deionised water and distilled methanol and were air dried before use. Chromatograms were later developed in the same direction as that in which the papers were eluted.

2.2.9.1.2. PREPARATION OF COLUMN PACKING:

ABA was measured using either a glass column packed with 3% OV-17 on Gaschrom Q (mesh size 100-120) or an SP-2250, support-coated open tubular (SCOT) capillary column.

In order to prepare 3% OV-17 column-packing, 30g of Gaschrom Q (Applied Science Lab., U.S.A) was silanised by treatment with distilled benzene containing 0.5% hexamethyldisilazane (HMDS; Applied Science Lab., U.S.A) to just cover the Gaschrom Q in a round bottom flask. The benzene and excess HMDS were removed under reduced pressure at 55°-60°C on a rotary evaporator. The Gaschrom Q was then heated to 60°C for about 30 min and the whole procedure was repeated twice. The silanised Gaschrom Q was then suspended in methylene chloride until the liquid was 6mm above the solid. To this, 30ml of anhydrous ethylene chloride containing 1g of OV-17 (Applied Science Lab., U.S.A.) was added. The slurry was thoroughly mixed by swirling and the methylene chloride was removed under reduced pressure at 55°-60°C on a rotary evaporator. The OV-17 coated support was then oven dried at 60°C.

The SP-2250, SCOT, capillary column was obtained from Scientific Glass Engineers, Australia.

2.2.9.1.3. SILANISATION AND PACKING OF COLUMN

The method for silanisation of the column was developed by Firn (1968). The glass column was cleaned by passing acetone and anhydrous benzene through it with a suction pump. Following drying in an oven, the column was rinsed thrice with 3% HMDS in benzene and dried at 60°C for 15 min after each rinsing.

The column was packed by plugging one end with 12mm of glass wool and sucking in the packing material with a suction pump. The coiled portion was packed with 3% OV-17 on Gaschrom Q and the straight portions with

Gaschrom Q alone. The column was then installed in the GLC and conditioned at 270°C for 24 hr.

2.2.9.2. EXTRACTION, PURIFICATION AND MEASUREMENT OF ABA:

Free ABA was extracted and purified by a method modified from that of Coombe and Hale (1973). The tissue was frozen in liquid N_2 at sampling time, stored at -20°C and freeze dried before extraction.

2.2.9.2.1. EXTRACTION AND PURIFICATION:

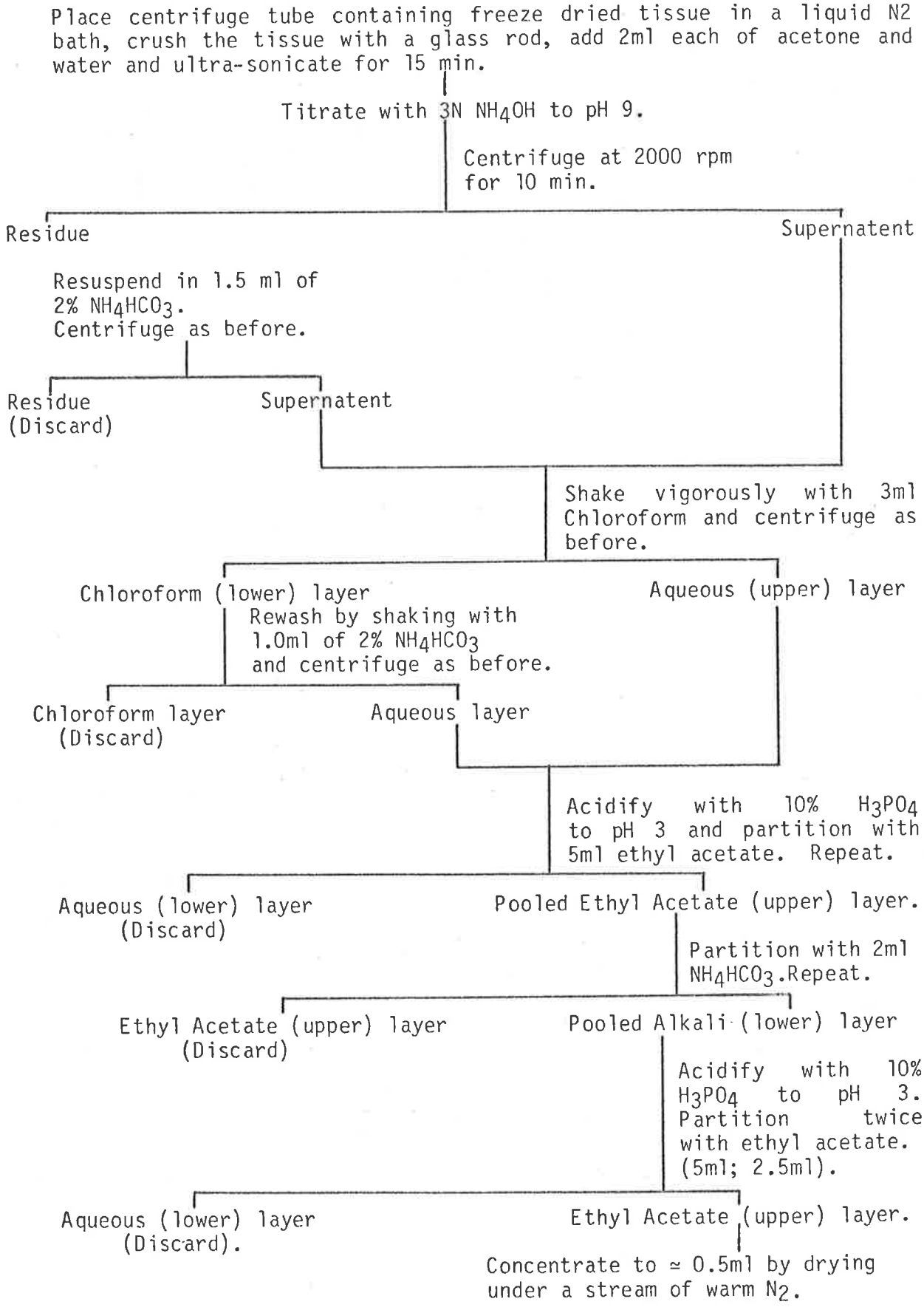
Fig 2.2.4 shows the flow diagram of the procedure for the extraction of ABA from samples comprising 1-2 spikelets. When the samples included greater number of spikelets or a leaf, the quantity of all reagents and solvents was doubled. Whenever it was necessary to interrupt the extraction procedure, the assay was stored for no more than 24h at 4°C at alkaline pH.

These partially purified extracts were applied as 40mm streaks to acid-washed Whatman No 3MM paper (see section 2.2.8.1.1.), using about 5µg of ABA as the marginal marker on each side of the paper. Following development for 4h in a descending mixture of isopropanol: water: NH_4OH (10:1:1 v/v) and drying for 30 min, the marker ABA spots were located as fluorescent areas under UV light and were outlined while the rest of the chromatogram was shielded with aluminium foil. The zones of extracts of equivalent Rf were excised and eluted overnight with 70% methanol. The eluates were dried under a warm stream of N_2, washed with anhydrous petroleum spirit and evaporated.

2.2.9.2.2. MEASUREMENT BY GAS-LIQUID CHROMATOGRAPH:

The ABA in the extracts was further separated, identified and quantified by GLC. The dried extract was dissolved in a mixture of 0.5ml ethyl acetate and 0.5ml methanol and methylated with diazomethane. The gas was prepared by reacting 2ml carbitol (diethylene glycol monoethyl ether), 2ml 60% (w/v) KOH and about 100mg p-tolylsulphonylmethylnitroso-amide (TONY). The resulting diazomethane was carried in a stream of N_2, saturated with methanol, through a small bore Teflon tubing with glass

Fig. 2.2.4. Flow diagram of the procedure for the extraction of ABA



nozzle and bubbled through the extract. Bubbling was stopped after the solution had turned yellow, indicating the presence of excess diazomethane. The extract was then dried under N_2 and stored at 0°C in the dark until injected on to GLC column within a few days.

Methyl-cistrans-abscisic acid was determined by electron capture detection on a GLC. Extracts were dissolved in ethyl acetate and aliquots of from 1/25 to 1/400 were injected in 0.5 to 2µl volumes, either on a 3% OV-17 packed column fitted in a Varian 2700 GLC with a ^{3}H-foil electron capture detector or an SP-2250, SCOT, capillary column fitted in a Packard 427 GLC with a ^{63}Ni-foil detector. The operating conditions of the two GLCs were as follows;

	Varian 2700	Packard 427
Temperatures:		
Injector	210°C	240°C
Column	180°C	205°C
Detector	245°C	300°C
Flow rates:		
Carrier gas	(N_2) 26ml/min	(He) 8ml/min
Make-up gas	-	(5% CH_4 in Ar) 50ml/min

Methyl-cistrans-abscisic acid peaks were identified by the coincidence of retention times with that of authentic compound and confirmed by the decrease in the height of this peak coupled with the increase in the methyl-transtrans-abscisic acid peak following UV irradiation (Fig 2.2.5). The amounts of ABA in the extracts injected into the Varian 2700 GLC were measured by comparison of peak area with a standard curve obtained from a series of known ABA (Sigma Chemical Co., U.S.A.) concentrations between 0.1 and 0.4ng. The extracts injected into the Packard 427 GLC were spiked with known amounts of aldrin and endrin. The amount of ABA was measured by comparing the peak-area ratios of ABA to aldrin and ABA to endrin with a standard curve of peak-area ratios obtained from a series of standard solutions with known ABA/aldrin and ABA/endrin concentration ratios.

Tissue samples spiked with 10-40ng abscisic acid gave recovery rates between 50-80 per cent in various experiments and the measured concentrations in extracts were corrected for recovery using calculations

from parallel spiked samples.

2.2.10. MEASUREMENT OF ETHYLENE:

The concentration of ethylene in the samples of air collected from around the plants was estimated using a GLC.

2.2.10.1. SILANISATION AND PACKING OF COLUMN:

The glass column was silanised and packed with Porapak Q (100-120 mesh; Waters Assoc. Inc., U.S.A) in a manner similar to that detailed in section 2.2.8.1.3.

2.2.10.2. MEASUREMENT BY GLC:

Ethylene was measured using a Varian 1400 GLC, fitted with a flame ionisation detector. 1.0ml samples of air around the plants were collected by piercing through the 'Suba Seal' septum with a hypodermic needle fitted to a syringe via a 1-way Luer-Lok valve. The valve was closed after the air was sucked into the syringe. The gas samples were then injected on to the Porapak Q column maintained under the following operating conditions;

Flow rates:

N_2 (carrier)	-	30ml min^{-1}
H_2	-	30ml min^{-1}
Air	-	300ml min^{-1}

Temperatures:

Injector	-	100°C
Column	-	40°C
Detector	-	100°C

The ethylene in the air samples was quantified by comparison of the peak heights with a standard curve obtained by a series of ethylene-air mixtures of known concentrations (Fig 2.2.6). The standard gas mixtures

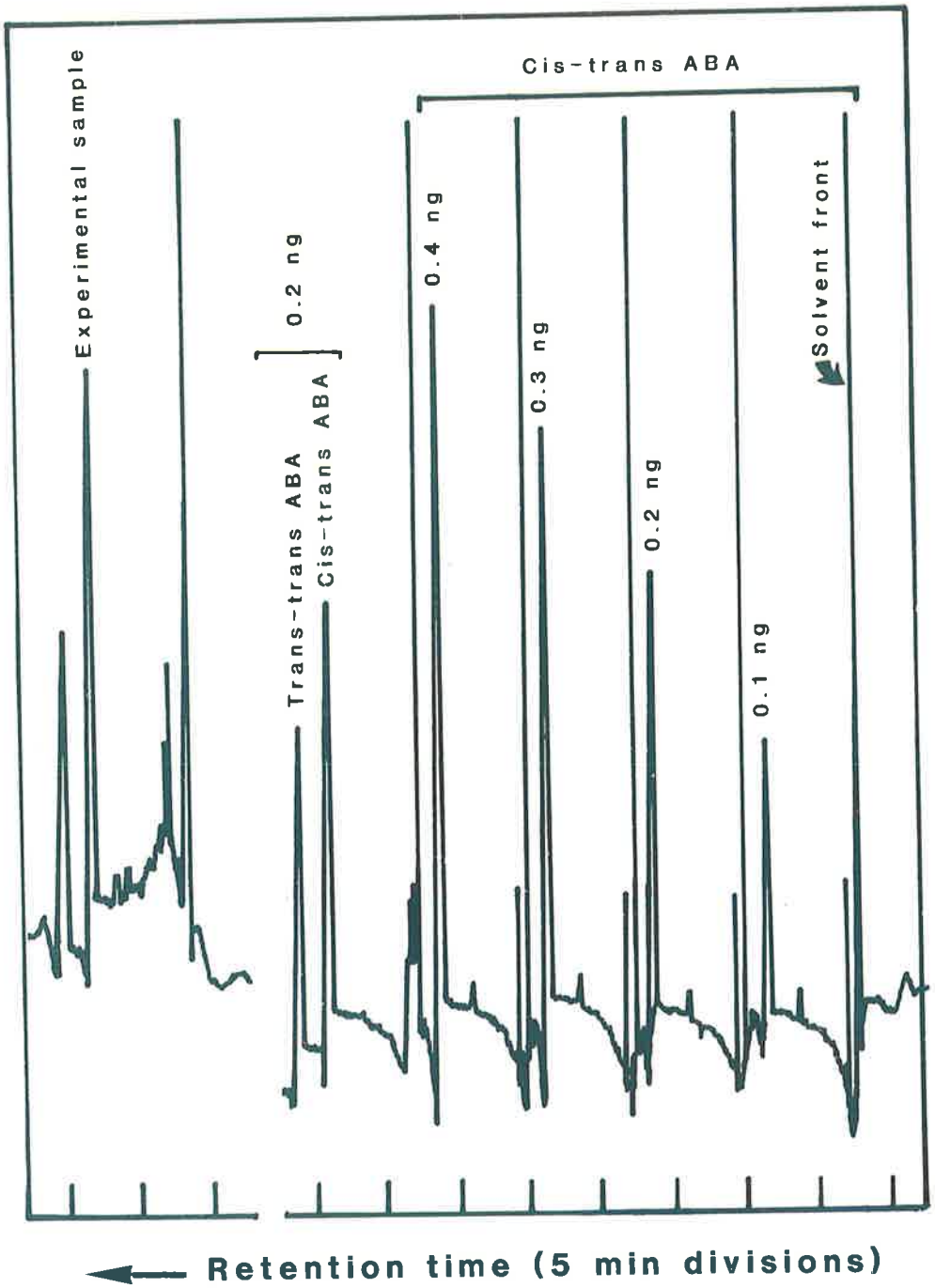


Fig. 2.2.5. GLC (Varian 2700) detection of abscisic acid.

were prepared by dilution of ethylene (C.I.G.) with air in 1 litre preserving jars fitted with 'Suba Seal' septa.

2.2.11. ASSESSMENT OF FEMALE FERTILITY:

The level of female fertility was assessed by emasculating primary and secondary florets of the central eight spikelets on a spike, cross-pollinating them with fertile pollen and recording percentage grain set. Florets were emasculated two days before the expected date of anthesis and covered with glassine bags. Stigmas were pollinated at anthesis with pollen obtained from the primary and secondary florets of well watered plants grown at 20°C. The covers were removed two weeks after anthesis and the grain set was recorded within a few days.

The details of specific crossing plans for various treatments are given where the relevant experiments are discussed. When a certain treatment was found to affect female fertility, the results were further confirmed by direct examination of the pistil.

2.2.12. ASSESSMENT OF MALE FERTILITY:

Male fertility was measured both by examining anthers for morphological abnormalities and by a staining technique for assessing pollen viability. Only the potentially fertile, lower three florets within a spikelet were considered for the evaluation of male fertility.

2.2.12.1. MACROSCOPIC ANTHER-EXAMINATION:

Normally, the filaments of the fertile anthers rapidly elongated at anthesis, pushing the anthers out of the floret while the thecae dehisced and released their pollen. Anthers that failed to extrude and dehisce at the time of anthesis were considered abnormal. Such anthers were frequently conspicuous by their smaller size and shrivelled or distorted appearance.

2.2.12.2. POLLEN VIABILITY:

Cereal pollen cannot be reliably germinated *in vitro*, so a staining

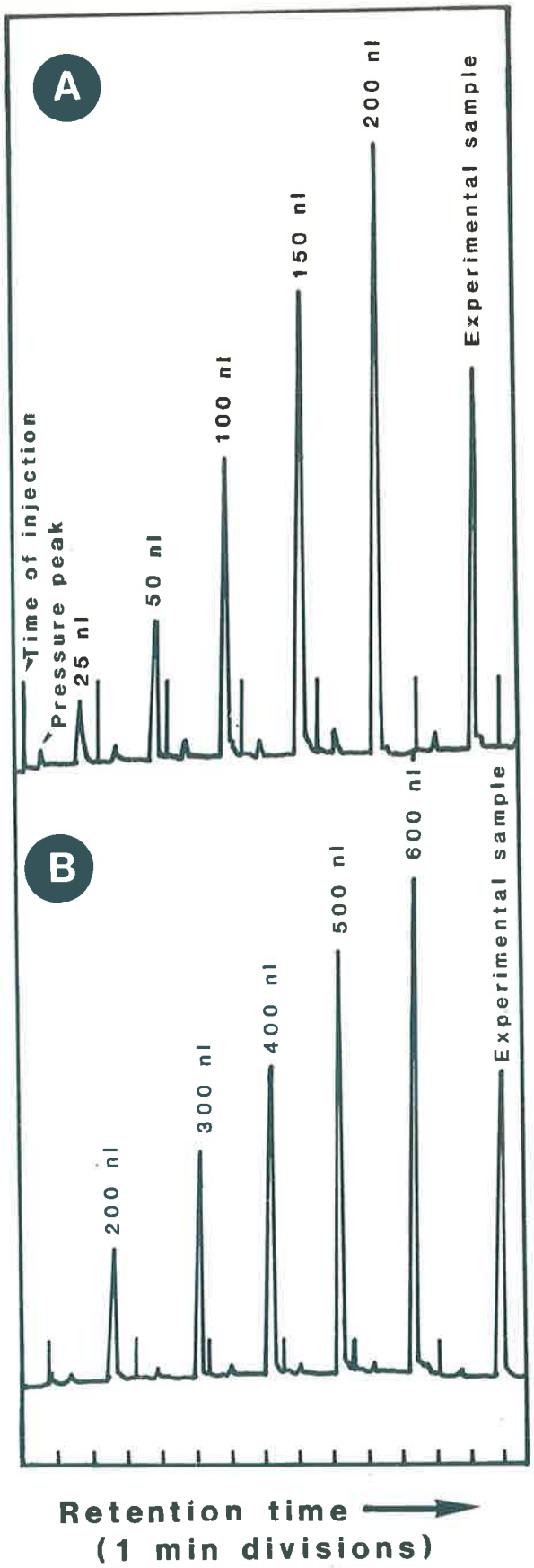


Fig. 2.2.6. GLC detection of ethylene

(A) Attenuation = 16

(B) Attenuation = 64

technique employing 2,3,5-triphenyl tetrazolium chloride (TTC) was adopted for the determination of pollen viability. The redox enzymes such as dehydrogenases, transfer protons to acceptor molecules. TTC, on reduction by a proton changes from a colourless to a red compound. Since active enzymes are necessary to transfer the protons, the change in the colour of the dye absorbed into pollen grains, serves as evidence for the presence of dehydrogenase enzyme activity and hence the viability of pollen (Stanley and Linskens, 1974).

A solution of the dye was prepared by dissolving 1.0g of TTC (BDH Chemicals, England) in 100ml of 0.15M tris-HCl buffer, pH 7.8. The solution was stored in the dark at 4°C and was used within 3 months of preparation.

Oxygen was excluded from the system since it can inhibit the reduction of the dye. Pollen was tapped directly from the anther on to a drop of the dye on a slide which was maintained in nitrogen within a cabinet. Non-dehiscent anthers were teased apart in the drop of dye with a dissecting needle to release the pollen. After gently placing the cover slip over the drop, the slide was transferred to a dark chamber in an atmosphere of N_2 and was observed 15 minutes later under an Olympus BHA-HL-413 research microscope. The pollen grains that stained red within this period were considered viable.

2.2.13. OBSERVATION OF *IN VIVO* POLLEN TUBE GROWTH:

In vivo pollen germination and pollen tube growth were studied by staining pistil squashes with Aniline blue fluorochrome and observing under a fluorescence microscope (Martin, 1959).

2.2.13.1. SAMPLING AND FIXATION:

All except the primary and secondary florets of approximately 12 central spikelets were removed, and the unexcised florets were emasculated two days prior to the expected date of anthesis and covered with glassine bags. At anthesis, the stigmas were pollinated with fertile pollen and 3h later pistils (ovary, styles and stigmas) were harvested and fixed in Carnoy's fluid (Absolute alcohol : Chloroform : Acetic acid, 6:3:1 v/v)

for at least 2h. The pistils were not stored in this fluid for more than a week.

2.2.13.2. HYDRATION, SOFTENING AND STAINING:

The tissue was hydrated by sequential immersion for a minimum of 10 min in each of 70% alcohol, 30% alcohol and two changes of distilled water. It was then softened by hydrolysing with 0.8N NaOH at 60°C for 45 min.

A 0.1% solution of water soluble Aniline blue (George T. Gurr Ltd, England) was prepared by dissolving the dye in 0.1N K_3PO_4 by shaking overnight and filtering. Softened pistils were transferred to a few drops of a mixture of glycerol and 0.1% aniline blue (1:1 v/v) and squashed by pressing under a cover slip. The edges of the cover slip were sealed with clear nail enamel. Slides were stored at 4°C in the dark and were observed within a few days of preparation as they tended to bleach with time.

2.2.13.3. MICROSCOPIC EXAMINATION:

The greenish-blue fluorescence of pollen grains and pollen tubes stained with Aniline blue fluorochrome was observed with the aid of a Carl Zeiss, standard WL, research microscope, fitted with a 50W high-pressure mercury vapour lamp and a IV FI Epifluorescence condenser. The filter combination used was;

Exciters	-	KP 490 and KP 500
Beam Splitter	-	FT 510
Barrier	-	LP 520

The slides were scored for number of pollen grains and pollen tubes on the stigma and of pollen tubes reaching the ovary. A pollen grain was scored as germinated when the length of the pollen tube equalled or exceeded the diameter of the grain.

2.2.14. ANATOMICAL OBSERVATIONS:

2.2.14.1. SAMPLING AND FIXATION:

Ovaries from primary and secondary florets of spikelets from the central region of the spike, sampled just prior to anthesis were used for anatomical observations. Anther development was examined by selecting matching plants at the onset of meiosis in the PMCs of the most advanced florets and sampling 1 or 2 plants from these on each day from meiosis to anthesis. Further details of the sampling procedures for various treatments are discussed along with the results.

Ovaries and anthers were fixed immediately upon excision in 3% glutaraldehyde solution in 0.025M phosphate buffer, pH 7.0. The tissue was stored at 4°C for a minimum of 24h.

2.2.14.2. DEHYDRATION AND EMBEDDING:

The tissue was dehydrated through alcohols and embedded in glycol methacrylate (GMA), (Feder and O'Brien, 1968). The tissue received sequential immersion at room temperature in the following;

Methoxy ethanol	-	2h
Ethanol	-	2h
Propanol	-	2h
Butanol	-	2h
Butanol + GMA (1:1 v/v)	-	2h
GMA	-	2h
GMA	-	2days

The GMA for embedding was prepared by dissolving 0.6g benzoyl peroxide in 93ml glycol methacrylate (Hartung Associates, U.S.A.) and 7ml polyethylene glycol (m.wt. = 400) by constant stirring for 2h. This mixture was stored at 4°C.

The specimens were then transferred to GMA in gelatine capsules (size 00) and were orientated in the required plane. The GMA was then polymerised by excluding air from the capsule and incubating at 60°C for two days.

2.2.14.3. SECTIONING:

Any excess GMA was trimmed from the specimens before the blocks were sectioned. Serial sections of 3.5µm thickness were obtained with a SORVALL type JB-4, Porter-Blum microtome, using freshly cut glass knives. Sections were placed in separate drops of water on glass slide, any folds in the sections were removed by straightening the sections with two fine dissecting needles observed through a stereo microscope and the slides were dried at 60°C for 24h before staining.

2.2.14.4. STAINING:

2.2.14.4.1. PERIODIC ACID SCHIFF'S REAGENT (PAS):

Details of PAS-staining procedure were adopted from Feder and O'Brien (1968). Slides were placed for 30 min in a saturated solution of 2,4 dinitrophenyl hydrazine (DNPH), prepared freshly by adding 0.5g of DNPH to 100 ml of 15% acetic acid, shaking for 1h and filtering. After rinsing for 1h in running water slides were transferred to a freshly prepared 1% solution of periodic acid. Slides were removed from periodic acid after 30 min, rinsed in tap water for 5 min, stained in fresh Schiff's reagent (BDH Chemicals [Australia], Ltd.) for 30 min and transferred to a mixture of 5ml of 10% sodium metabisulphite solution, 5ml of 1N HCl and 90ml distilled water for 2 min. The final step was repeated twice. When no other staining was required, slides were washed in distilled water (3 changes), oven dried at 60°C and mounted in Dammer Xylene.

2.2.14.4.2. TOLUIDINE BLUE O:

The stain was prepared by dissolving 290mg of sodium benzoate and 250mg of benzoic acid in 200ml distilled water by constant shaking with gentle heating (Feder and O'Brien, 1968). 100mg of Toluidine blue O (Hopkin and Williams, England) was then added to this solution, shaken for 30 min and allowed to stand overnight. The mixture was then filtered and the pH of the filterate adjusted to 4.5.

Slides with unstained or PAS stained sections were kept in Toluidine blue O solution for 5 min, removed and washed for 10 min in running

water. They were then rinsed for 2 minutes each in three changes of distilled water, dried and mounted in Dammer Xylene.

2.2.14.4.3. COOMASSIE BRILLIANT BLUE:

Slides were immersed for 5 min in 0.25% (w/v) solution of Coomassie brilliant blue (Sigma Chemical Co., U.S.A.) in 7% acetic acid and then dipped briefly in 7% acetic acid to remove excess of the dye (Fisher, 1968). Slides were then air dried and mounted in glycerol containing 5% acetic acid.

2.2.14.4.4. ANILINE BLUE:

0.1% Aniline blue stain was prepared as described in section 2.2.12.2. Drops of a mixture of glycerol and 0.1% aniline blue (1:1 v/v) were placed on sections, covered with a glass cover slip and observed with a fluorescence microscope (section 2.2.12.3).

2.2.14.4.5. AURAMINE O:

0.01% (w/v) solution of auramine O (National Biological Stains Dept., U.S.A) was prepared in 0.025M phosphate buffer pH 7.0 (Heslop-Harrison, 1976). Slides were mounted in 1:1 (v/v) mixture of the stain and glycerol and observed with a fluorescence microscope (section 2.2.12.3).

2.2.14.4.6. AUTOFLUORESCENCE:

Sections mounted in glycerol were examined for autofluorescence in the same way as those stained with aniline blue or auramine O.

2.2.14.5. MICROSCOPIC EXAMINATION:

Stained sections were observed through an BHA-HL-413 research microscope fitted with Plan-achromatic optics and a 100W halogen lamp, or a Carl Zeiss microscope equipped for fluorescence microscopy (Section 2.2.12.3).

2.2.15. SCANNING ELECTRON MICROSCOPY:

Anthers fixed in 3% glutaraldehyde solution in 0.025M phosphate buffer, pH 7.0 for a minimum of 24h were washed in distilled water and placed for 2 min. in 2% potassium iodide-iodine solution prepared by dissolving 2.0g potassium iodide and 0.2g crystalline iodine in 100ml distilled water. After rinsing in several changes of distilled water until the supernatant was clear, the anthers were immersed in 3 changes of lead acetate solution prepared by dissolving 200mg lead acetate and 6.67g sodium hydroxide in 100ml distilled water. Anthers were then infiltrated with 50% glycerol for 24h, critical point dried, split longitudinally and mounted on a metal boat with DAG 915 high conductivity paint. Following coating with Pt/Pd in vacuum (~ 10^{-4} tor), the anthers and pollen grains therein were observed through a JEOL, JEM 100CX scanning electron microscope.

2.2.16. PHOTOGRAPHY:

Whole anthers were photographed on Ilford FP-4, 125 ASA, monochrome film with a Leica camera fitted on Leitz Aristophot apparatus. Stained pollen grains and sectioned material were photographed with an Olympus PM-10 photomicrographic system fitted on an Olympus, BHA-HL-413 microscope and aided by an Olympus EMM-7 exposure-meter. In this case, Ilford FP-4, 125 ASA and Kodak Ektachrome 64 ASA or Kodacolor-II, 100 ASA, daylight (balanced for colour temperature using filters) or film was used for monochrome or coloured photographs respectively. Coloured fluorescence photomicrographs were obtained with Kodacolor 400 ASA film with an Olympus PM-10 system fitted on to a Carl Zeiss, standard WL, research Microscope, with attachments for fluorescence microscopy as described in section 2.2.12.3. Scanning electron-micrograph was obtained through a photomicrographic system built-in the JEOL, JEM 100CX microscope, using Ilford FP4, 125ASA film.

2.2.17. STATISTICAL ANALYSIS

Completely randomised or randomised block experimental designs were employed throughout this investigation. The data were analysed using the computing facilities at the Waite Agricultural Research Institute, with

the help of staff biometricians. Most of the data were analysed by parametric statistical methods, with the exception of the data on pollen viability which were analysed using non-parametric, Mann-Whitney 'U' test. All percentages were subjected to angular transformation whenever they were analysed by a parametric method.

3. RESULTS AND DISCUSSION

3.1. EFFECTS OF STRESS ON GROWTH AND GRAIN YIELD

3.1.1. WATER DEFICIT:

3.1.1. SENSITIVE STAGE OF DEVELOPMENT AND GRAIN SET:

Two similar experiments were conducted in order to identify the stage of maximum sensitivity to water dificit during the latter part of floral development. Both experiments yielded similar results, hence only one of these experiments is discussed in detail here and the second alternative is presented only in the cases where the two sets of results differed.

Single drying cycles were imposed on different groups of plants by withholding water in such a way that a group was rewatered only after the succeeding group had wilted, thus providing a series of seven partially overlapping episodes of water deficit (treatments 1-7) commencing a few days before meiosis in the PMCs and continuing until a few days after anthesis (Fig. 3.1.1). The control pots were watered to field capacity whenever their soil water content fell below 75 per cent, thus maintaining the control plants at a high water regime throughout the experiment. The results of this experiment are summarised in table 3.1.1.

The leaf RWC decreased from 92.5 to 65.9-67.5% on the last day of each of the treatments. Water deficit in treatment 2 and 3 resulted in a significant reduction in both the number of grains per spike and percent grain set when compared to the well watered plants. These attributes were also reduced in response to treatment 4, but to a lesser extent (significant at P=0.05). Water deficit at any other time had no significant effect on either the number of grains or grain set. The extent of the reduction in grain set induced by water stress in this period varied widely between individual spikes, the number of grains in a spike ranging from 0 to 33, while the number of grains in control plants ranged from 15 to 35.

The reduction in grain set by treatment 2 or 3 was due mainly to infertility in the primary and secondary florets within each spike (Table

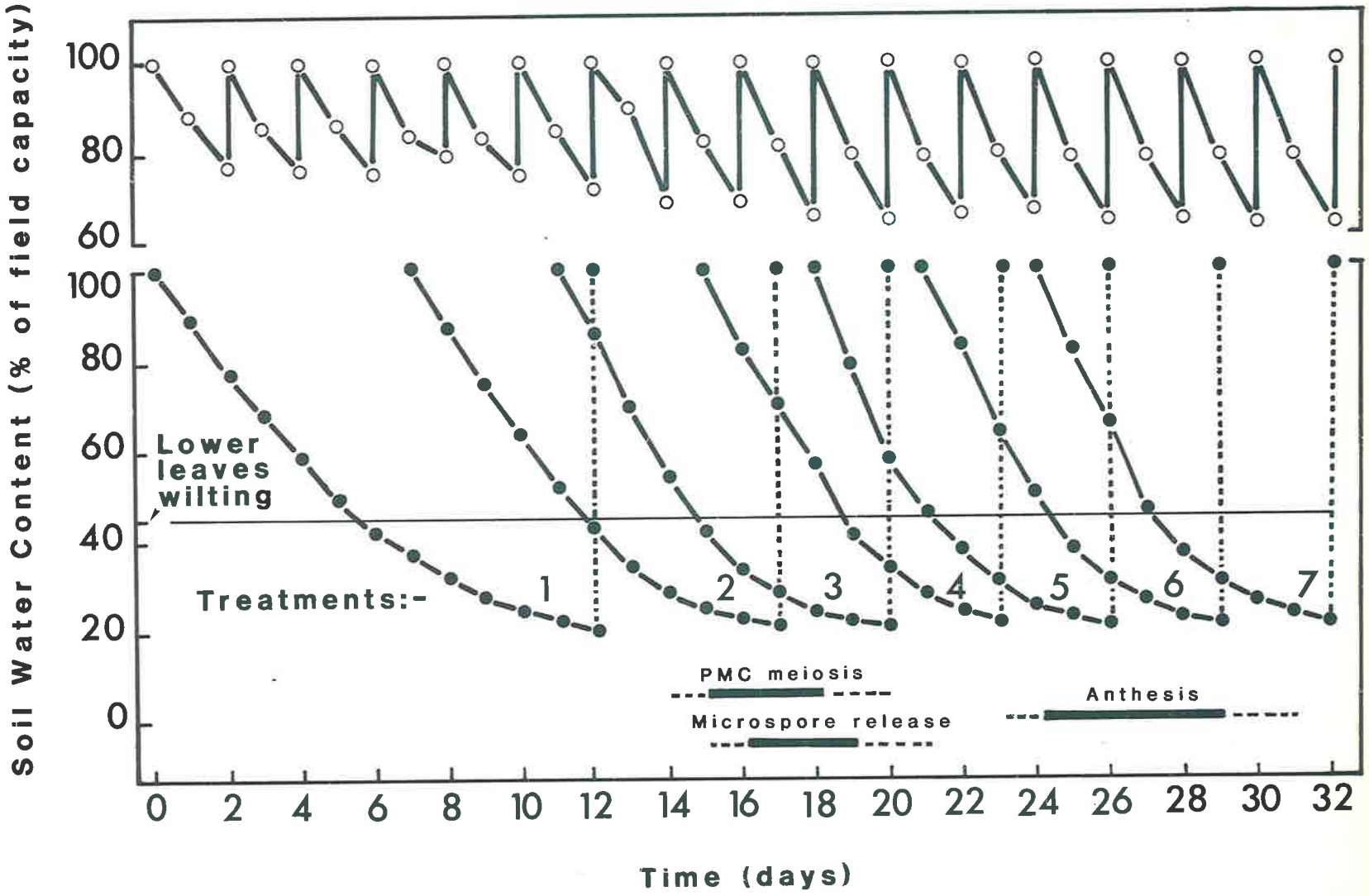


Fig. 3.1.1. Soil water content during episodes of water stress imposed by withholding water at different stages of floral development. Control (○), Water stress (●). Thick areas on the bars represent the period during which 90 per cent of the observed florets were at the corresponding developmental stage.

Table 3.1.1. Effects of water stress treatments imposed at various stages of development (See Fig. 3.1.1.)

Attribute	Treatments							
	Control	1	2	3	4	5	6	7
RWC (%)	92.5^{A}	67.4^{B}	67.5^{B}	67.1^{B}	65.9^{B}	66.5^{B}	67.0^{B}	67.1^{B}
Number of grains per spike	26.5^{A}	24.1^{A}	17.9^{B}	16.7^{B}	21.8^{A}	25.6^{A}	25.2^{A}	25.5^{A}
Grain set (%)	55.0^{A}	53.2^{A}	39.7^{B}	36.1^{B}	49.4^{A}	54.1^{A}	51.7^{A}	51.7^{A}
Weight per grain (mg)	43.3^{BCD}	42.8^{BC}	46.7^{CDE}	$50.^{E}$	47.7^{DE}	44.0^{BCD}	40.1^{B}	34.5^{A}
Grain yield per spike (mg)	1128^{A}	1018^{A}	839^{BC}	816^{B}	1023^{AC}	1105^{A}	971^{AC}	828^{BC}
Plant height at maturity (cm)	70.1^{A}	58.5^{B}	61.1^{B}	57.6^{B}	59.4^{B}	60.8^{B}	63.4^{BC}	67.5^{AC}
Shoot weight at maturity (g)	7.99^{A}	6.45^{B}	6.36^{B}	6.85^{B}	6.56^{B}	6.46^{B}	6.14^{B}	6.20^{B}

Each value is a mean of 18-20 plants except RWC values which represent 4 plants each.
Within a row, values not marked by the same letter are significantly different (P = 0.01).

3.1.2). Treatment 4 caused similar effects, although to a diminished extent. The remaining treatments did not alter the grain set in either of these classes of florets. Although the grain set in tertiary florets varied considerably between the treatments, this was apparently random as no specific trend was detected in the response. Where grain set was reduced, therefore, it was consistently reduced in those florets most likely to bear grains in the normal course of events.

The spike was divided into three equal regions to assess grain set in different parts of the spike. Grain set in spikelets in the lower and upper regions of the spike was significantly reduced by treatments 2,3 and 4 but that in the middle region was not (Table 3.1.3). Although this difference was clear in this experiment, the results of the second experiment did not indicate that water stress reduced grain set only in the extremities of the spike. In this experiment, where treatments 1, 2 and 3 corresponded approximately to the first three treatments respectively, in Fig. 3.1.1, treatment 3 caused a significant reduction in grain set and the loss of grain set was spread equally in all regions of the spike (Table 3.1.4).

The first indication of plant-water-stress was the beginning of wilting in lower leaves about 3-5 days after the watering was first suspended. As is clear in the following section (3.1.1.2), the onset of wilting in the lower leaves coincided with the beginning of drop in Ψ of the uppermost fully expanded leaf. Hence, the plants were under water stress from the commencement of wilting to the resumption of watering. If this criterion of water stress is applied to treatments 2, 3 and 4 that caused the reduction in grain set (Fig. 3.1.1 and Table 3.1.1) it is apparent that the period of plant water stress in these treatments covers the phase of meiosis in the PMCs, the subsequent release of microspores from the tetrads together with the initial stages of pollen development. The maximum sensitivity to water stress seems to exist between the commencement of meiosis and tetrad break up. At the onset of meiosis, the spike was still enclosed by the sheaths of the penultimate and the flag leaves, and the flag leaf sheath had extruded about 35-40 mm above the auricle of the penultimate leaf (Fig. 3.1.2).

Table 3.1.2. Effect of water stress treatments imposed during various stages of development (See Fig. 3.1.1) on grain set in florets in the primary, secondary and tertiary postions.

Position of floret	Treatments							
	Control	1	2	3	4	5	6	7
	Grain set (percentage of fully formed florets)							
Primary	81.5^{CD}	85.1^{D}	53.7^{A}	57.4^{AB}	70.3^{BC}	75.1^{CD}	79.0^{CD}	82.1^{D}
Secondary	62.0^{B}	59.7^{B}	35.3^{A}	34.0^{A}	53.1^{B}	58.0^{B}	57.0^{B}	60.2^{B}
Tertiary	13.4^{ABC}	6.0^{A}	25.7^{C}	12.6^{AB}	18.2^{BC}	23.6^{BC}	10.5^{A}	4.8^{A}

Within a row, values (means of 18-20 plants) not marked by the same letter are significantly different (P = 0.01).

Table 3.1.3. Effect of water stress imposed during various stages of development (See Fig. 3.1.1) on grain set in the lower, middle and upper regions of the spike

Region of Spike	Treatments							
	Control	1	2	3	4	5	6	7
	Grain set (percentage of fully formed florets)							
Lower third	46.7^{D}	43.9^{CD}	25.6^{AB}	14.7^{A}	31.1^{B}	34.6^{BCD}	38.5^{BCD}	42.0^{CD}
Middle third	69.1^{ABC}	65.0^{AB}	65.2^{AB}	59.7^{A}	71.4^{BC}	75.8^{C}	68.8^{ABC}	64.5^{AB}
Upper third	47.6^{B}	48.5^{B}	25.1^{A}	26.9^{A}	42.0^{B}	46.1^{B}	44.5^{B}	46.3^{B}

Within a row, values (means of 18-20 plants) not marked by the same letter are significantly different (P = 0.01).

Table 3.1.4. Effect of water stress imposed during various stages of development (See page 4) on grain set in the lower, middle and upper 1/3rd regions of the spike.

Region of Spike	Treatments			
	Control	1	2	3
	Grain set (percentage of fully formed florets)			
Lower third	40.4^{A}	33.4^{A}	27.2^{A}	10.2^{B}
Middle third	55.4^{A}	57.5^{A}	60.9^{A}	31.5^{B}
Upper third	34.7^{A}	38.6^{A}	32.0^{A}	15.0^{B}

Within a row, values (means of 15-20 plants) not marked by the same letter are significantly different (P = 0.05).

Fig 3.1.2. A wheat plant at the onset of meiosis in the PMCs of the main shoot.

a - flag leaf sheath, b - auricle of the penultimate leaf, c - position of spike.

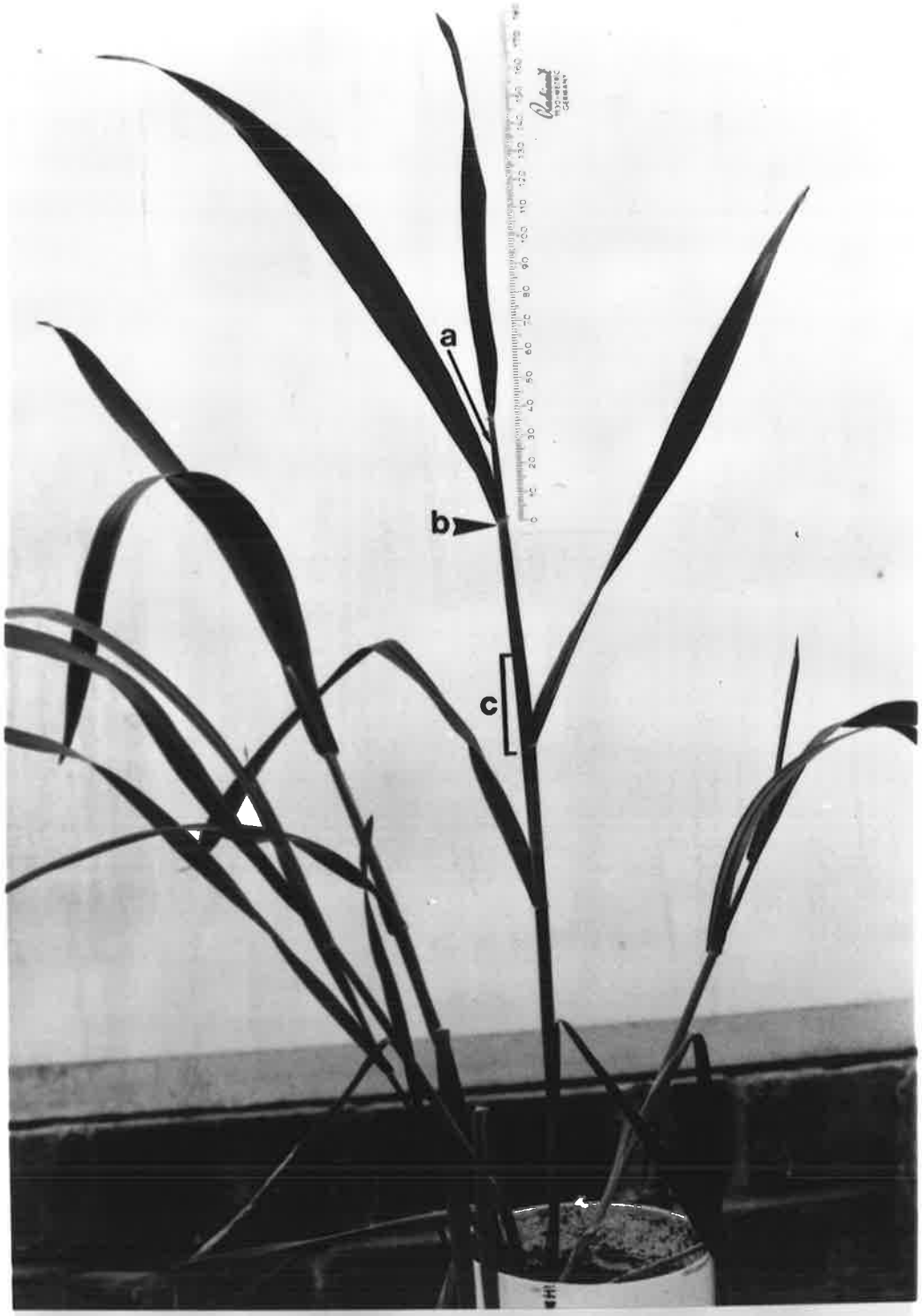


Fig. 3.1.2.

3.1.1.2. WATER STATUS:

Leaf Ψ and RWC of the well watered (control) plants ranged from -3 to -9 bar and 88.2 to 95.8 per cent respectively and both declined slightly as the leaf matured (Fig. 3.1.3). During a drying cycle that duplicated treatment 3 in Fig. 3.1.1, leaf Ψ and RWC decreased following the imposition of stress and both were clearly lower than the control levels 4-5 days after watering ceased. At this time soil water content had declined to approximately 40-45 per cent of field capacity and wilting had just commenced in the lower leaves. Leaf Ψ had dropped to -23.2 bar and RWC to 73.6 per cent before the pots were rewatered, having reached soil water content of about 20 per cent. The Ψ of the spike that ranged between -0.5 and -4.5 bar, always remained higher than that of the leaf. Despite a substantial drop in the leaf Ψ the spike Ψ did not decline during the episode of water deficit.

3.1.1.3. GRAIN WEIGHT:

The decreased grain set following water stress at the sensitive stage markedly lowered grain yield per spike (Table 3.1.1). The yield per spike was also reduced in response to the water stress treatment 7 that occurred immediately after anthesis. This decline in grain yield was entirely due to a reduction in grain weight and, in contrast to the stress imposed during the sensitive stage, water deficit during and after anthesis did not significantly reduce grain set.

The average weight per grain was the highest in response to treatment 3 which caused greatest decline in both grain set and yield (Table 3.1.1). A comparison of the frequency distributions of the weights of individual grains from treatment 3 and control plants shows that the distribution was shifted to higher weights in treatment 3 (Fig. 3.1.4). Some grains in treatment 3 were heavier than any grains in the control plants. The most likely explanation for this is that when grain set was reduced by water stress there was a compensatory increase in the weight of the surviving grains.

3.1.1.4. VEGETATIVE GROWTH:

All water stress treatments shown in Fig. 3.1.1 resulted in a

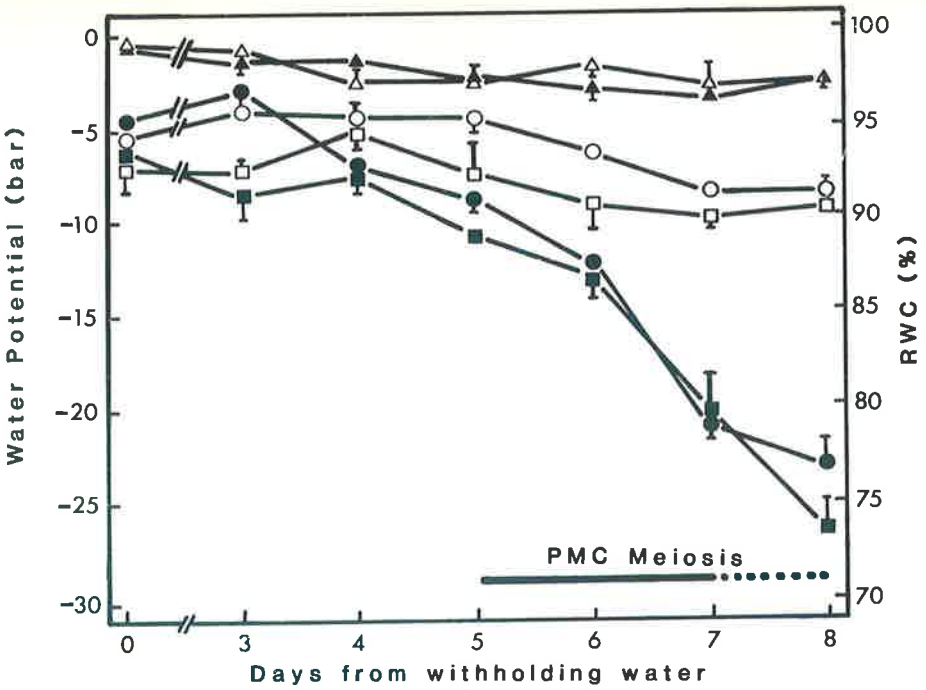


Fig. 3.1.3. The effect of withholding water supply during the sensitive period on the relative water content (■) and water potential (●) of the uppermost fully expanded leaf and on the water potential of the spike (▲). Open symbols represent the corresponding control values. Standard errors of means are shown by vertical bars (where no bars exist they fell within the point).

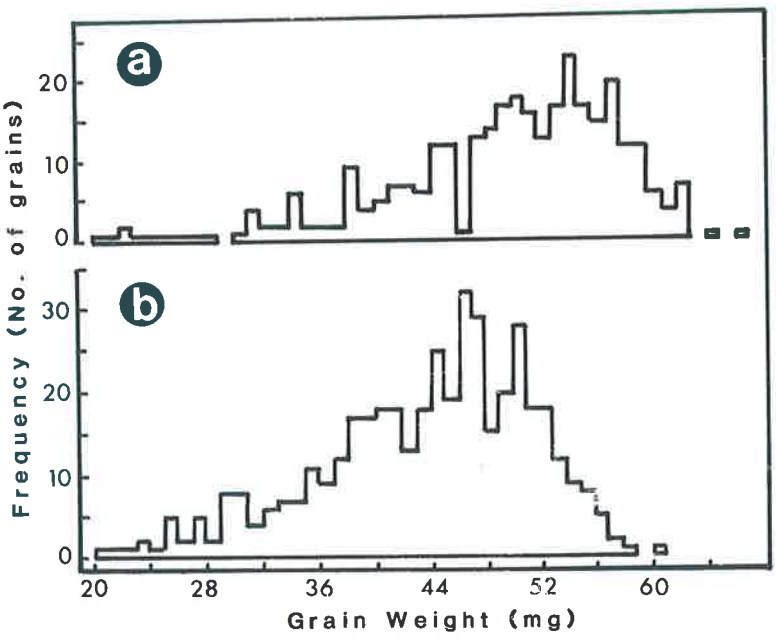


Fig. 3.1.4. The frequency distribution of the weight of individual grains from control plants (b) and plants subjected to water stress (a) during the sensitive stage, treatment 3 (see Fig. 3.1.1.).

significant reduction in final shoot weight, and water deficit before anthesis also caused a decrease in plant height (Table 3.1.1). Height was not affected by water stress imposed after anthesis, possibly since stem elongation had slowed or ceased by this stage.

3.1.1.5. DISCUSSION:

Water stress during the period from the beginning of meiosis in the PMCs to the release of microspores and their early development caused significant reduction in grain set. In female tissue, this period is coincident with meiosis in the MMC and the degeneration of three redundant megaspores (Bennett *et al*, 1973b). This identification of developmental stages is not absolute, however, as a considerable inherent asynchrony existed between different florets of a spike and developmental differences of up to 3 days were frequently observed between the most advanced primary floret and the least developed tertiary floret on an ear. There was also some asynchrony between spikes despite efforts to eliminate it. It is indeed possible that only a very short and specific stage of development is susceptible to water deficit; if such is the case the number of florets that are affected in any set of plants depends on the proportion of florets that experienced stress at this critical stage. The period most sensitive to water deficit would appear to be that when microspores are released from tetrads since (a) this is the only stage of development covered by all treatments that reduced grain set and (b) this stage is coincident with the highest level of stress in treatment 3 which caused greatest decline in grain set (Fig. 3.1.1. and Table 3.1.1). This stage of male development also corresponds with the beginning of the degeneration of three redundant megaspores in the gynaeceum. According to Bingham (1966) the sensitive period to water stress occurs during or a "few days" before the PMC meiosis. In the present investigation, however, water deficit occurring during the week preceding meiosis had no effect on grain set. Further, the data indicate that the most sensitive stage is after meiosis, rather than before as suggested by Bingham (1966). In the investigations by Bingham, replication was minimal and the timing of stress was apparently determined by observations of meiosis in control plants alone. It is conceivable that the development of the water - stressed plants varied from that of the control plants monitored, and this may be the reason for the apparent disagreement between the conclusions of the two

studies. It is also possible, however, that the stage of maximum sensitivity differs among cultivars. The compensatory grain set in the distal florets of a spikelet when the basal florets are barren (Bingham, 1966) was not confirmed in the present investigation.

Water stress did not affect the spike Ψ despite a substantial drop in the leaf Ψ and RWC. Reduction in grain set in the absence of water stress in the spike, also observed by Morgan (1980), suggests that the grain set is reduced not as a direct consequence of desiccation of the floral organs but because of some physiological changes triggered by the drop in Ψ elsewhere in the plant, possibly the leaves.

When grain set was lowered by water deficit, the surviving grains tended to be heavier (Fig. 3.1.4). Other workers have also reported a similar increase in the weight of remaining grains when the number of grains on the spike has been artificially reduced, either by controlled pollination or the removal of grains after anthesis (Bingham, 1969; Pinthus and Millet, 1977; Fischer and HilleRisLambers, 1978). Such results have been commonly interpreted to suggest that the total photosynthetic supply to the spike limits final grain weight, but Jenner (1980) has disputed this conclusion since grain removal did not increase the inflow of sucrose to remaining grains although it did increase the amount of amino nitrogen which entered those grains. Singh and Jenner (1982) have found either no relationship or an inverse relationship between grain weight and concentration of carbohydrates in the grain or the rachis, indicating that the availability of assimilates does not limit grain weight.

No loss in grain set was observed when water deficit occurred during or after anthesis (Fig. 3.1.1, and Table 3.1.1). This contrasts with the observations of some other workers (Wardlaw, 1971; Brocklehurst *et al*, 1978) who found that water stress at this time caused a reduction in grain set. These variations in response may be due either to differences between the cultivars or to the severity of stress imposed in various experiments.

A reduction in grain weight encountered here, in response to a water deficit immediately after anthesis, during the endosperm-cell production

phase, has been attributed to a reduction in the storage capacity of the grain following a reduction in the number of endosperm cells (Brocklehurst *et al*, 1978).

3.1.2. HEAT STRESS:

3.1.2.1. SENSITIVE STAGE OF DEVELOPMENT AND GRAIN SET:

In the first experiment described here, each of six groups of plants was exposed to a high temperature regime of 30°C (day and night) for 3 days, such that upon a group being returned to the lower temperature (20°C) the succeeding group was transferred from the lower to the higher temperature. This provided a continuous series of groups of plants (treatments 1-6) each exposed to high temperature for periods of 3 days; the first period commenced just before the onset of meiosis and the last after the completion of anthesis (Fig. 3.1.5). Ears exposed to high temperature during anthesis were enclosed in glassine bags to prevent cross-pollination and hence their temperature may have been slightly higher than the ambient temperature. During all other treatments, however, the ears were unenclosed. The control plants were grown throughout at 20°C. The results are shown in Table 3.1.5 and Fig 3.1.5. Heat treatment at any time from initiation of meiosis until conclusion of tetrad break-up (treatments 1, 2 and 3) resulted in a drastic reduction in the number of grains per spike and per cent grain set, the greatest decline being registered in response to the high temperature in the middle of this period (Treatment 2). There was no comparable effect of heat at any other treatment period. The extent of the decrease in grain set in treatments 1, 2 and 3 varied widely between spikes, the number of grains ranging from zero to 31 per spike, with most values below 15. Comparable figures in control plants varied between 16 and 39, the majority of ears having more than 30 grains.

Almost all of the reduction in grain set caused by treatments 1, 2 and 3, occurred in the florets at primary and secondary positions, where, given favourable conditions most grains are normally set (Table 3.1.6). Grain set in tertiary florets was low even at 20°C and was unaffected by exposure to 30°C.

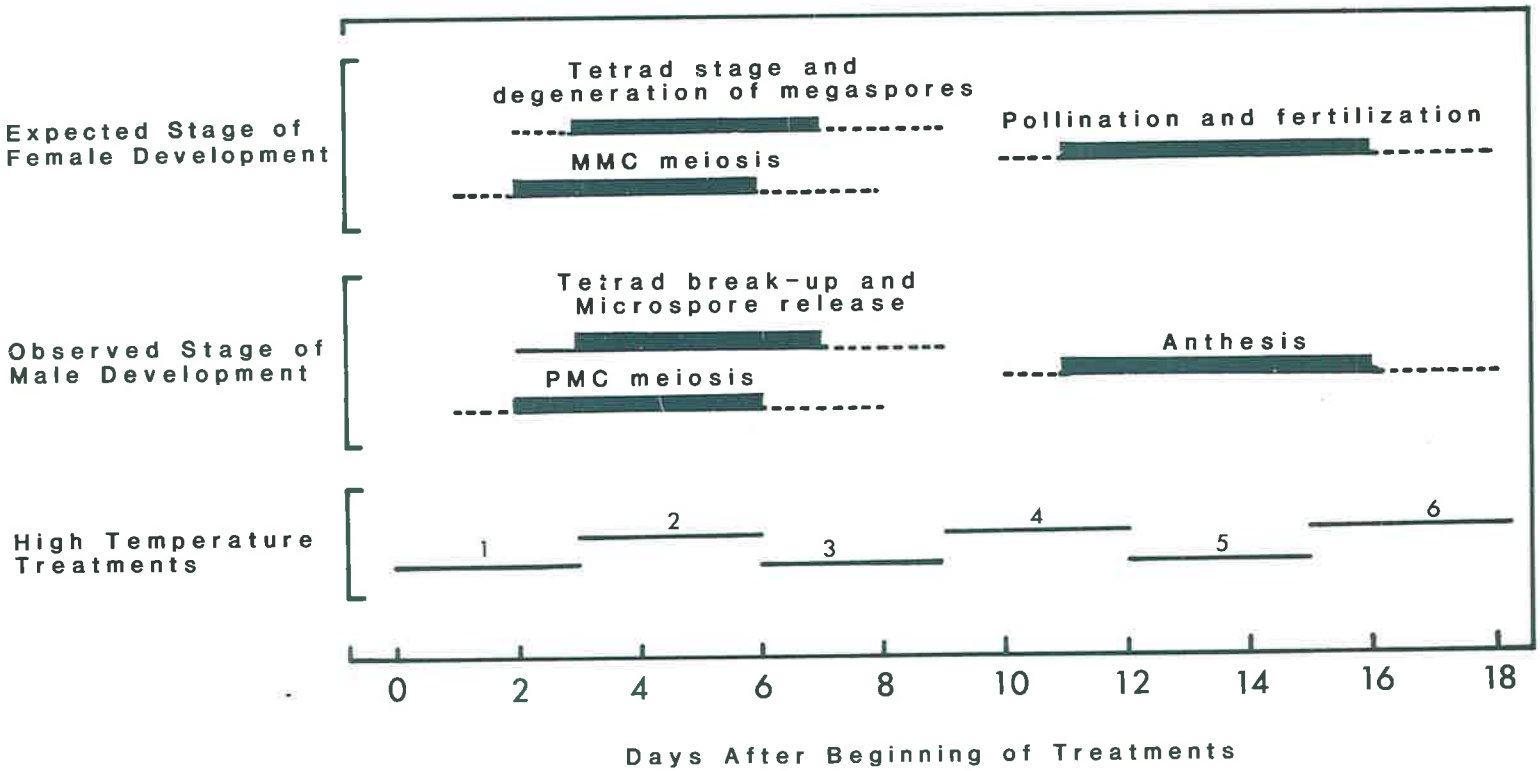


Fig. 3.1.5. Relationship between the periods of exposure to 30°C and the observed development of male and expected development of female reproductive tissues. Thick areas on the bars represent the period during which 90 per cent of the observed florets were at the corresponding developmental stage.

Table 3.1.5. Effect of exposure to 30°C for periods of 3 days at various stages of development of wheat (see Fig. 3.1.5)

Attribute	Control (20°C)	High temperature (30°C) treatments					
		1	2	3	4	5	6
Relative Water Content (%)	89.1^{A}	86.9^{A}	87.4^{A}	90.3^{A}	90.3^{A}	89.3^{A}	89.4^{A}
Number of grains per spike	29.1^{A}	16.4^{B}	9.1^{B}	15.0^{B}	26.9^{A}	29.1^{A}	26.9^{A}
Grain set (%)	54.6^{A}	31.5^{BC}	17.2^{C}	28.3^{C}	50.1^{AB}	54.8^{A}	50.7^{AB}
Weight per grain (mg)	44.1^{ABC}	46.5^{AC}	47.9^{A}	47.9^{A}	43.9^{ABC}	42.2^{BC}	40.2^{B}
Grain yield per spike (mg)	1251^{A}	721^{BC}	433^{C}	699^{BC}	1147^{A}	1210^{A}	1064^{A}
Shoot weight at maturity (g)	6.13^{A}	6.62^{A}	7.42^{A}	6.30^{A}	6.16^{A}	6.39^{A}	6.89^{A}
Plant height at maturity (cm)	75.6^{A}	71.3^{A}	66.4^{A}	65.8^{A}	69.5^{A}	73.2^{A}	73.6^{A}

Each value is a mean of 19-20 plants except Relative Water Content which was measured on 4 plants per treatment. Within a row, values not marked by the same letter are significantly different (P = 0.01).

Table 3.1.6. Effect of 30°C imposed for 3 days at various stages of development on grain set in florets in the primary, secondary and tertiary postions (see Fig. 3.5.1).

Position of floret	Control (20°C)	High temperature (30°C) treatments					
		1	2	3	4	5	6
	Grain set (percentage of fully formed florets)						
Primary	78.0^{A}	35.2^{B}	23.1^{B}	35.9^{B}	67.9^{A}	76.7^{A}	79.1^{A}
Secondary	57.9^{A}	29.2^{BC}	16.5^{C}	31.0^{BC}	53.0^{AB}	63.5^{A}	54.0^{AB}
Tertiary	21.5^{A}	23.1^{A}	10.4^{A}	15.6^{A}	25.0^{A}	19.0^{A}	11.1^{A}

Within a row, values (means of 19-20 plants) not marked by the same letter are significantly different (P = 0.01).

Table 3.1.7. Effect of 30°C imposed for 3 days at various stages of development on the grain set in the lower, middle and upper 1/3rd regions of the spike (see Fig. 3.5.1).

Region of Spike	Control (20°C)	High temperature (30°C) treatments					
		1	2	3	4	5	6
	Grain set (percentage of fully formed florets)						
Lower third	56.8^{A}	36.9^{ABC}	21.2^{C}	31.3^{BC}	53.2^{AB}	55.1^{A}	53.2^{AB}
Middle third	65.2^{A}	36.7^{BC}	23.8^{C}	39.7^{BC}	62.4^{AB}	66.0^{A}	58.1^{AB}
Upper third	41.5^{A}	14.9^{B}	6.1^{B}	12.8^{B}	33.4^{A}	43.0^{A}	41.1^{A}

Within a row, values (means of 19-20 plants) not marked by the same letter are significantly different (P = 0.01).

Grain set in the lower, middle and upper regions of the spikes was assessed separately in order to ascertain whether different regions displayed similar sensitivity to heat. The results presented in Table 3.1.7. demonstrate that all three regions of the spike contributed equally to the overall decline in grain set.

The effects of temperature regimes of 30/20°C (day/night) for 3 days and 30°C (day and night) for 1 day during the sensitive period of development on grain set were also assessed. The longer treatment was started at the onset of meiosis in the most advanced florets whereas, when the temperature treatment lasted for only a day, plants at the commencement of meiosis and those where meiosis had started about 24h earlier were treated in roughly equal proportion. This was done to ensure that florets at the critical stage at most of the positions in a spike were represented in the treatment. Both these treatments caused significant reductions in the number of grains per spike as well as per cent grain set (Table 3.1.8).

3.1.2.2. WATER STATUS:

Atmospheric relative humidity during exposure to high temperature was maintained above 70% and no differences between the RWC of the uppermost leaf of control plants and of those subjected to heat stress during various developmental stages were found (Table 3.1.5). RWC and Ψ of the uppermost fully expanded leaf and the spike Ψ were measured every day during a 3-day spell of 30°C temperature (imposed to duplicate treatment 2, Fig 3.1.5) during the sensitive period. The leaf RWC, the leaf Ψ nor the spike Ψ of the plants under this temperature regime were at no time significantly lower than those of the plants at 20°C (Fig. 3.1.6). Spike Ψ, presumably the most important parameter with respect to floral fertility, did not fall below -3 bar in response to the heat treatment.

3.1.2.3. GRAIN WEIGHT:

Treatments 1, 2 and 3 (Fig 3.1.5) significantly lowered grain yield per plant, apparently due entirely to effects on grain set (Table 3.1.5). No other treatment reduced grain yield significantly and no treatment, whether or not it affected grain set, had any significant influence on the

Table 3.1.8. Effects of two temperature regimes imposed during the sensitive stage of development.

	Temperature regimes			
	20°C (Control)	30°C day/20°C night for 3 days	20°C (control)	30°C (day & night) for 1 day.
Number of grains per spike	19.2	12.3*	24.0	18.3*
Grain set (%)	68.9	43.9*	65.8	54.2*

* Significantly different from control (P = 0.05). Treatments means based on 10-12 plants.

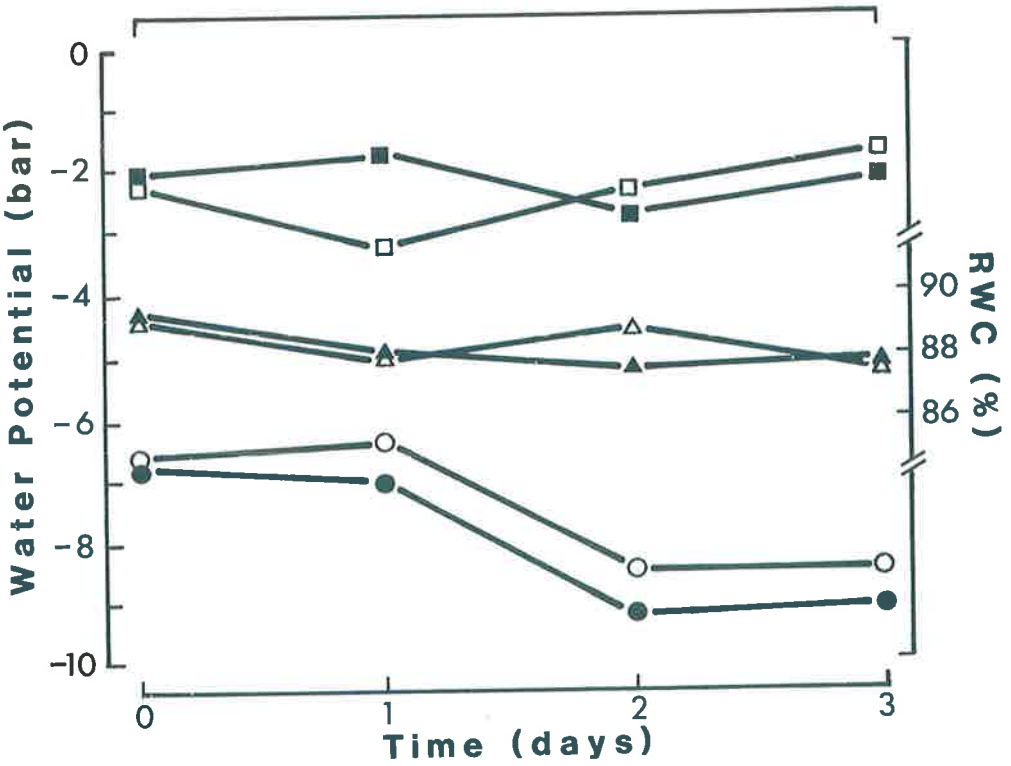


Fig. 3.1.6. Effect of exposure to 30°C for 3 days during PMC meiosis (similar to treatment 2, Fig. 3.1.5.) on the relative water content (▲) and water potential (●) of the uppermost fully expanded leaf and on the water potential of the spike (■). Open symbols represent the corresponding control (20°C) values. The treatment means (of 3 replicates each) were not significantly different (P = 0.01) on any occasion for any of the attributes.

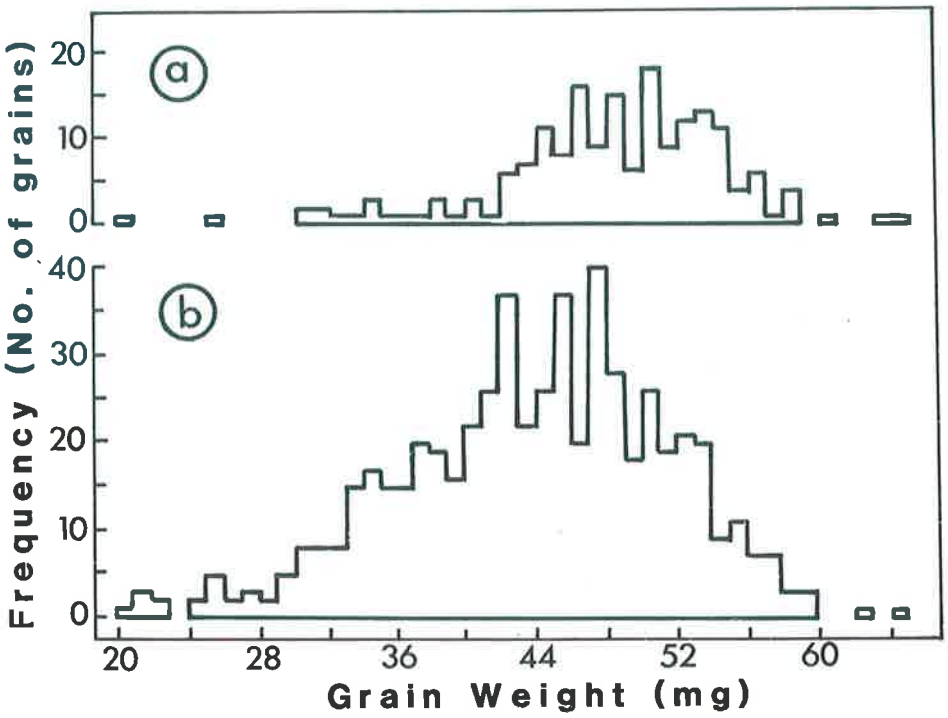


Fig. 3.1.7. Frequency distribution of the weight of individual grains (a) from plants exposed to 30°C for 3 days during PMC meiosis (treatment 2, see Fig. 3.1.5.) and (b) those retained at a lower temperature (20°C).

average weight per grain when compared with grains on plants grown at 20°C throughout. Nevertheless, treatments which reduced grain set (treatments 1-3) produced grains with higher average weight compared to those (treatments 5 and 6) where florets would have undergone early grain development at the elevated temperature (Table 3.1.5).

Although frequency distribution of the weights of individual grains on plants exposed to treatment 2 (Fig 3.1.5) was shifted slightly towards the higher weights, when compared to the similar distribution from control plants (Fig 3.1.7), there was no significant difference between the average grain weight on heat affected and control plants (Table 3.1.5). The data, therefore, provided no convincing evidence for a compensatory increase in grain weight upon reduction in grain set.

3.1.2.4. VEGETATIVE GROWTH:

None of the heat treatments (Fig 3.1.5) significantly affected final shoot weight or height of the plants. There was a small negative effect (significant at P=0.05) of treatments 2 and 3 on plant height, presumably because these treatments coincided with the phase of most rapid stem elongation.

3.1.2.5. DISCUSSION:

A drastic decline in grain set was observed when plants were exposed to 30°C during the period of male development from the onset of meiosis to tetrad break-up; no effects of these treatments were observed at other stages of development. In female tissue this period is concurrent with meiosis in MMC and the degeneration of three redundant megaspores in the tetrad. (Fig. 3.1.5. after Bennett *et al.*, 1973b). It is probable that the stage of development most sensitive to heat stress may be of short duration. Although treatments within a 9-day period had a significant effect on grain set (Fig. 3.1.5, Table 3.1.5), this may not indicate a corresponding period of sensitivity. Because of a high level of asynchrony between florets within a spike and between spikes (Section 3.1.1.5) the stage of development responding to heat stress may have been more specific and of shorter duration than the data presented in Table 3.1.5 would suggest. This notion is further supported by the observation

that exposure to 30°C for only 24h could significantly reduce grain set (Table 3.1.8). Variable reductions in grain set among different spikes and treatments might then have reflected the number of florets at the critical stage when exposed to the damaging temperature. If this interpretation is correct, then the later stages of meiosis and the break-up of tetrads releasing the microspores is the most likely temperature-sensitive event. This stage was subjected to high temperature in some florets in all treatments showing reduced grain set (Table 3.1.5) and most florets would have been heated at this stage in treatment 2 (Fig. 3.1.5) which had the greatest effect on grain set. The stages of female development occurring during this period include the latter part of meiosis and the beginning of the degeneration of redundant megaspores.

Although this response bears a marked resemblance to the effect of water deficit, where the same sensitive period was identified (Section 3.1.1.1), heat stress did not affect grain set through tissue desiccation. The conditions of the experiments were chosen to avoid excessive evapo-transpiration and hence there was no decline in the water potential of either the spike or the leaf (Fig. 3.1.6). Thus, although water deficit may have contributed to previously reported heat-induced abnormalities in sporogenesis (Randolf, 1932; Sax, 1937; Smika and Shawcroft, 1980) a more direct role of elevated temperature *per se* also appears to occur. Indeed, a rise in leaf temperature may play some role in the damage caused by water stress, since stomatal closure induced by water deficit could cause an increase of several degrees in leaf temperature (Gates *et al.*, 1968).

Wardlaw (1970) has reported a decrease in the number of grains per spike when wheat (cv. Gabo) was exposed to high temperature, comparable to that in the present study, during the 10 days following anthesis. Present observations with the same cultivar have shown that exposure to heat for 3 days immediately following anthesis had no significant effect on grain set. This difference suggests that persistent high temperature may interfere with the development of the fertilized embryo sac, but an early return to a lower temperature permits its eventual development to a mature grain.

A reduction in grain set induced by water stress was partially offset

by an increase in the mean weight of surviving grains (Section 3.1.1.3) but no comparable compensation was apparent in heat-stressed plants. Compensatory increases in grain weight following surgical removal of grains has been reported (Bingham, 1969; Pinthus and Millet, 1977; Fischer and HilleRisLambers, 1978) but Lupton and Ali (1966) observed that, whereas removal of a few spikelets led to an increase in mean surviving grain weight, a drastic pruning depressed grain growth. A similar response may have occurred here, as heat-stress reduced grain number more than did water stress. It is also possible that the high temperature inhibited later grain growth and thus prevented the expression of increase in grain weight in response to the reduced grain number.

3.2. EFFECTS OF STRESS ON FEMALE- AND MALE-FERTILITY

Following delineation of the sensitive stage during which water deficit and heat caused a reduction in grain set (section 3.1) further experiments were conducted to identify the tissue that was affected and the nature of injury that occurred. Water stress and heat during the period of development under study could reduce grain set in any of a number of ways, including:

(1) Production of sterile pollen or embryo sac.

(2) Modification of the normal function of somatic tissue in such a way as to interfere with pollination or fertilization either by preventing the transfer of pollen to the stigma or by inhibiting normal pollen tube growth and eventual gametic union.

(3) Inhibition of early cell divisions in the fertilized embryo sac thus impairing its development to the seed.

The second and third possibilities are considered unlikely to be the cause as stress during the period when these events are in train did not alter grain set. However, water dificit or heat encountered at an earlier stage could possibly produce delayed effects which would subsequently prevent these processes. This possibility, together with the effects of stress on female and male fertility were investigated by a series of experiments.

3.2.1. WATER DEFICIT:

3.2.1.1. EFFECTS ON FEMALE FERTILITY AND SOMATIC TISSUE:

In one experiment, two sets of plants were grown in identical conditions; one set was supplied with adequate water throughout whereas the other was subjected to water stress at the sensitive stage (see section 3.1.1.1). These two sets were then further sub-divided to provide a total of four groups (I to IV, Table 3.2.1). All but the primary and secondary florets on the central eight spikelets were removed two days before the anticipated date of anthesis. The spikes on one set from each of the water-stressed and well-watered plants were covered with glassine bags and allowed to self-pollinate naturally, whilst similar sets of

spikes were emasculated two days prior to anthesis and hand-pollinated at anthesis using pollen from well-watered plants and covering to prevent uncontrolled pollination.

Table 3.2.1. Comparative Seed set among Water-stressed and Well-watered Plants of Wheat following Self-pollinationn or Cross-pollination with Pollen from Well-watered Plants.

	Watered Male			
	X	X		
Treatment	Watered Female (I)	Stressed Female (II)	Watered Selfed (III)	Stressed Selfed (IV)
Grain Set (%)	72.4^{A}	74.8^{A}	88.7^{B}	59.5^{C}

Values (means of 21-30 plants) bearing different letter are significantly different (P = 0.01)

A comparison between the self-pollinated spikes of the water-stressed and well-watered plants (III and IV) shows that grain set was significantly reduced by water deficit (Table 3.2.1). However, upon cross-pollination with pollen from well-watered plants, grain set was identical in the well-watered and water-stressed female parents (I and II) and, moreover, was close to that in well-watered self-pollinated plants (III). The 16.3% difference in grain set between self-pollinated and cross-pollinated well-watered plants is likely to have been due to the lower efficiency of hand-pollination. These results show that female

fertility was unaffected by water stress. The results also eliminate any possible role of the non-sporogenous tissue of the stressed plants in preventing pollen germination, pollen tube growth, fertilization or subsequent grain development. The likely reason for the reduction of grain set by water stress, therefore, is the induction of male sterility.

3.2.1.2 EFFECTS ON MALE FERTILITY:

The indirect evidence from the above-mentioned experiment, indicating that male fertility was affected by water dificit, was substantiated by direct observations of anthers and pollen from stressed plants. Well-watered plants bore anthers that were about 3-4mm in length at maturity. These anthers generally extruded from the florets and dehisced through longitudinal slits along both sides, releasing their pollen during anthesis (Fig. 3.2.1). However, 41% of the florets from plants subjected to water stress during the sensitive period contained anthers that were abnormal (Table 3.2.2 and Fig 3.2.1). These anthers were small and frequently shrivelled; they extruded only partially and either did not dehisce at all or dehisced abnormally at the apex alone. Viable pollen

Table 3.2.2. Effect of water stress imposed during the sensitive stage on anther morphology and pollen viability

Attribute	Control	Water Stress
Florets containing abnormal anthers (%)	3.8	41.0**
Pollen viability in normal anthers (%)	91.1	57.8**

** Significantly different from control (P = 0.01).

Fig 3.2.1. Water-stress-affected (a) and heat affected (b) anthers in comparison to the anthers from control plants.

nc - normal anther from a control plant, n - apparently normal anther from stressed plant, ab - abnormal anther, pg - pollen grain.

Bar represents 0.5mm.

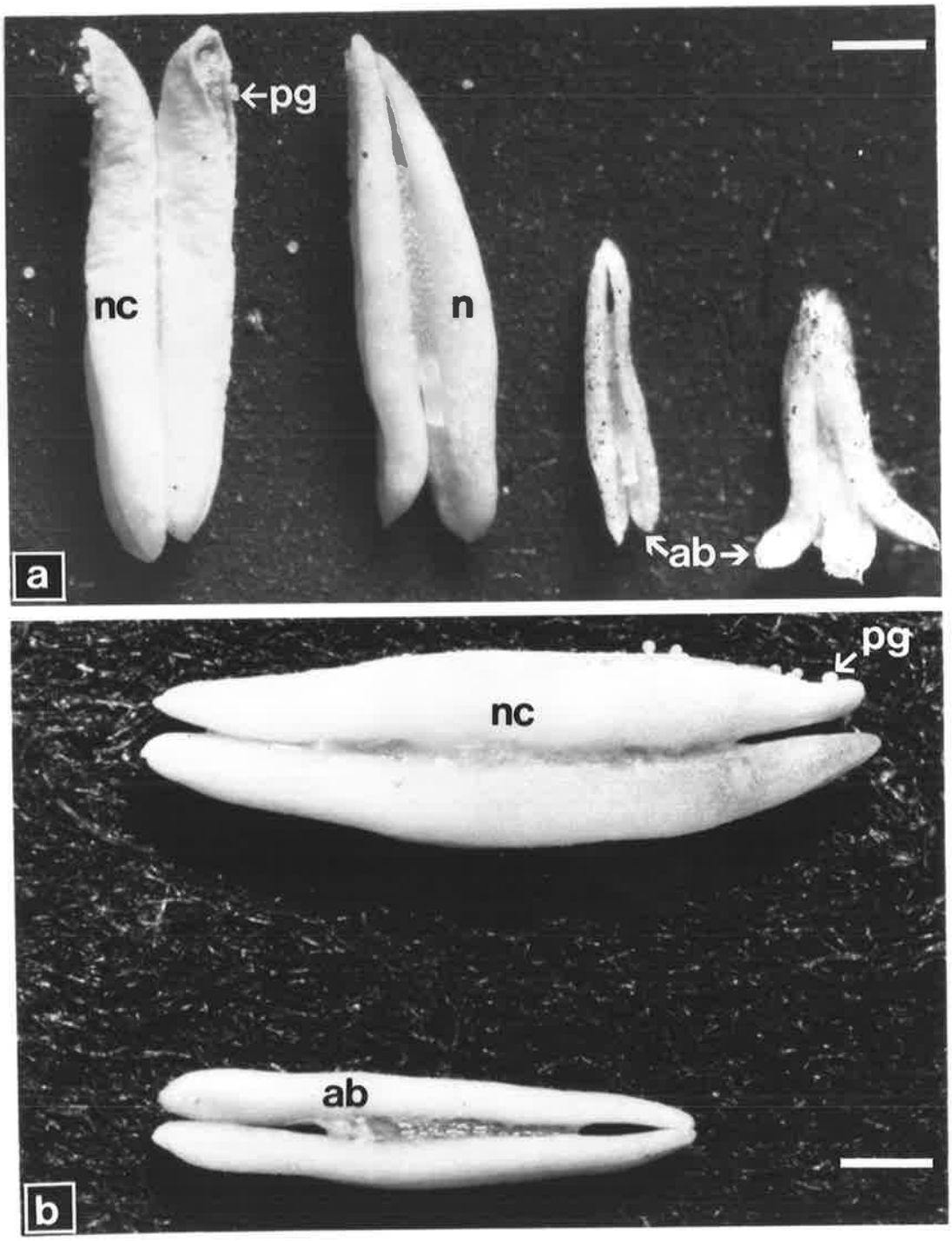


Fig. 3.2.1.

Fig 3.2.2. Effects of water stress during the sensitive stage on pollen viability assessed by staining with TTC.

(a) Stained pollen from a well-watered plant.
(b) Pollen from an abnormal anther from a water-stressed plant.
(c) & (d) Pollen from apparently normal anther from a water-stressed plant.

v - vaible pollen stained with TTC, iv - inviable, and shrivelled pollen lacking staining, ns - non-staining pollen grain that appears normal otherwise.

Bar represents 50μ.

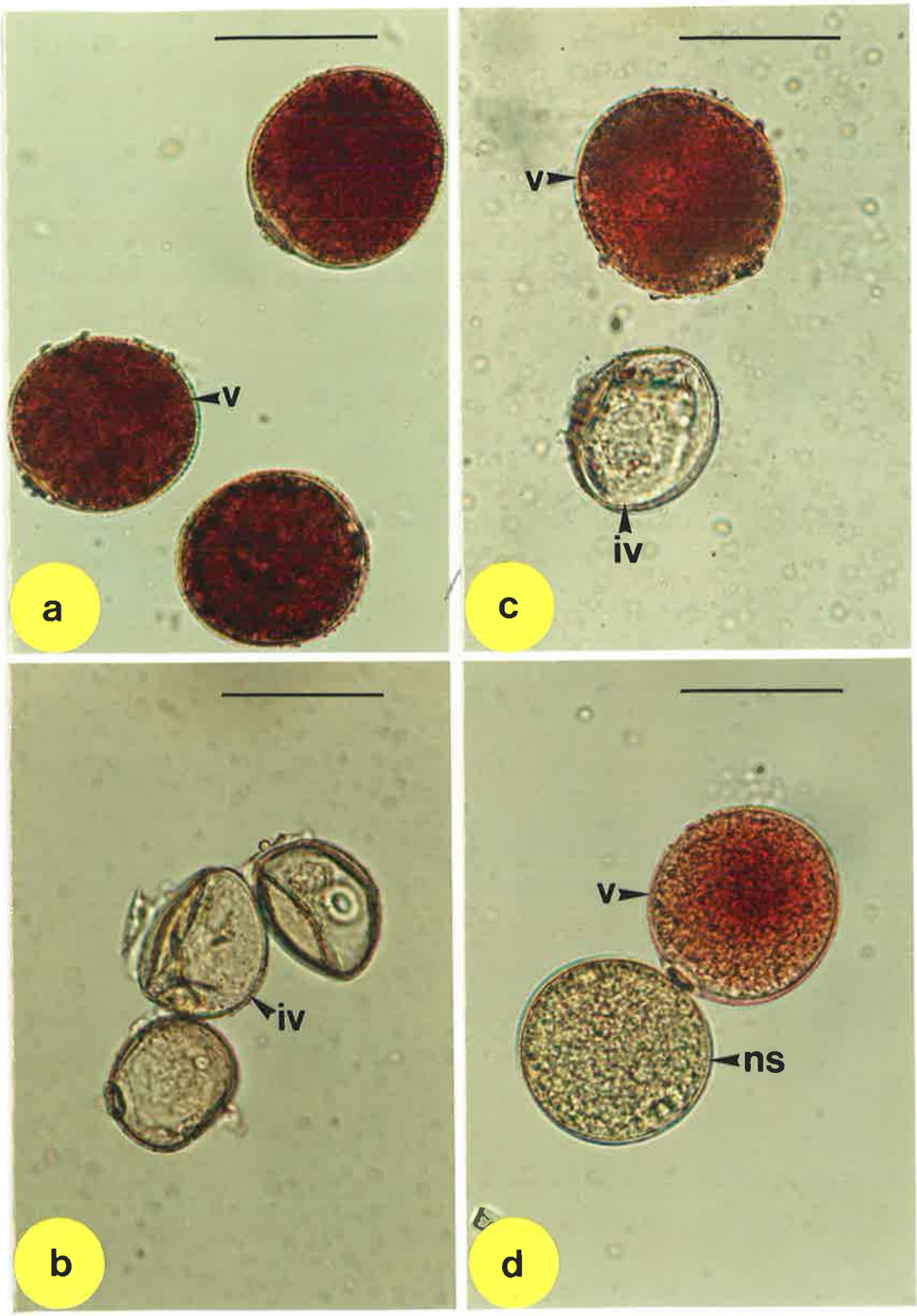


Fig. 3.2.2.

from control plants stained dark red with TTC (Fig. 3.2.2a), but pollen obtained from abnormal anthers failed to show any such staining, indicating their inviability. These pollen grains were mostly shrivelled and had 'dilute' cytoplasm that contained very little or no starch granules. (Fig. 3.2.2b). Only 3.8% of the florets on control plants contained such anthers.

The mean pollen viability in apparently normal anthers from stressed plants was 57.8% which was considerably below the control level of 91.1% (Table 3.2.2). In individual florets from water-stressed plants, the proportion of viable pollen in apparently normal anthers varied between 0 and 99.3%, whereas in well-watered plants it was almost invariably above 75% (Fig. 3.2.3). The inviable pollen grains from the apparently normal anthers on stressed plants varied greatly in appearance, ranging from pollen grains that resembled those of the abnormal anthers (Fig. 3.2.2c), through grains with variable density of cytoplasms, up to some grains that looked structurally normal but did not stain with TTC (Fig. 3.2.2d).

3.2.2. HEAT STRESS:

3.2.2.1. EFFECTS ON FEMALE FERTILITY AND SOMATIC TISSUE:

An experiment having a similar format to that described in section 3.2.1.1, was designed to ascertain the role of male sterility, female sterility and somatic tissue in the reduction of grain set in response to heat during the sensitive stage of development. The details of this experiment were same as described earlier for water stress (Section 3.2.1.1) except that:-

- an exposure to 30°C for 3 days replaced the water stress treatment,
- control treatment comprised plants grown at 20°C and
- the latter were used also as source for pollen.

The results of this experiment are summarised in Table 3.2.3. A comparison between the self-pollinated spikes of heat-treated plants (IV) and those retained at 20°C (III) showed that grain set was significantly reduced by the high temperature. Upon cross-pollination with pollen from control plants, the grain set of plants exposed to the high temperature (II) was still significantly lower than among those retained at the lower

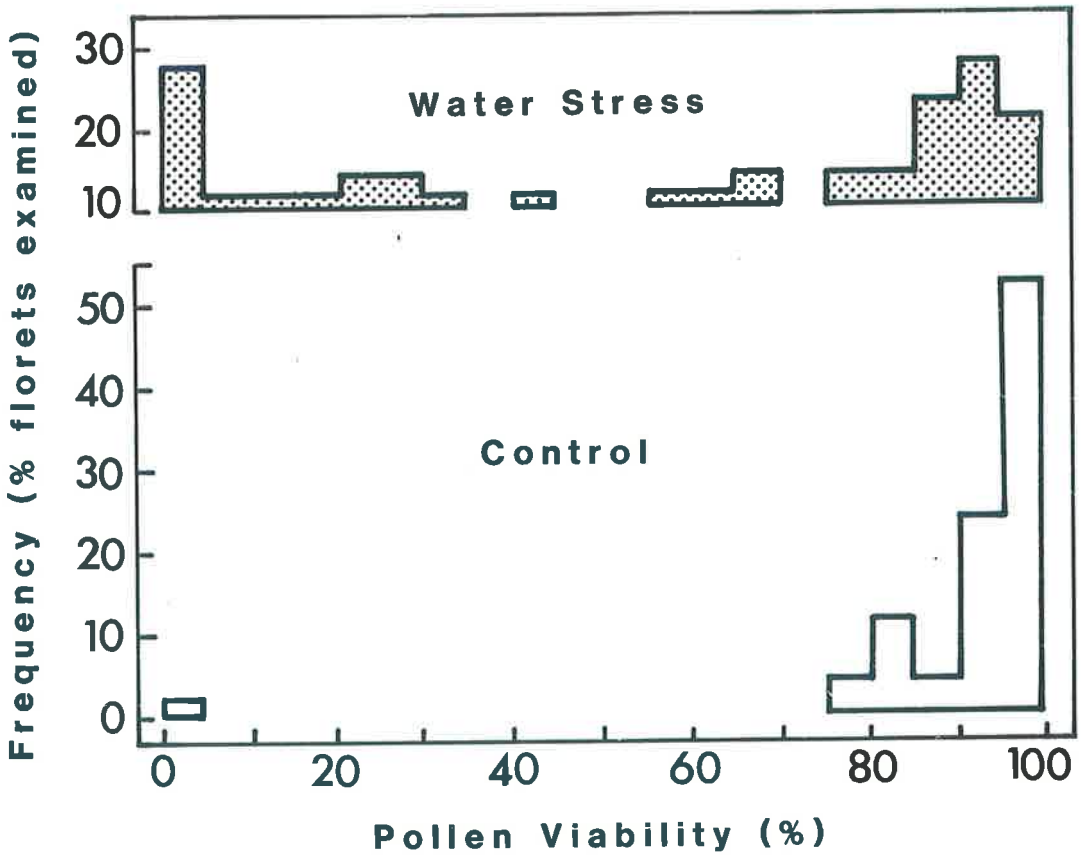


Fig. 3.2.3. The effect of water stress during the sensitive period on pollen viability in apparently normal anthers.

temperature (I). Hand-pollination was not as successful as self-pollination in producing grains in control plants, but these results suggest that female fertility was reduced by exposure to heat. It is also probable that heat may have affected grain set by its effect on non-sporogenous tissues, such as stigma and style, of the female parent. Pollen germination and pollen tube growth through the pistils on heat-stressed plants together with the effects of heat on the structure of the ovary, are discussed in section 3.3.

Table 3.2.3. Comparative grain set among plants exposed to 20°C or 30°C for 3 days during the sensitive stage and either self-pollinated or cross-pollinated with pollen from plants grown at 20°C.

	20°C (control) Male X		20°C (control) Male X	
Treatment	(I) 20°C (control) Female	(II) 30°C Female	(III) 20°C (control) Selfed	(IV) 30°C Selfed
Grain Set (%)	73.3	57.9	89.3	42.2

Means represent 11-17 replicates.

I Vs II*, I Vs III**, III Vs IV**, II Vs IV*

* significantly different (P = 0.05)

** significantly different (P = 0.01)

The grain set in heat-treated plants (II) pollinated with pollen from control plants improved significantly when compared to plants subjected to heat and allowed to self-pollinate (IV), indicating that the reduction in grain set at high temperature cannot be attributed solely to female sterility and suggesting that an effect of heat on male fertility is also highly likely (Table 3.2.3).

3.2.2.2. EFFECTS ON MALE FERTILITY:

Direct observation of anthers and pollen grains substantiated the indications from the cross-pollination experiment that male tissue is affected by exposure to heat. When plants were exposed to 30°C for 3 days during the sensitive stage, anthers in 80% of the florets were abnormal, both structurally and functionally (Fig. 3.2.1b; Table 3.2.4). Most of these anthers remained relatively small, were in some cases shrivelled, and rarely extruded or dehisced. A temperature of 30°C for 1 day or 30°C day/20°C night for 3 days also resulted in the production of such abnormal anthers, although in reduced numbers (Table 3.2.4). Only about 3-8% of florets, situated almost exclusively at the extremities of the spike, contained such anthers in the control plants.

Viable pollen from plants grown at 20°C stained dark red with TTC. Abnormal anthers from plants grown at 30°C contained sterile pollen with similar characteristics to that from anthers rendered abnormal by water stress (Fig. 3.2.2b). Even in those anthers which looked apparently normal and dehisced as usual, pollen viability was significantly lowered by each of the higher temperature regimes, the most extreme damage being caused by exposure to 30°C for 3 days (Table 3.2.4). Florets from plants grown at 20°C had a pollen viability above 85% in the apparently normal anthers, but in a large proportion of florets on plants exposed to higher temperature, such anthers contained pollen of lower viability (Fig. 3.2.4a, b). The largest effect was again caused by 3 days of exposure to 30°C. The pollen grains in these apparently normal anthers were similar in appearance to those in comparable anthers from water-stressed plants (Fig 3.2.2c,d).

3.2.3. DISCUSSION:

Pollination of water-stressed female parents with fertile pollen

Table 3.2.4. Effect of various temperature regimes imposed during the sensitive stage on anther morphology and pollen viability.

Attribute	20°C (control)	30°C for 3 days	20°C (control)	30°C day/20°C night for 3 days	20°C (control)	30°C for 1 day
Florets containing abnormal anthers (%)	2.9	80.3**	8.4	28.1**	8.4	23.9**
Pollen viability in normal anthers (%)	93.3	58.7**	95.9	80.5**	95.9	82.4**

** significantly different from control (P = 0.01)

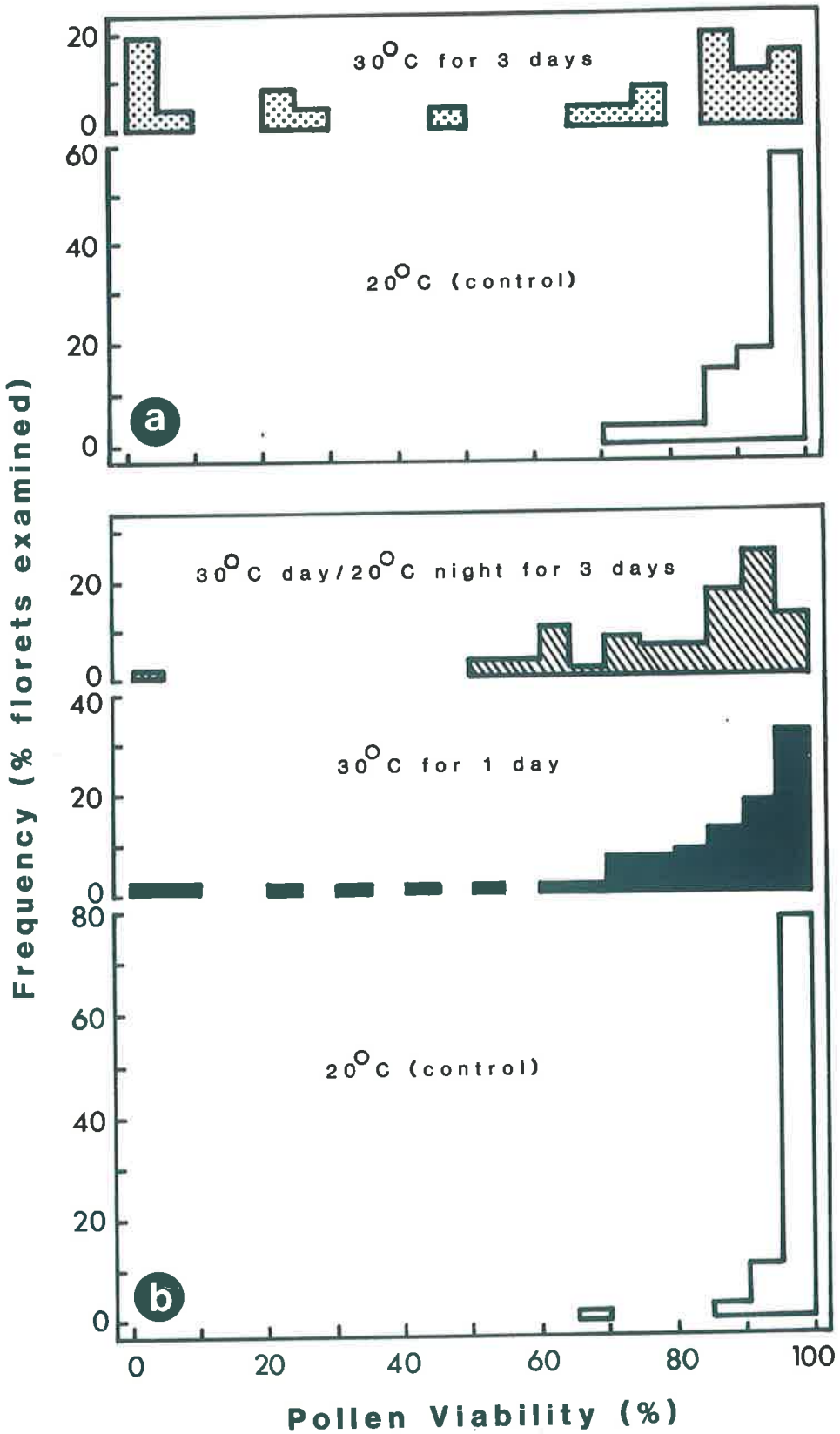


Fig. 3.2.4. Pollen viability in florets containing apparently normal anthers in plants grown at 20°C and in those exposed during the sensitive stage of development to temperature regimes of (a) 30°C for 3 days, (b) 30°C day/20°C night for 3 days or 30°C for 1 day.

restored grain set to the level of similarly pollinated control plants (Table 3.2.1), confirming that the female fertility was unaffected by water stress (Bingham, 1966). Skazkin and Lukomskaya (1962) found that water deficit caused abnormalities in ovules of oats but in these experiments, stress (of unspecified magnitude) was "severe" and "prolonged". It is possible, therefore, that a more drastic reduction in Ψ in the present investigation could have caused female sterility. On the other hand, oats may be inherently more susceptible to drought than wheat. Contrary to the effects of water deficit, heat-stress caused a significant reduction in the female fertility, though not all of the male sterile florets were also female sterile. This effect of heat on female fertility that was later confirmed through anatomical studies on ovaries and pollen tubes, is discussed in section 3.3.

Although heat-affected anthers were generally not as distorted as those affected by water deficit, the effects of two types of stress on pollen were similar. Most of the stress-affected pollen grains, by their appearance, seemed to have ceased development soon after the release of microspores from tetrads. This appeared to impair the accumulation of materials in the cytoplasm, leading to death. This phase of pollen development follows the period apparently most sensitive to water deficit and heat, and it is suggested that stress imposed during the earlier period causes a lesion which is manifested during the later phase of pollen maturation.

The appearance of both viable and inviable pollen grains within a single anther (Figs. 3.2.2; 3.2.3; 3.2.4) may have been due to the asynchronous development of PMCs. Bennett *et al.*, (1971) have reported some degree of asynchrony between PMCs within an anther, and my own observations corroborate this. Such asynchrony may account for the heterogeniety of response, given that the sensitive stage is very short-lived. Alternatively, some cells may be better protected from stress than others by virtue of their position within the anther.

The anatomical events that lead to the abortion of pollen grains in response to stress and the possible mechanism of the process are dealt with in section 3.4.

3.3. EFFECTS OF HEAT STRESS ON OVARY ANATOMY AND POLLEN TUBE GROWTH

When pistils from plants subjected to heat stress at the sensitive stage (Section 3.2.2.1) were pollinated with fertile pollen, the resultant grain set was 21% lower than that in the similarly pollinated unstressed pistils, suggesting an effect of heat on female fertility (Section 3.2.2.1). These results were confirmed by the observations of ovary anatomy and pollen tube growth in the pistils from plants exposed to 30°C for 3 days during the sensitive stage.

The heat-stressed pistils were macroscopically identical to the control pistils.

3.3.1. CONTROL OVARIES:

Twelve control ovaries (from plants grown at 20°C) were serially sectioned and all showed similar anatomy. The ovary wall consisted of 16-20 layers of loose, parenchymatous cells containing starch grains and was bounded by an epidermis (Fig. 3.3.1). The ovary contained a single ovule with an outer integument, an inner integument which formed the micropyle, a nucellus and an embryo sac (Fig. 3.3.1; 3.3.2a). Each integument consisted of two layers of somewhat radially flattened cells. The ovule had a wide chalaza of tightly packed parenchymatous cells. The nucellus consisted of parenchymatous cells which became larger and looser towards the centre of the tissue. The integuments, chalaza and nucellus were devoid of starch grains but had darkly staining cytoplasm.

The pyriform embryo sac was approximately 570 µm in length. The egg apparatus was situated at the narrower, micropylar end of the embryo sac, and consisted of an egg cell which contained starch grains and two synergids with poorly-developed filiform apparatus (Fig. 3.3.2b and c). The egg nucleus was larger than the more faintly staining synergid nuclei. The synergids had smaller and fewer starch grains than the egg cell. The walls of egg cell and synergids were thicker towards the micropyle. In some cases the central cell was located adjacent to the egg apparatus (Fig. 3.3.2c), in others it was nearer to the antipodal cells

Fig 3.3.1. T.S. of a normal ovary of wheat grown at 20°C.

ep - epidermis, es - embryo sac, o - ovule, ow - ovary wall, v - vascular bundle.

Bar represents 100µm

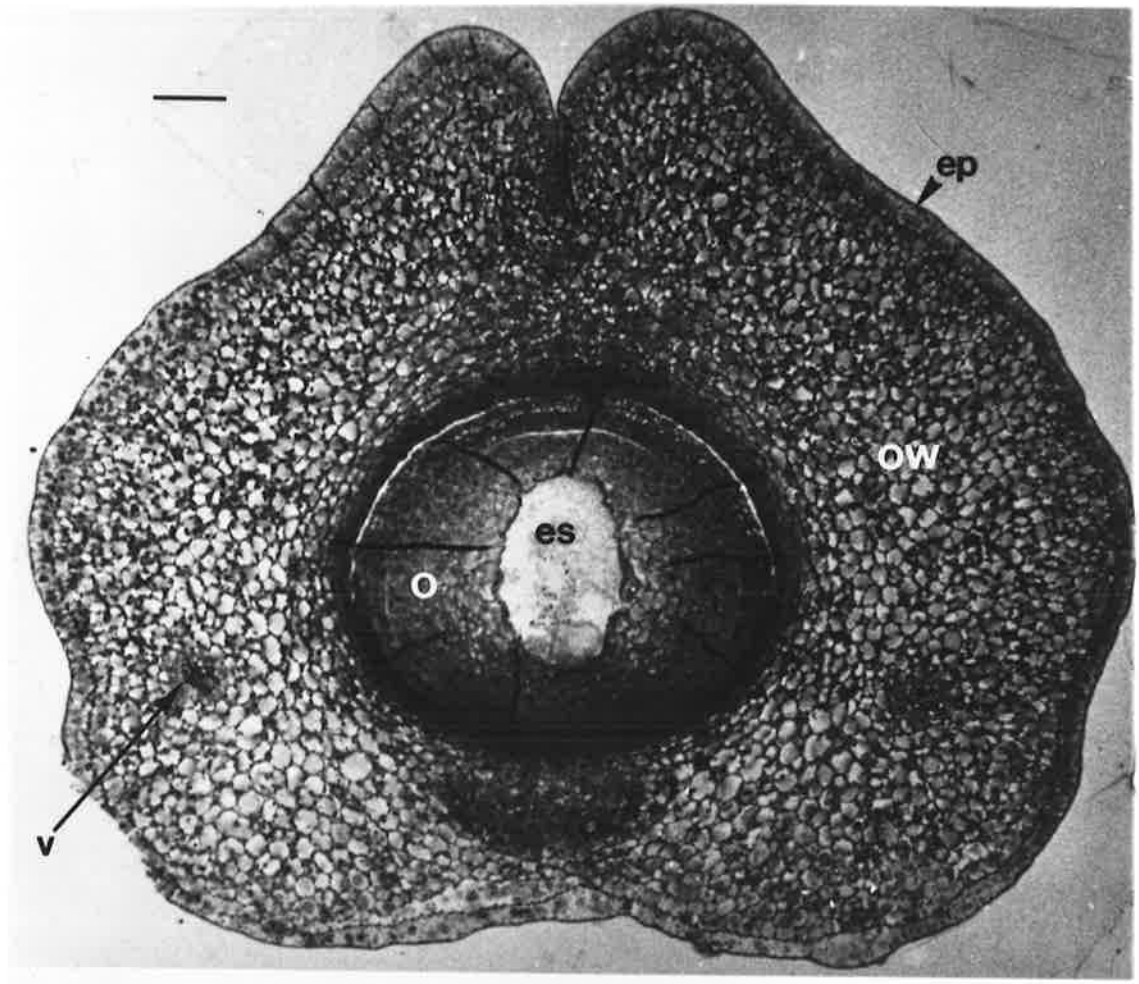


Fig. 3.3.1.

Fig 3.3.2. Normal ovule and embryo sac of wheat grown at 20°C

(a) L.S. of ovary showing the ovule.
(b) L.S. of ovary showing the embryo sac.
(c) L.S. of ovary showing details of egg apparatus and central cell.

a - antipodal cell, c - central cell, e - egg cell, es - embryo sac, ii - inner integument, m - micropyle, ch - chalaza, n - nucellus, oi - outer integument, ow - ovary wall, pn - polar nucleus, s - synergid, st - starch grain, f - filiform apparatus, d - granular deposit in embryo sac, en - egg nucleus.

Bar represents 50μm

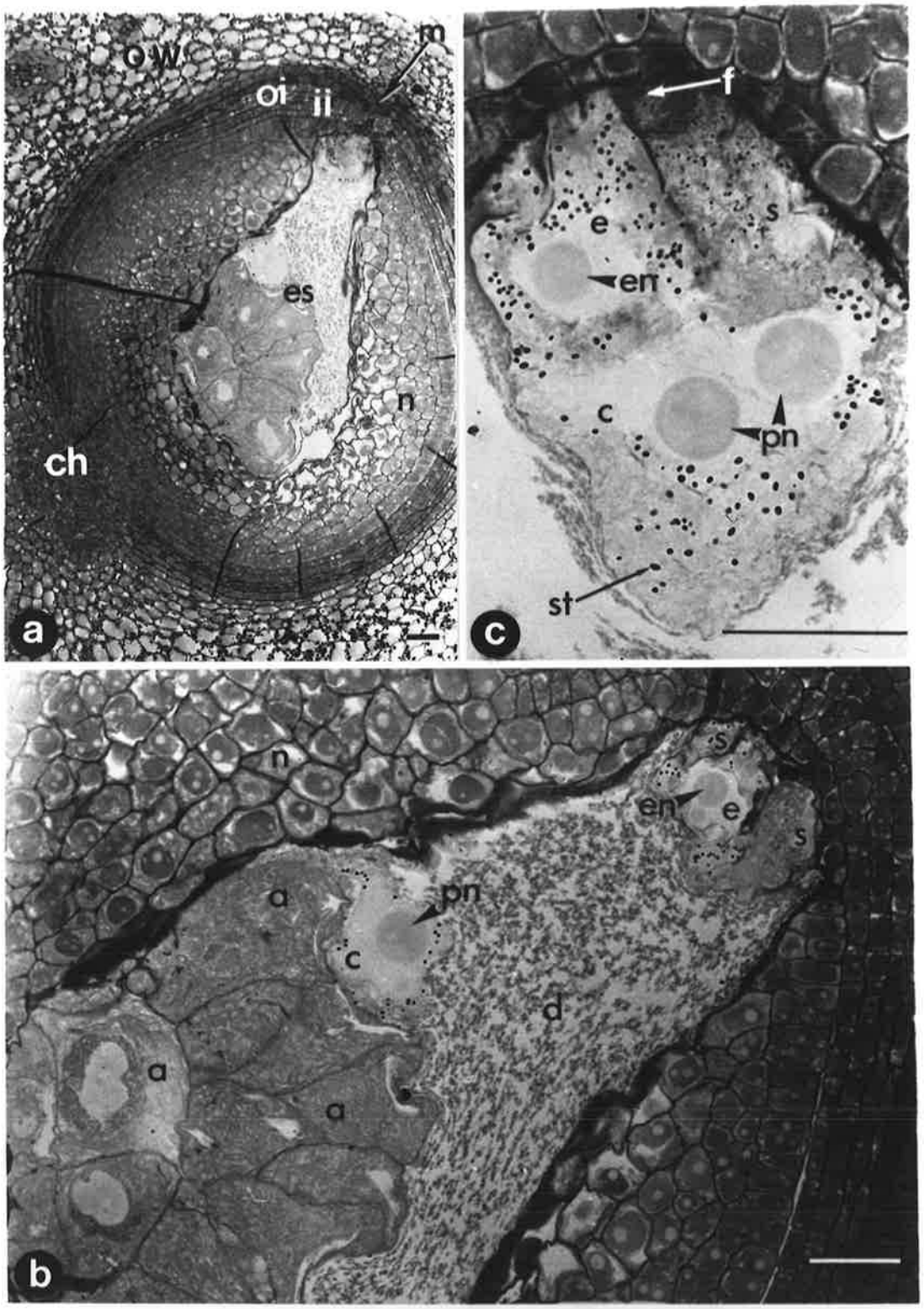


Fig. 3.3.2.

(Fig. 3.3.2b). It contained starch grains and generally two large polar nuclei. Some embryo sacs, however, had a uninucleate central cell. At the broader, chalazal end of the embryo sac there were betwwen 11 and 31 antipodal cells (Fig 3.3.2b) with an average of 19 per embryo sac. The antipodal cells contained no starch and had large, irregularly shaped and faintly staining nuclei. The lumen of the embryo sac contained granular material.

3.3.2. HEAT-STRESSED OVARIES:

Of the 18 ovaries that were serially sectioned, 12 had the same anatomy as the control ovaries (Table 3.3.1). The other 6 ovaries had abnormalities in their nucellus or embryo sac. No ovary showed any abnormality in the ovary wall.

Table 3.3.1 Anatomy of 18 ovaries subjected to heat stress (30°C for 3 days) during the sensitive stage of development.

Anatomical Features	Number of Ovaries
Normal	12
Reduced development of nucellus and no embryo sac	1
Small embryo sac with no cellular organization	2
Small embryo sac with incomplete cellular organization	2
Small embryo sac with complete cellular organization	1

One ovary showed a drastic reduction in nucellus development with some degeneration (Fig. 3.3.3a). The inner integument was highly convoluted and there was proliferation of the outer integument. No embryo sac was found.

Two ovaries had small embryo sacs which lacked any recognizable cells (Fig 3.3.3b). There was some proliferation of the nucellus into the embryo sac area and some convolution of the outer integument.

Two ovaries had small embryo sacs with incomplete cellular organization. One of these had four cells at the micropylar end (Fig. 3.3.3c) and six at the chalazal end (Fig. 3.3.3d). The cytoplasm of these cells lacked starch and nuclei stained faintly. The other ovary had three cells at the micropylar end of the embryo sac; two of these cells resembled synergids but the third lacked the characteristic starch granules of an egg cell (Fig. 3.3.4a). There was a central cell with some starch (Fig. 3.3.4b) but the chalazal end of the embryo sac was filled with un-walled cellular contents and had no organised antipodal cells (Fig. 3.3.4c). There was some convolution of the integuments.

Finally, one ovary had an embryo sac with the full complement of its constituent cells. However, the cells lacked detail as the nuclei were either absent or inconspicuous and there was a reduction in starch accumulation in the egg and central cells (Fig. 3.3.4d). In addition, the embryo sac was only half the length of the control embryo sac (cf. Figs. 3.3.2b, 3.3.4d).

3.3.3. POLLEN TUBE GROWTH:

In vivo pollen germination and pollen tube growth were studied following pollination of emasculated florets with fertile pollen. The number of pollen grains and pollen tubes in 62 control and 81 experimental pistils that were exposed to 30°C for 3 days during the sensitive period of development are shown in Table 3.3.2. Similar numbers of pollen grains germinated on the stigmas but on the average, fewer pollen tubes reached the ovary of the heat-stressed than the control pistils. The distribution of pollen tube numbers was skewed in the experimental group as the percentage of ovaries reached by more than 20 pollen tubes was decreased

Fig 3.3.3. Effect of exposure to 30°C for 3 days during the sensitive stage on the structure of ovule and embryo sac of wheat

(a) L.S. of ovary showing ovule with abnormalities of nucellus and integuments
(b) L.S. of ovary showing ovule with small embryo sac lacking cellular organisation
(c) & (d) T.S. of ovary showing respectively, four cells at the micropylar end and four of the six cells at the chalazal end of an embryo sac with incomplete cellular organisation.

cv - convolution in outer integument, d - degenerate nucellus tissue, es - embryo sac, ii - inner integument, n - nucellus, oi - outer integument, ow - ovary wall, p - proliferation, mc - cells at the micopylar end, chc - cells at the chalazal end.

Bar represents 50µm

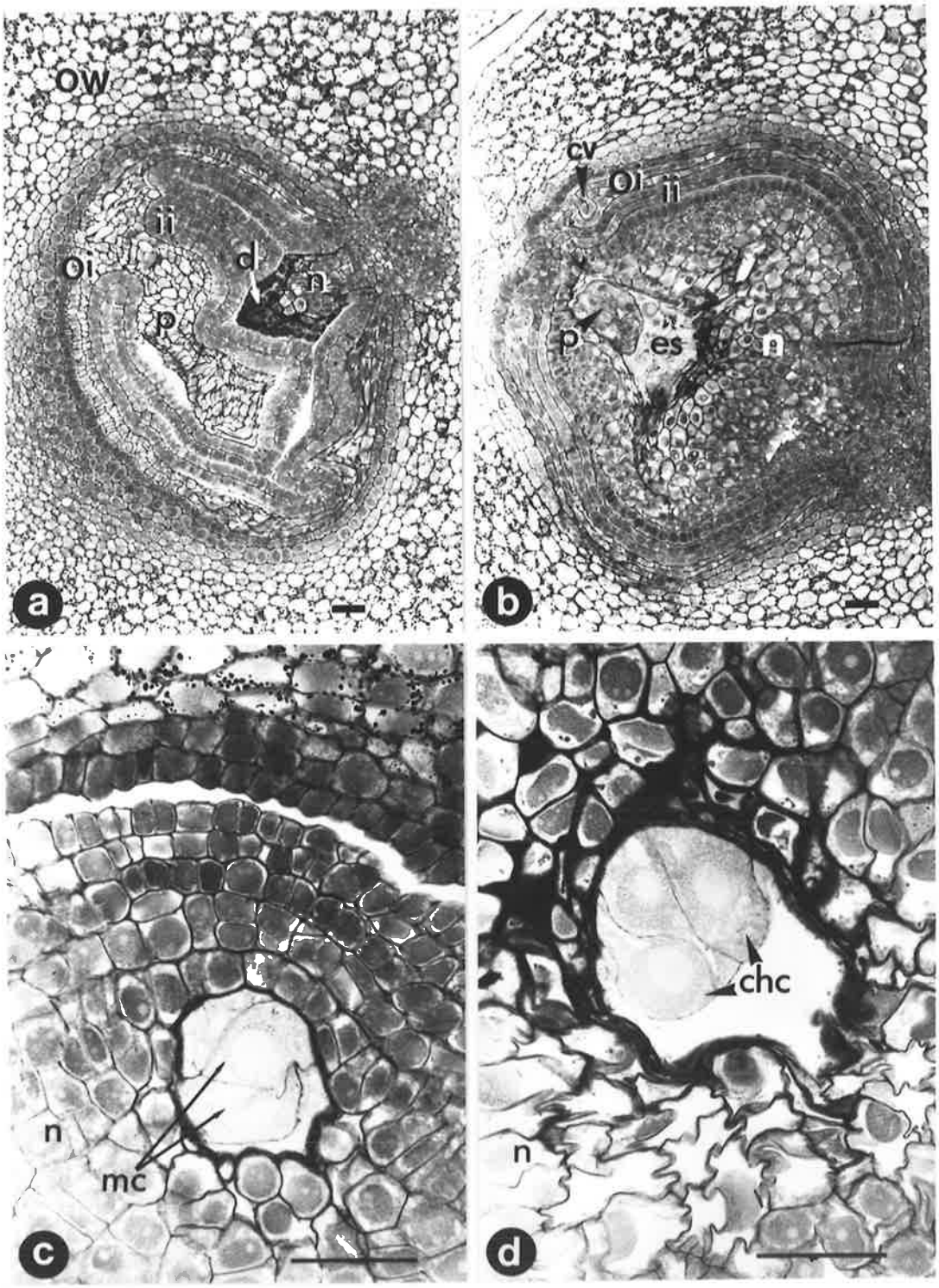


Fig. 3.3.3.

Fig 3.3.4. Effect of exposure to 30°C for 3 days during the sensitive stage on the structure of ovule and embryo sac of wheat

(a), (b) and (c) Oblique T.S. of ovary showing respectively, egg apparatus, central cell and chalazal end of a small embryo sac with incomplete cellular organisation.
(d) L.S. of ovary showing ovule with a small embryo sac with complete cellular organistion.

a - antipodal cell, c - central cell, cv - convolution in integuments, ii - inner integument, n - nucellus, oi - outer integument, ow - ovary wall, s - synergid, e - egg cell, da - degenerate antipodal cells, st - starch, ea - egg apparatus

Bar represents 50µm

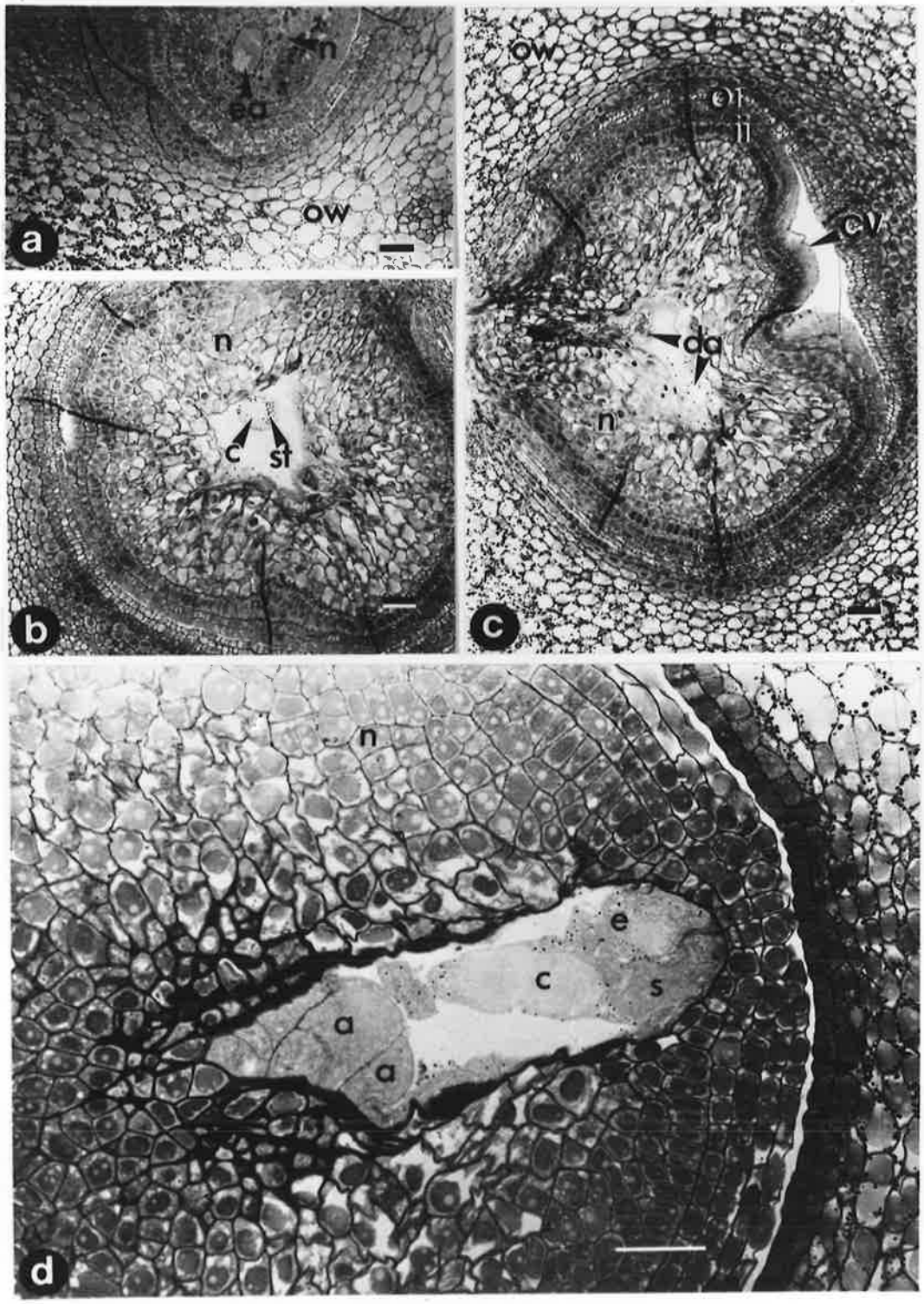


Fig. 3.3.4.

whereas those reached by less than six pollen tubes was increased by heat stress (Table 3.3.3). No pollen tube reached 7.4% of the experimental ovaries. Pollen tubes in the control pistils showed directional growth towards the ovary (Fig. 3.3.5a) whereas many pollen tubes in the experimental pistils grew only short distances (Fig. 3.3.5b). Aniline-blue-positive material (callose) was deposited in some of the pollen tubes which showed abnormal growth (Fig. 3.3.5c).

Table 3.3.2 Pollen germination and pollen tube growth in 62 control (20°C) and 81 heat-stressed (30°C for 3 days during the sensitive stage) pistils following pollination with control pollen.

	Mean (±S.E.) number of;		
	Pollen grains per pistil	Germinated pollen grains per pistil	Pollen tubes reaching the ovary
Control	133.9 ± 11.0	84.1 ± 6.9	19.1 ± 1.3
Heat-stressed	154.5 ± 11.5	84.4 ± 6.8	14.1 ± 1.0 **

** significantly different from control (P = 0.01)

Table 3.3.3. Number of pollen tubes reaching control (20°C) and heat-stressed (30°C for 3 days during the sensitive stage) ovaries following pollination with control pollen

	Percentage of ovaries reached by x pollen tubes: x=						
	0	1→5	6→10	11→20	21→30	31→40	>40
Control	0	6.6	18.0	31.1	31.1	13.1	1.6
Heat-stressed	7.4	13.6	19.8	33.3	23.5	2.5	0

The Pearson's X^2 test applied to the data arranged in a contingency table showed that the pollen tube distribution in the heat stressed ovaries was different from that in the controls at the 1% level of significance.

Fig 3.3.5. Pollen tube growth in pistils exposed to 30°C for 3 days at the sensitive stage of development and those maintained at 20°C following pollination with fertile pollen from plants grown at 20°C

(a) Normal pollen tube growth in a control pistil
(b) Haphazard and suppressed growth of pollen tubes on a heat stressed stigma
(c) Detail of abnormal pollen tube growth on a heat stressed stigma.

pg - pollen grain, pt - pollen tube, c - callose, o - ovary, s - stigma, h - hair on ovary surface, cv - convoluted pollen tube.

Bar represents 50μm

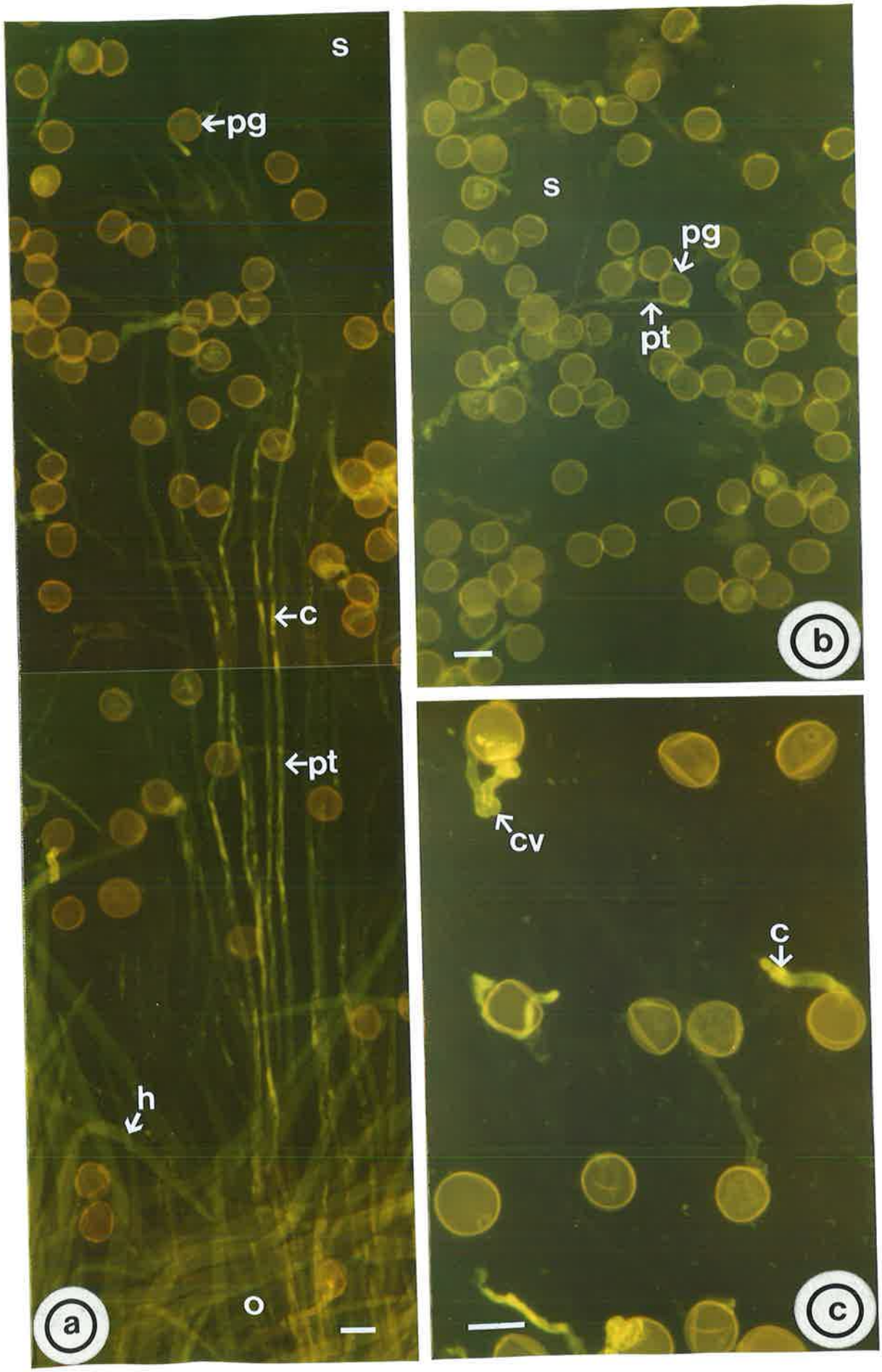


Fig. 3.3.5.

3.3.4 DISCUSSION:

The structure of the normal embryo sacs was similar to that described by previous authors (Percival, 1921; Morrison, 1955; Hoshikawa and Higuchi, 1960; Bennett *et al.*, 1973b). The presence of only one polar nucleus in some embryo sacs supports the view that the polar nuclei fuse before fertilization (Bennett *et al.*, 1973b).

Of the 18 heat-stressed ovaries observed, a third were structurally abnormal. Abnormalities were varied but fell into four broad categories; variation may reflect asynchrony in the developmental stages of the ovules at the time when heat stress was applied. The onset of meiosis in the PMCs coincides approximately with meiosis in the megaspore mother cell (Bennett *et al.*, 1973a) and there is variation in timing between the florets within a spike. Alternatively, it is possible that florets at certain positions within a spike may be more vulnerable to heat stress than others.

There appeared to be two major effects of heat stress on ovule anatomy. One was an effect on the nucellus leading to reduced development and degeneration. The integuments had proliferated to occupy the space that would normally have contained nucellus. The ovary wall appeared to develop normally. The other effect was on the embryo sac, resulting in reduced cellular organization or curtailed extension. In the most severe cases, nucellus tissue had proliferated into the empty embryo sac cavity. This has also been described in oat plants which were subjected to "prolonged" and "severe" water stress (Skazkin and Lukomskaya, 1962). The reason for the lack of cellular detail in the small embryo sac with the full complement of cells is not known but may indicate that the embryo sac cells were degenerating. Of heat stressed embryo sacs, 27.7% showed incomplete cellular organization, corresponding well with the 21% reduction in female fertility estimated by crossing with fertile pollen (Section 3.2.2.1). The small embryo sac with the full complement of cells may have been fertile, despite the reduced cellular detail. Insufficient number of ovaries was serially sectioned for the numbers to be subjected to statistical analysis, but differences in pollen tube growth and seed set (section 3.2.2.1) were both highly significant. The investigation of ovary anatomy illustrates the reasons for the reduced fertility.

Heat stress resulted in a reduction in the mean number of pollen tubes entering the ovary. The wheat ovary contains a single embryo sac and theoretically only one pollen tube is required for grain set. Only 7.4% of pistils had no pollen tubes reaching the ovary, yet female fertility was reduced by 21% (section 3.2.2.1). Thus some of the ovaries which attracted pollen tubes were not successfully fertilised, due probably to the structural abnormalities in the ovule. The poorer pollen tube growth in the experimental ovaries resulted in shorter tubes which showed abnormal growth and the deposition of callose, a ß-1,3-glucan cell wall component, in the pollen tube and pollen grain. This has also been observed in incompatible grass pollen inhibited at the stigma surface (Heslop-Harrison, 1978) and in pollen tubes that ceased growing in avocado pistils grown at high temperature (Sedgley, 1977). Callose plugs are a regular feature of normal pollen tubes and also occur in wheat. However, callose occluding pollen tubes which show abnormal growth probably indicates metabolic disruption. In wheat the inhibition of tube-growth may be attributable to an effect of the heat stress on stigma and style development since the pollen tube uses nutrients from the pistil for its growth to the ovary (Loewus and Labarca, 1973). As heat-stressed stigmas appeared macroscopically identical to the controls, it is also possible that the poor pollen tube growth was due to a lack of attraction from the structurally abnormal embryo scas. Some authors have suggested that directional pollen tube growth results from an attraction, possibly chemotropic, from the embryo sac (Rosen, 1964; Glenk *et al.*, 1971; Sedgley, 1976) and in particular from the synergids (Jensen, 1965; Jensen and Ashton, 1981).

In the field, heat stress commonly occurs in combination with water stress. The reduction in grain set under water-stress conditions is associated exclusively with male sterility (Bingham, 1966; Section 3.2.1.1). It is possible that a water deficit more severe than that imposed in the present investigation may induce female sterility. The heat-induced abnormalities in the female tissue may, however, represent a qualitative difference between the effects of heat and water stress on sporogenesis in wheat. Under field conditions, effects on yield of partial pollen sterility induced by stress could possibly be offset by cross-pollination by pollen from unaffected florets (Hoshikawa, 1961; De Vries, 1971). However, such compensatory ability is lost completely by

the loss of female fertility. For this reason, high temperature during the late reproductive development of wheat is likely to be a more serious threat to yield than is water deficit.

3.4. ANTHER- AND POLLEN-DEVELOPMENT IN RESPONSE TO WATER-STRESS AND HEAT

3.4.1. CONTROL ANTHERS:

Well-watered plants grown at 20°C were selected and labelled at the onset of PMC-meiosis in the most advanced floret of the spike. Anthers from the primary and secondary florets of approximately 8 spikelets from the middle region of the spikes of 1-2 plants were excised on each successive day between meiosis and anthesis. Following fixation, dehydration and embedding, 3.5µm thick sections of these anthers were stained with periodic acid-Schiff's reagent (PAS), toluidine blue O (TBO), coomassie brilliant blue, auramine O and aniline blue, and examined microscopically. Fluorescence of aniline blue and auramine O was checked against autofluorescence of unstained sections.

Development of a normal wheat anther is summarised in table 3.4.1. The premeiotic wheat anther consisted of two lobes, each with two thecae or locules, connected by the filament with a single vascular bundle (Fig. 3.4.1a). The anther wall consisted of four layers - the innermost 'tapetum', the 'middle layer', the 'endothecium' and the outermost 'epidermis' which had a thin cuticle. The epidermal and endothecial cells contained starch grains but cells of the inner two layers did not. After completion of the last premeiotic mitosis in the archesporium, each anther locule contained 4-10 columns of pollen mother cells (PMCs).

During the premeiotic prophase, the PMC nuclei enlarged and the tapetal nuclei completed their last mitotic division before the onset of PMC meiosis (Fig. 3.4.1a). No wall formation accompanied this division and the cells remained binucleate until their degeneration during pollen development. A PAS-positive deposit was present in the anther lumen (Fig. 3.4.1a).

During meiosis, each PMC was enclosed by a callose wall (Fig. 3.4.2) and the nuclei of tapetal cells were prominent (Fig. 3.4.1b).

Following tetrad break-up and the disappearance of the callose layer,

Table 3.4.1. Summary of the development of control anthers.

Stage of microspore development.	Anther anatomy
1) Pre-meiotic interphase.	4-10 columns of PMCs with large darkly-staining cytoplasm. Tapetal cells binucleate and darkly staining.
2) Meiosis	Each PMC enclosed within a callose wall.
3) Young microspores	Callose wall broken down. Microspores aporate, thin walled, with large nuclei. Degeneration of tapetum commences with darkly-staining cytoplasm.
4) Vacuolated microspores	Exine and pore formation in progress. Intine thin and faintly-staining. Microspores irregularly shaped. Orbicules appear on the inner walls of degenerating tapetal cells. Pore always in contact with tapetum. Anther diameter starts to increase.
5) PGM-1	Microspore nuclei enlarge before dividing to form vegetative and generative nuclei. Intine stains PAS-positive. Tapetal degeneration continues. Radial thickenings appear in endothecium. PAS- and TBO-positive granular material appears in anther lumen. Pollen grains begin to accumulate starch and become round in shape.
6) PGM-2	Pollen grains filled with starch. Generative nucleus divides to form two ovoid and darkly staining sperms. Vegetative nucleus stains faintly. Tapetal cell walls broken down.
7) Mature pollen grain	Walls between thecae of each anther lobe break down. Only two outer layers of anther wall remain. Tapetum degenerated. Pollen grain vacuolate, spherical, trinucleate and have starch. Pollen wall has an outer 3-layered exine, middle z-layer and an inner 2-layered intine.

Fig. 3.4.1. Normal development of anthers and pollen grains of wheat.

(a) T.S. of an anther showing pre-meiotic interphase in the pollen mother cells.

(b) T.S. of an anther with PMCs at meiosis.

(c) T.S. of an anther at the young microspore stage.

(d) T.S. of an anther showing vacuolate microspores undergoing exine development.

st - starch, ep - epidermis, en - endothecium, m - middle layer, t - tapetum, pmc - pollen mother cell, l - anther lumen, vb - vascular bundle, ms - microspore, tn - tapetal nucleus, mn - microspore nucleus, ex - exine, in - intine, pr - pore, v - vacuole, f - filament.

Bar represents 50μm.

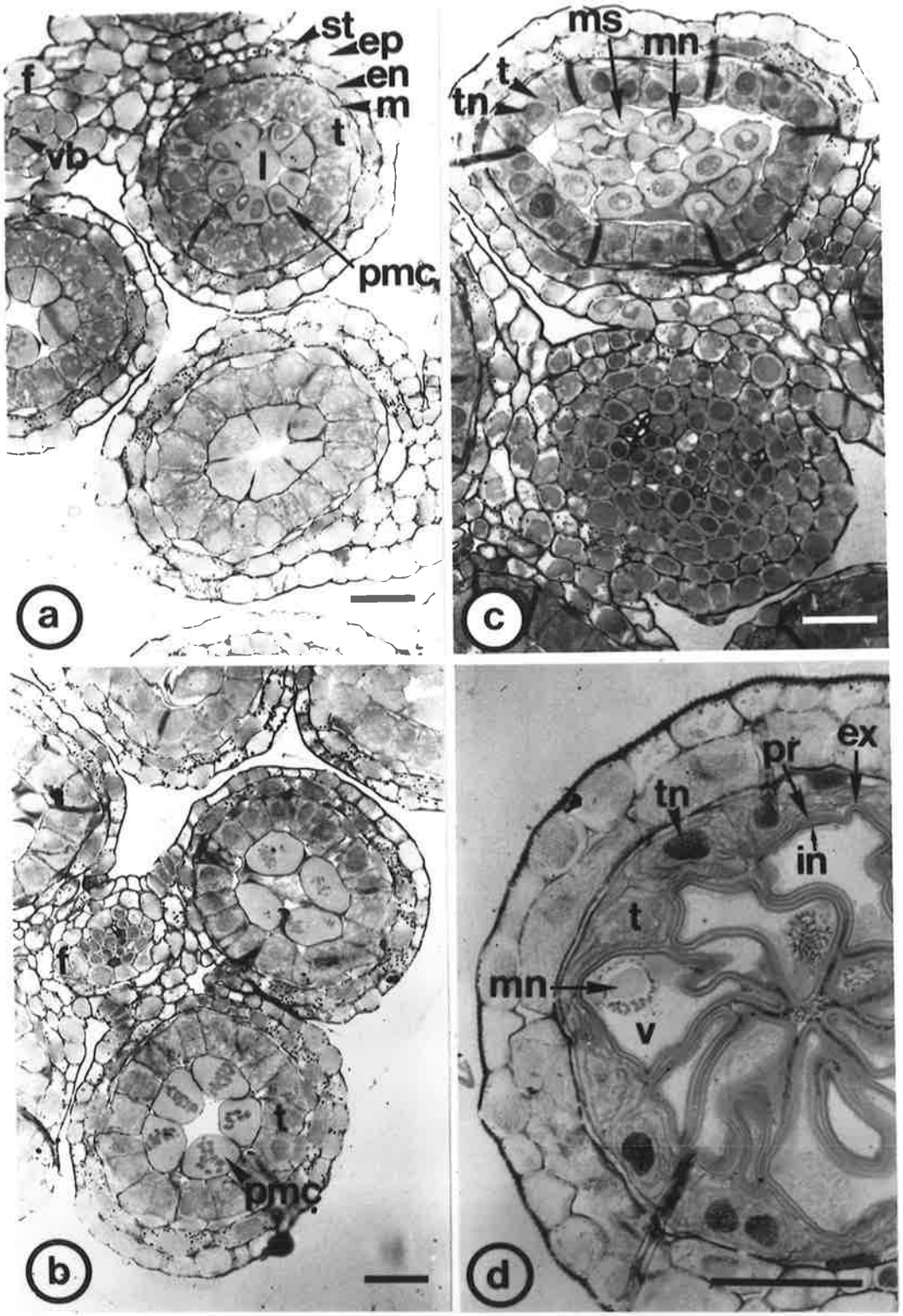


Fig. 3.4.1.

Fig. 3.4.2. T.S. of a control anther stained with aniline blue showing callose wall around each pollen mother cell.

c - callose, pmc - pollen mother cell

Bar represents 50μm.

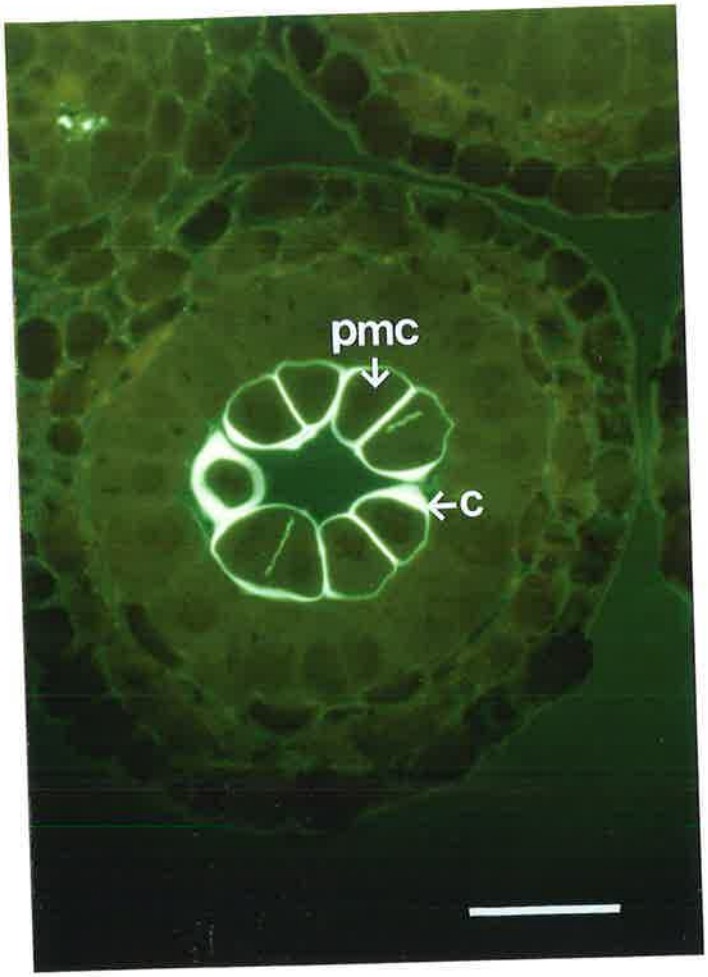


Fig. 3.4.2.

the microspores had thin walls and prominent nuclei (Fig. 3.4.1c). Degeneration of the tapetum coincided with pollen wall development (Fig. 3.4.1d). The exine stained with TBO, coomassie blue and ouramine O, indicating the presence of phenolics, proteins and lipids respectively. The thin intine was thickened in the pore region and showed faint staining with PAS indicating carbohydrate and heavy staining with coomassie blue showing the presence of proteins. The inner walls of the tapetum had numerous orbicules with similar staining characteristics to the exine. All of the developing microspores were in contact with the tapetum and, whenever a pore was observed, it was facing towards and in contact with the tapetum (Fig. 3.4.1d). The microspores, that had a prominent vacuole at this stage, were irregularly shaped, due possibly to the limited space in the anther lumen. Anther diameter remained constant until the young microspore stage ('3'-table 3.4.1) and increased thereafter (Fig. 3.4.3). This, coupled with the degeneration of the tapetum, resulted in an approximately 3-fold increase in the diameter of the anther-lumen from the young microspore stage to maturity (Fig. 3.4.3). The increase in lumen-diameter was paralleled by the gradual rounding-off of the developing microspores.

Microspore nuclei enlarged before the first pollen-grain-mitosis (PGM-1). The PGM-1 produced two nuclei: the 'vegetative nucleus' that became larger and more diffuse, and the 'generative nucleus' that became darkly-staining (Fig. 3.4.4a). A wall between the two nuclei divided the pollen grain asymmetrically into a larger 'vegetative cell' and a smaller 'generative cell'. The intine now stained distinctly PAS-positive and was thickened in the pore region. The exine increased in thickness with no change in staining characteristics. Granular PAS- and TBO- positive material, which first appeared in the anther lumen following the beginning of exine formation, was abundant at the time of PGM-1. The starch disappeared from the endothecium and epidermis, and thickenings, that stained with PAS, TBO, aniline blue and auramine O, appeared on the endothecial cell walls (Fig. 3.4.4a). Orbicules and tapetal-cell-walls stained with auramine O.

Starch deposition in the pollen grains commenced following PGM-1, and at the same time the granular material disappeared from the anther lumen (Fig. 3.4.4b). Degeneration of the tapetal cells was complete by the end of the second pollen-grain-mitosis (Fig. 3.4.4c).

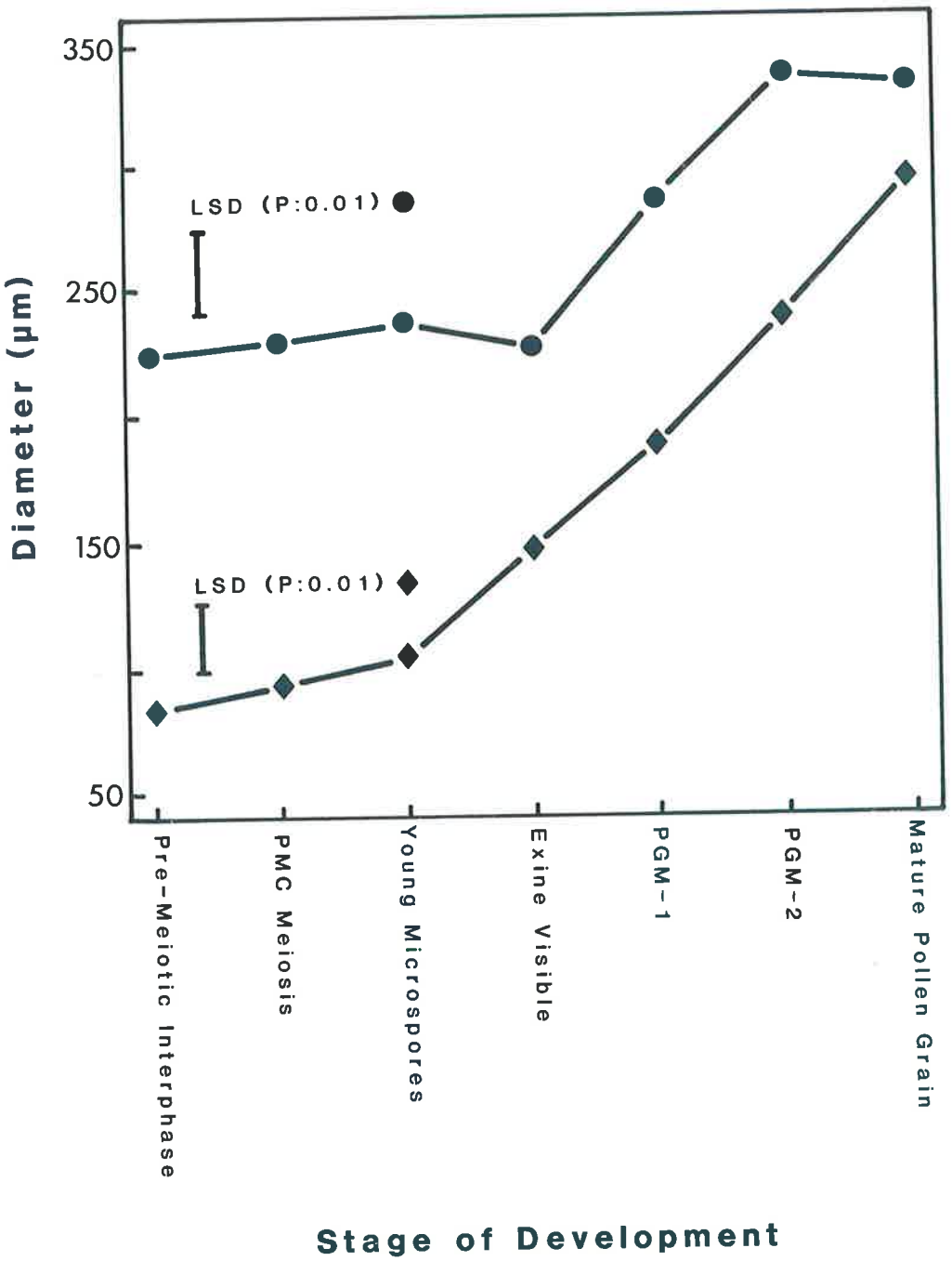


Fig. 3.4.3 Diameter of anther (●) and anther-lumen (◆) at various stages of development. Each mean is derived from 6 measurements. The abscissa refers to the order of developmental stages, not a time scale.

Fig. 3.4.4. Normal development of anther and pollen grains of wheat.
(a) T.S. of an anther showing binucleate pollen grains.
(b) T.S. of an anther showing pollen grains at metaphase of PGM-2.
(c) and (d) T.S. of anthers showing trinucleate pollen grains.

m - middle layer, vc - vegetative cell, gc - generative cell, vn - vegetative nucleus, gn - generative nucleus, g - granular deposit in anther lumen, pr - pore, th - thickenings in endothecial cells, st - starch, t - tapetum, sp - sperm, tr - tapetal residue.

Bar represents 50μm.

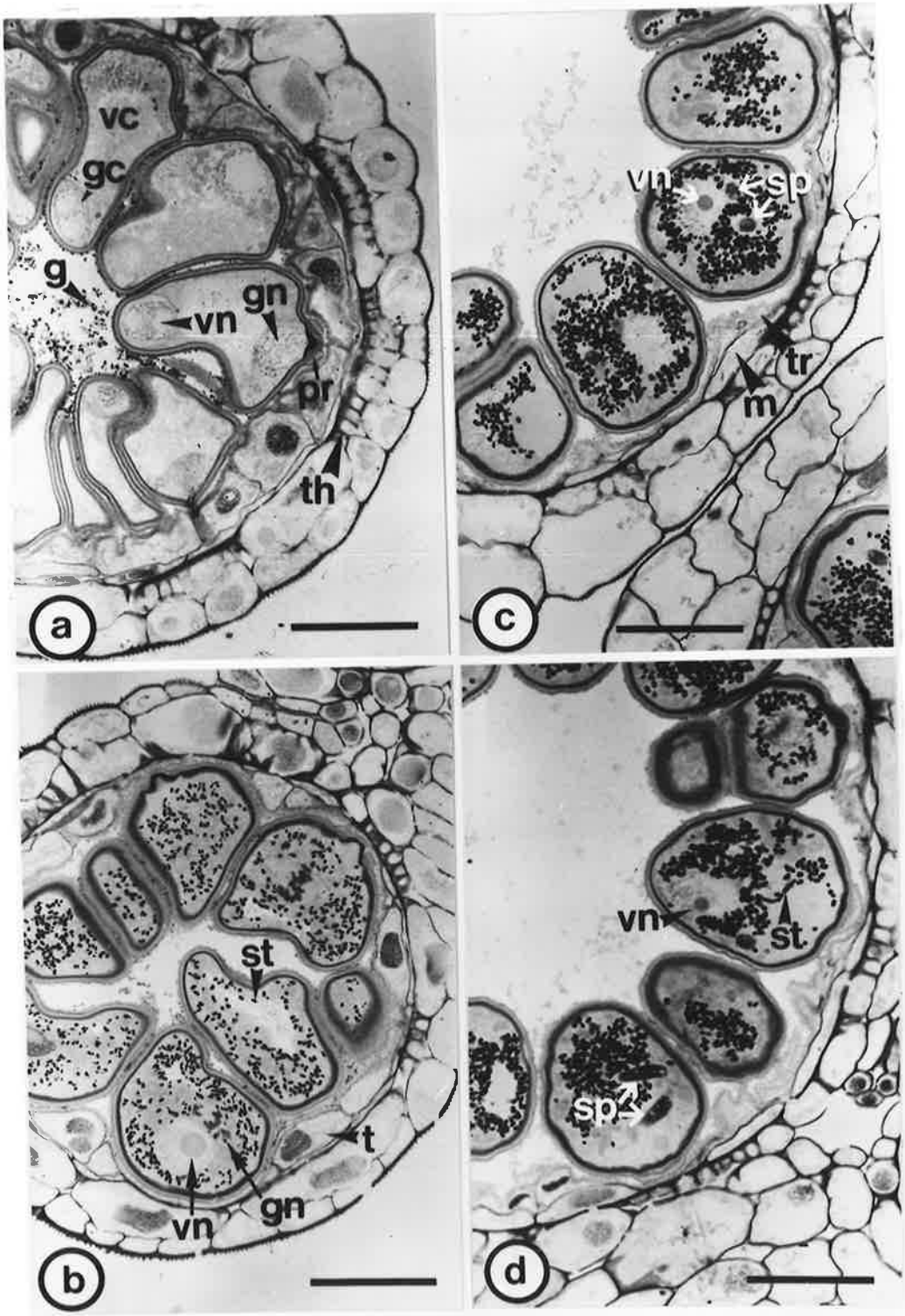


Fig. 3.4.4.

The generative nucleus divided during PGM-2 producing two ovoid sperm nuclei (Fig. 3.4.4c,d). The vegetative nucleus became very diffuse and stained faintly.

At maturity, the wall separating the two thecae of an anther lobe was broken down and the latter dehisced as a single locule (Fig 3.4.5a). The anther wall consisted of the two outer layers only, with a few residual cells of the middle layer still remaining (Fig. 3.4.5b). All that was left of the tapetum was a thin layer of orbicules and some PAS-positive material lining the anther lumen. The pollen grains contained starch, a vacuole, one generative nucleus and two sperms (Fig. 3.4.5b). The pollen wall consisted of an outer 3-layered, exine, a middle Z-layer that was more prominent in the pore region, and an inner 2-layer intine that was thickened in the pore area (Fig. 3.4.5b; 3.4.7a; terminology as described by Heslop-Harrison, 1979). The exine was sculptured giving a granular texture to the surface of the pollen grain (Fig. 3.4.6). An operculum of exine material was present in the pore region (Fig. 3.4.5b; 3.4.6). The staining characteristics of various components of anther and pollen grain at maturity are summarised in table 3.4.2. and are also depicted in fig. 3.4.7 to 3.4.9.

3.4.2. WATER-STRESSED ABNORMAL ANTHERS:

Plants were subjected to water stress by withholding water until the leaf water potential dropped to -25.4±0.5 bar, when watering was resumed. One day before the resumption of watering spikes at the onset of PMC-meiosis in the most advanced floret were selected and labelled. Anthers from these plants were periodically harvested and studied in the manner described in section 3.4.1.

Water-stressed anthers developed normally up to about the time of PGM-1 following which abnormalities started appearing. Two types of abnormal development that were observed are summarised in table 3.4.3.

3.4.2.1 TYPE-1:

In 58.4% of the abnormal thecae, developing microspores became

Fig. 3.4.5. Normal development of anthers and pollen grains of wheat.
(a) T.S. of a mature anther. Bar represents 100µm.
(b) T.S. of a mature anther showing details of pollen grain and anther-wall. Bar represents 10µm.

w - anther wall, pg - pollen grain, f - filament, in - intine, op - operculum, or - orbicule, en - endothecium, ep - epidermis, c - cuticle, st - starch grain, sp - sperm, ex - exine, z - z layer, m - middle layer.

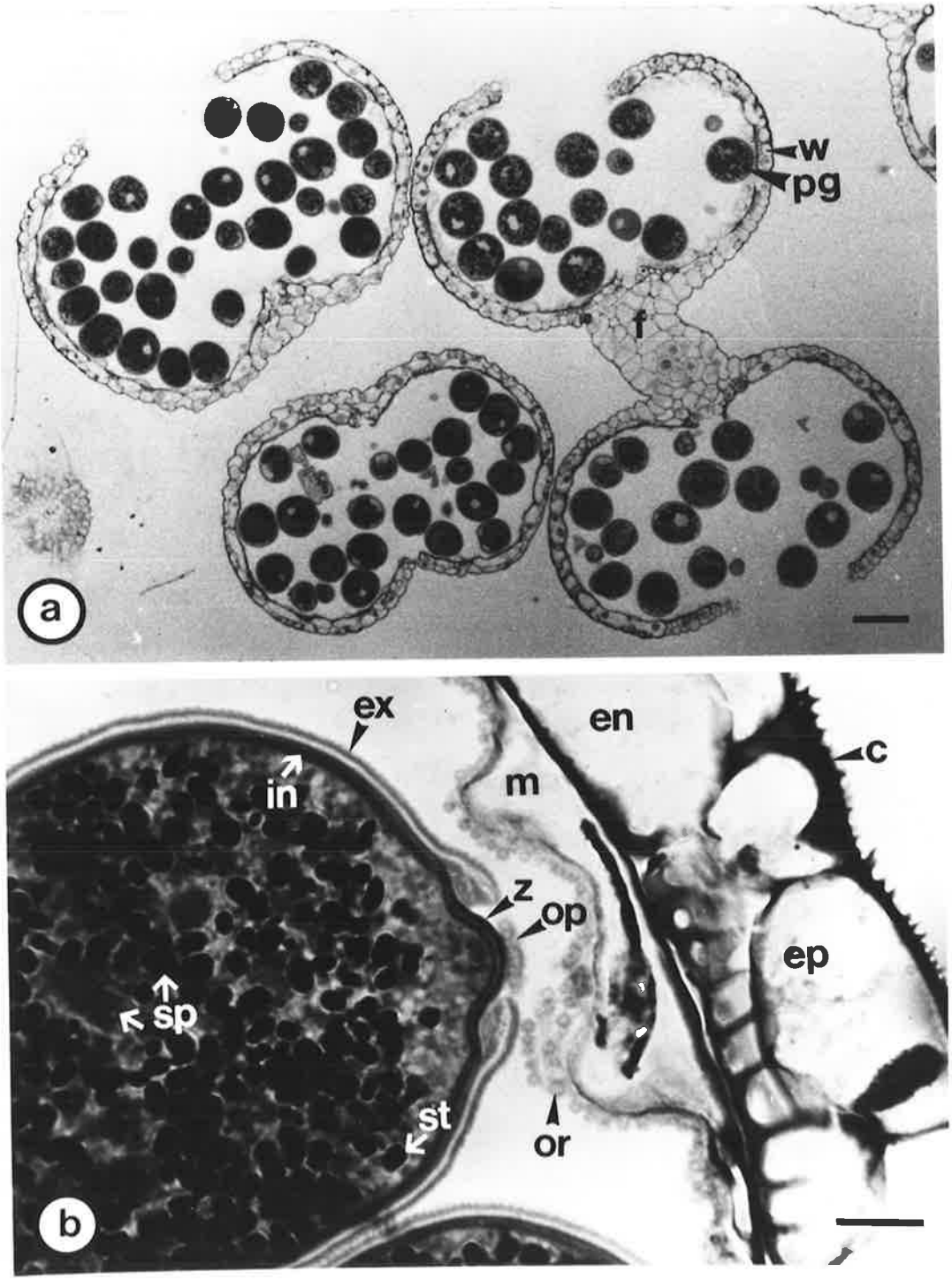


Fig. 3.4.5.

Fig. 3.4.6. Scanning electron microscopic view of the pore region of mature pollen.

op - operculum, pr - pore, ex - exine.

Bar represents 1µm.

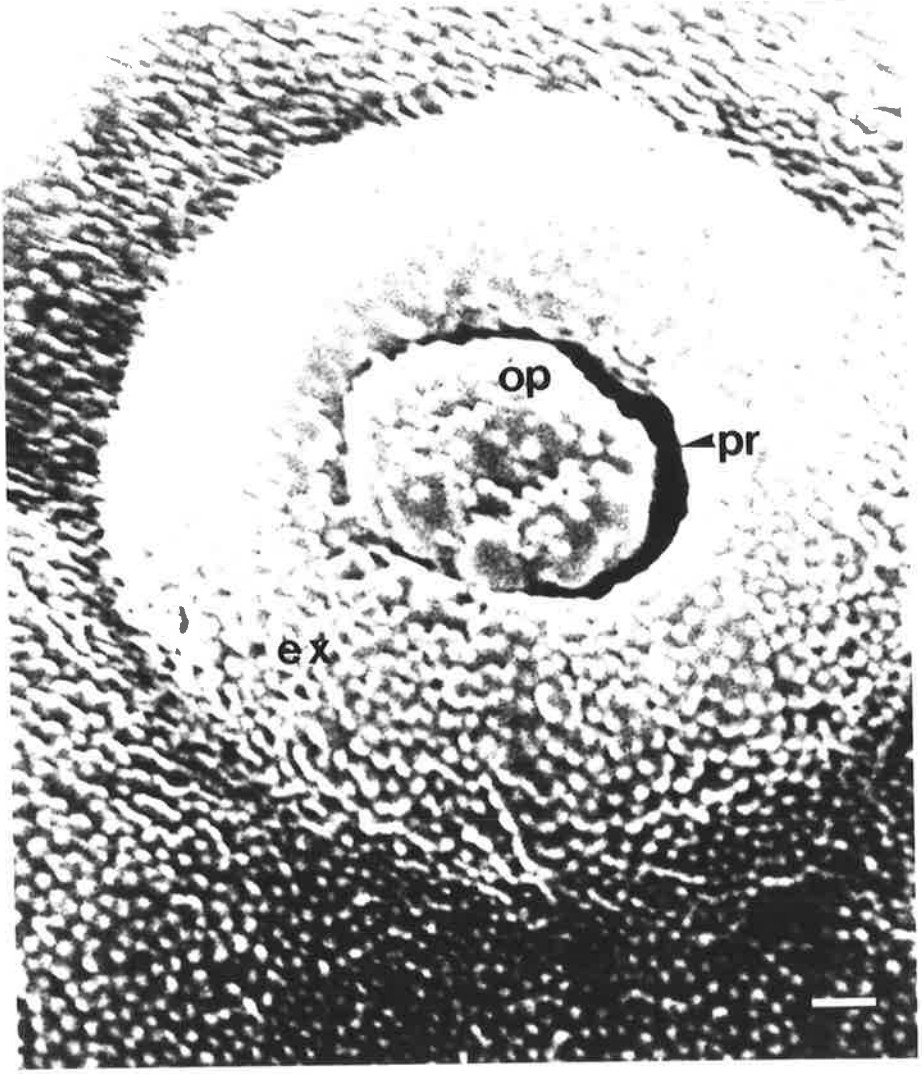


Fig. 3.4.6.

Table 3.4.2. Staining characteristics of different components of mature control (c) anthers and those containing abnormal pollen (Ab.)

Anther-Components	Stain Specificity	STAIN											
		PAS		TBO		Coomassie brilliant blue		Auramine O		Aniline blue		Autofluorescence	
		Carbohydrates (vicinal glycol groups)		Phenolics, acidic polysaccharides, acidic polyanionic groups		Proteins		Lipids		Callose		Phenolics or lignin like compounds	
		C	Ab	C	Ab	C	Ab	C	Ab	C	Ab	C	Ab
1) Anther wall:													
(a) Cuticle		+	+	-	-	+or-	+or-	+	+	-	-	-	-
(b) Cell walls		+	+	+	+	+	+	-	-	+or-	+or-	+(weak)	+(weak)
(c) Endothecial thickenings		+	+	+	+	-	-	+or-	+or-	+	+	+(weak)	+(weak)
(d) Cytoplasm		-	-	+	-or+	+	+	-	-	-	-	-	-
(e) Nuclei		-	-	+	+	+	+	-	-	-	-	-	-
2) Tapetal residue (orbicules)		-	-	+	+	+	+	+	+	-	-	+	+
3) Anther lumen		+	+	-	-	-	-	-	-	-	-	-	-
4) Pollen grain:													
(a) Exine		-	-	+	+	+	+	+	+	-	-	+	+
(b) Z-layer		+	+	-	-	+	+	-	-	-	-	-	-
(c) Intine		+	+or-	-	-	+	+or-	-	-	-	-	-	-
(d) Cytoplasm		+	+*	+	+*	+	+*	-	-	-	-	-	-
(e) Cytoplasmic granules (starch)		+	+*	-	-	-	-	-	-	-	-	-	-
(f) Vegetative nucleus		-	-	+	+*	+	+*	-	-	-	-	-	-
(g) Sperms.		-	-	+	-	+	-	-	-	-	-	-	-

* Present only in rare cases

Fig. 3.4.7. T.S. of mature anthers stained with (a) PAS/TBO combined, and (b) PAS, showing details of anther wall and pollen grain.

ex - exine, in - intine, z - z layer, or - orbicules, st - starch grain, sp - sperm, ep - epidermis, en - endothecium, op - operculum, m - middle layer, th - thickenings on endothecial cell walls, c - cuticle.

Bar represents 10μm.

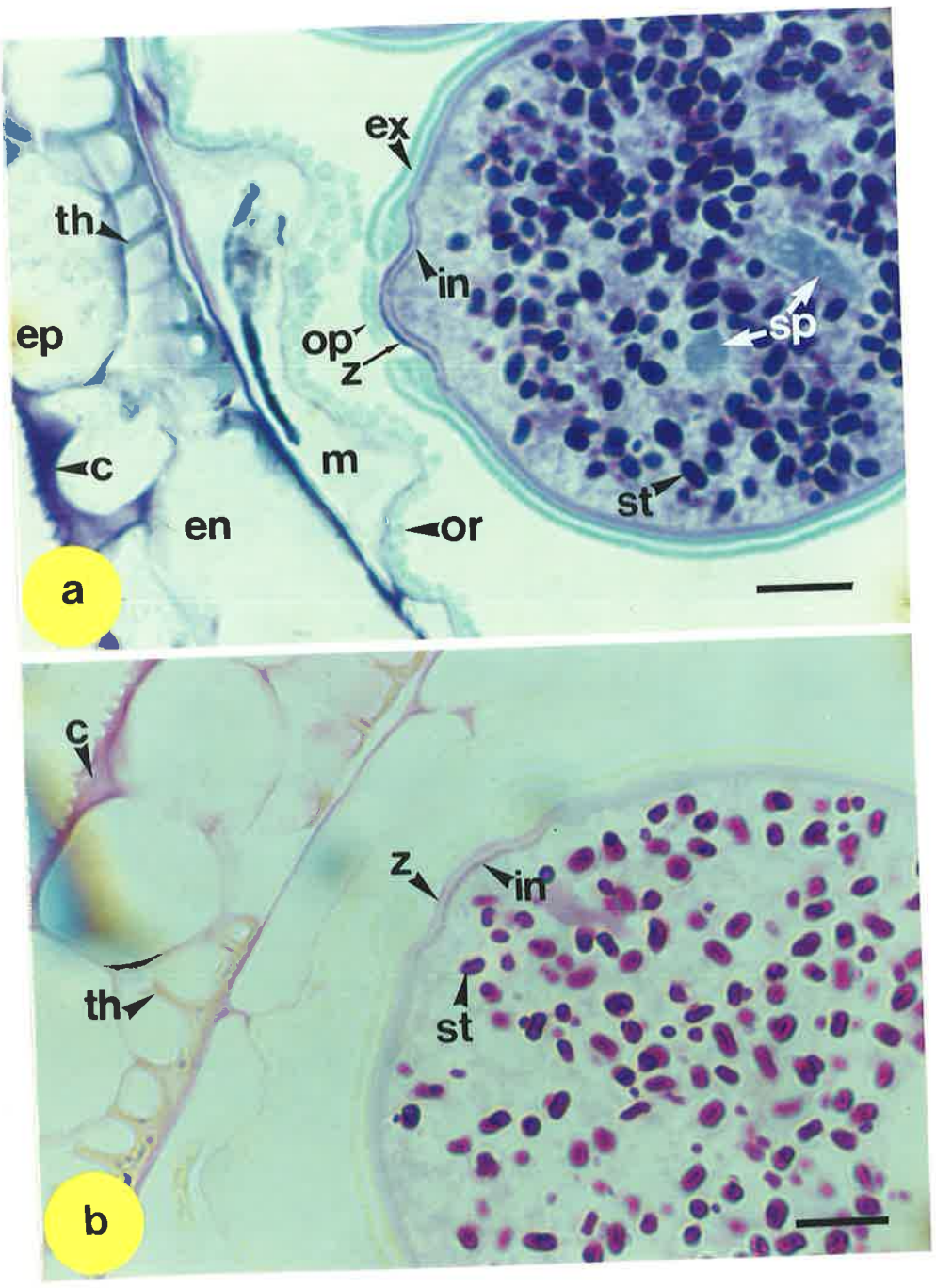


Fig. 3.4.7.

Fig. 3.4.8. T.S. of mature anthers stained with (a) TBO and (b) coomassie brilliant blue, showing details of anther wall and pollen grain.

th - thickenings on endothecial cell walls, or - orbicule, ex - exine, sp - sperm, in - intine.

Bar represents 10μm.

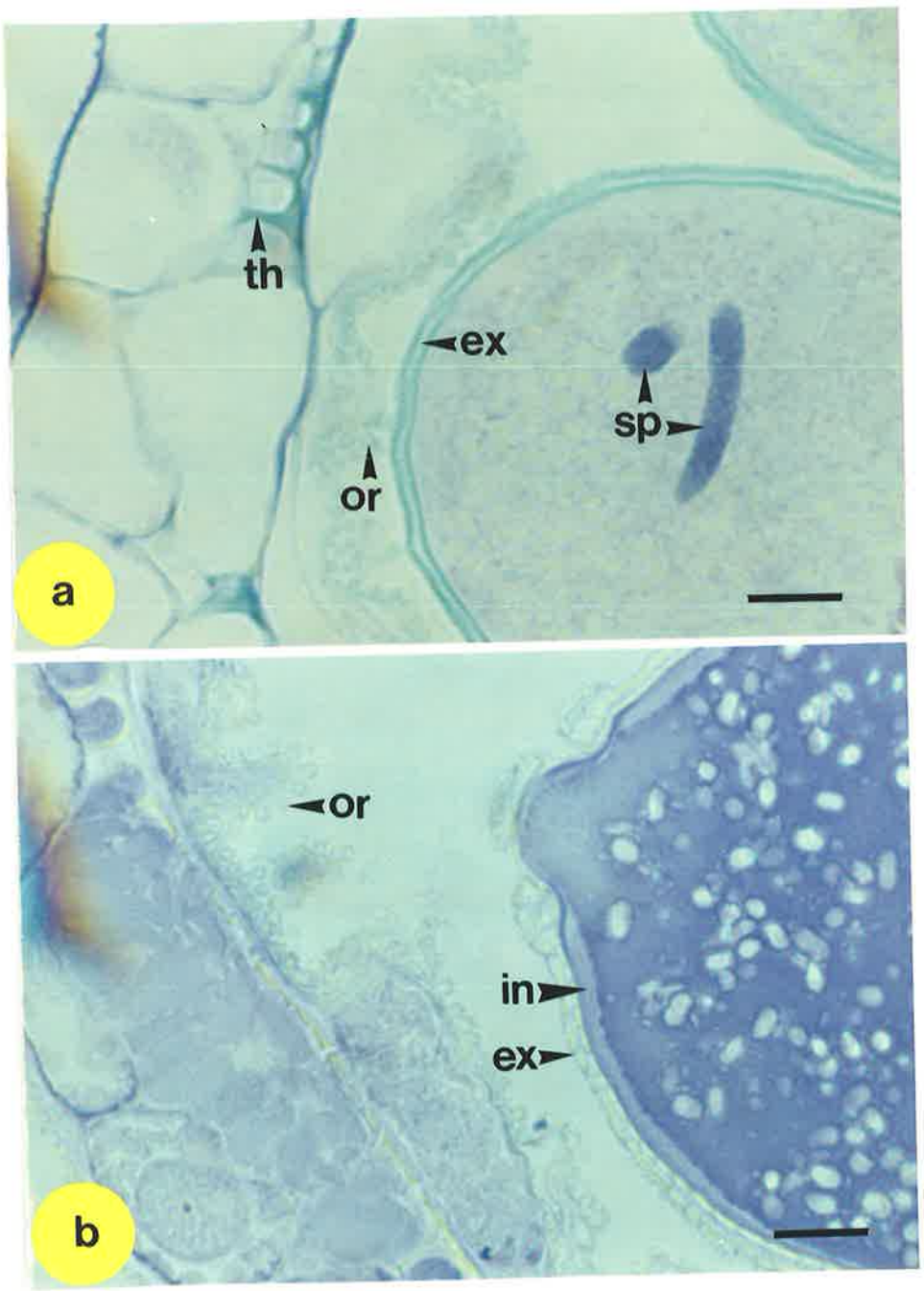


Fig. 3.4.8.

Fig. 3.4.9. T.S. of mature (a) autofluorescent anther and those stained with (b) auramine O and (c) aniline blue.

ex - exine, pg - pollen grain, th - thickenings on the endothecial cell walls, aw - anther wall.

Bar represents 50μm.

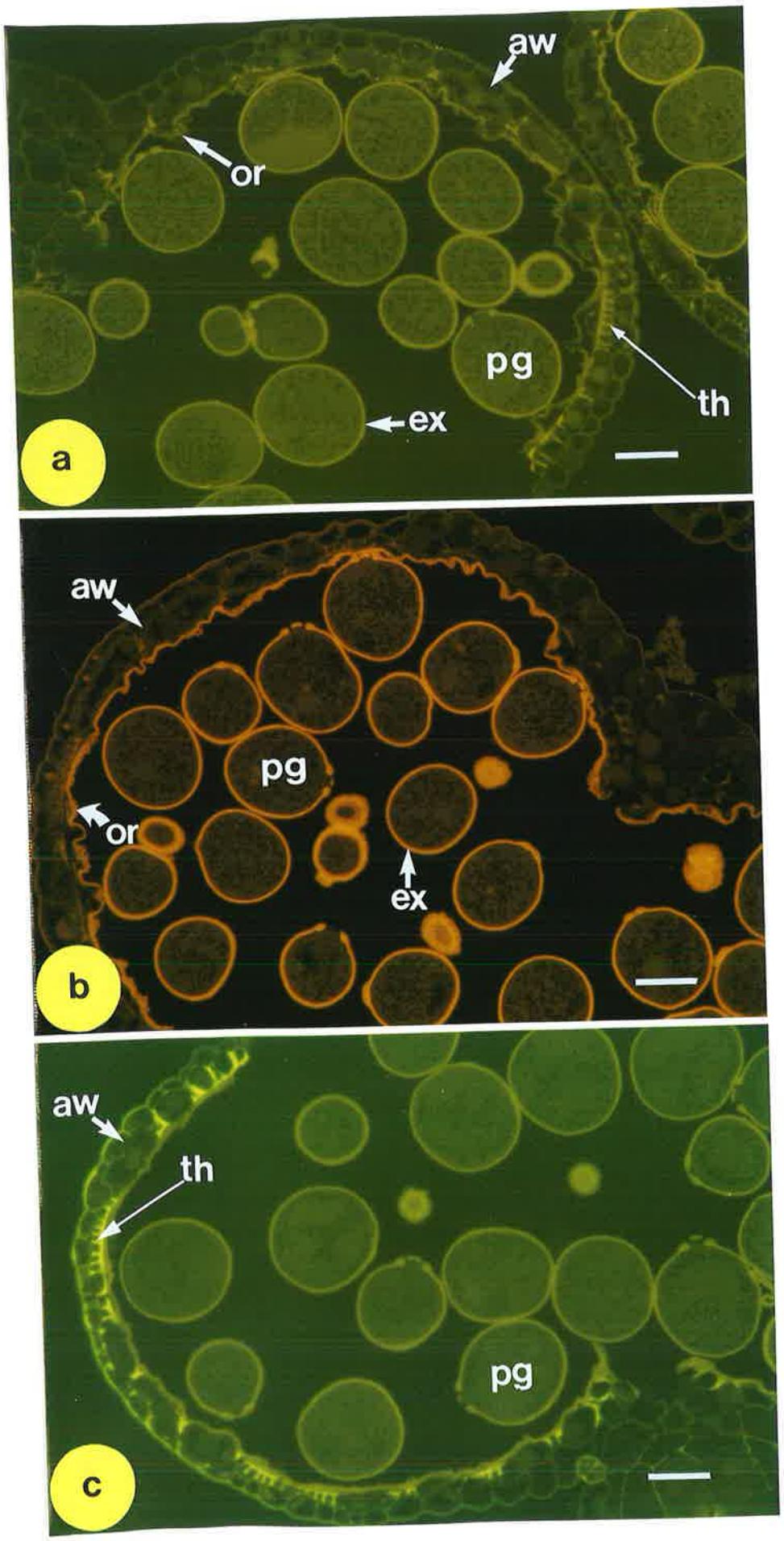


Fig. 3.4.9.

Table 3.4.3. A summary of abnormal anther- and pollen-development in response to water-stress and heat (30°C for 3 days) applied during the sensitive stage of development.

Type of Development	Proportion of abnormal thecae %	Abnormalities	Extent of sterility
Water Stress:			
Type-1	58.4	Abnormalities appeared near PGM-1. Filament and vascular bundle degenerated. Microspores lost contact with tapetum and no starch accumulation occurred.	Complete
Type-2	41.6	Abnormalities appeared near PGM-1. No degeneration of filament or vascular bundle occurred. Microspores lost contact with tapetum and no starch accumulation occurred.	Partial or Complete
Heat Stress:			
Type-1	51.8	Meiosis was abnormal. Tapetum degenerated prematurely.	Complete
Type-2	48.2	Abnormalities appeared after PGM-1. Tapetal degeneration and filament were normal. Microspores lost contact with tapetum and no starch accumulation occurred.	Partial or Complete

disoriented and lost contact with the tapetum at or close to the time of PGM-1 (Table 3.4.3; Fig. 3.4.10a). Microspore nuclei frequently stained unusually heavily. At the same time cells of the filament started to stain heavily and show signs of degeneration (Fig. 3.4.10a,b). The tapetum degenerated in the normal manner but the pollen grains remained shrivelled and accumulated no or very little starch. Both the vascular bundle and the filament eventually degenerated (Fig. 3.4.10c,d.). In some cases PGM-1 was completed but it remained uncertain whether or not PGM-2 occurred. The mature anthers of this type were smaller in diameter than normal anthers (Table 3.4.4). and contained shrivelled pollen grains (Fig. 3.4.10d). The exine of these pollen grains had developed normally but the intine was relatively thinner. There was very little cytoplasm or starch. All of the pollen grains within these anthers were sterile (see section 3.2.1.2. for evidence). The staining characteristics of various components of abnormal anthers are given in table 3.4.2.

Table 3.4.4. Mean external diameter of mature normal anthers and abnormal anthers induced by water-stress or heat (30°C for 3 days) during the sensitive stage of development.

		Water Stress		Heat Stress	
	Control	Type 1	Type 2	Type 1	Type 2
Diameter (µm)	333±6.1 ^{A}	247±13.5 ^{C}	289±11.0 ^{B}	226±7.7 ^{C}	310±13.2 ^{AB}

Values (means of 6 measurements) bearing different letters are significantly different (P = 0.01)

3.4.2.2. TYPE-2:

The remaining 41.6% of the abnormal thecae aborted in a fashion very similar to type-1, except that pollen abortion was not accompanied by filament degeneration (Table 3.4.3; Fig. 3.4.11a,b.). PGM-1 and PGM-2

Fig. 3.4.10. Anthers from wheat plants subjected to water-stress during the sensitive period of development, showing abnormal development of type-1.

(a) T.S. of an anther showing beginning of pollen- and filament degeneration.

(b) T.S. of an anther showing pollen abortion and filament degeneration and pollen abortion.

(c) T.S. of an anther showing advanced stages of filament degeneration and pollen abortion

(d) T.S. of a mature anther with degenerate filament and aborted pollen grains.

mn - microspore nucleus, t - tapetum, vb - vascular bundle, f - filament, apg - abnormal pollen grain, d - degenerated cells, st - starch, in - intine, ex - exine.

Bar represents 50µm.

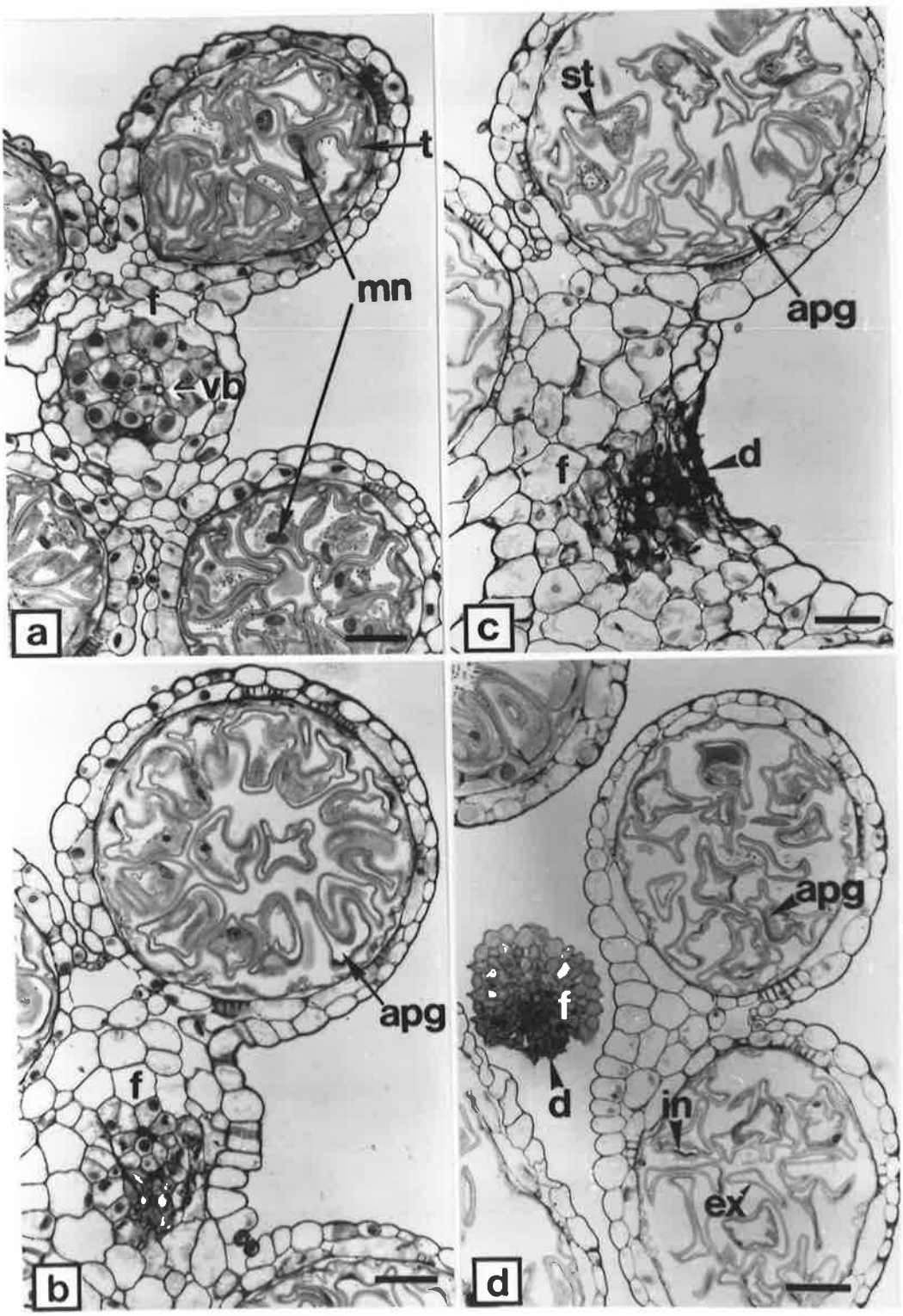


Fig. 3.4.10.

Fig. 3.4.11. Anthers from wheat plants subjected to water-stress during the sensitive period of development, showing abnormal development of type-2.

(a) T.S. of a mature anther showing normal filament and partial pollen sterility
(b) T.S. of a mature anther showing normal filament and complete pollen sterility.

apg - abnormal pollen grain, f - filament, in - intine, ex - exine, st - starch, mpg - normal pollen grain.

Bar represents 50μm.

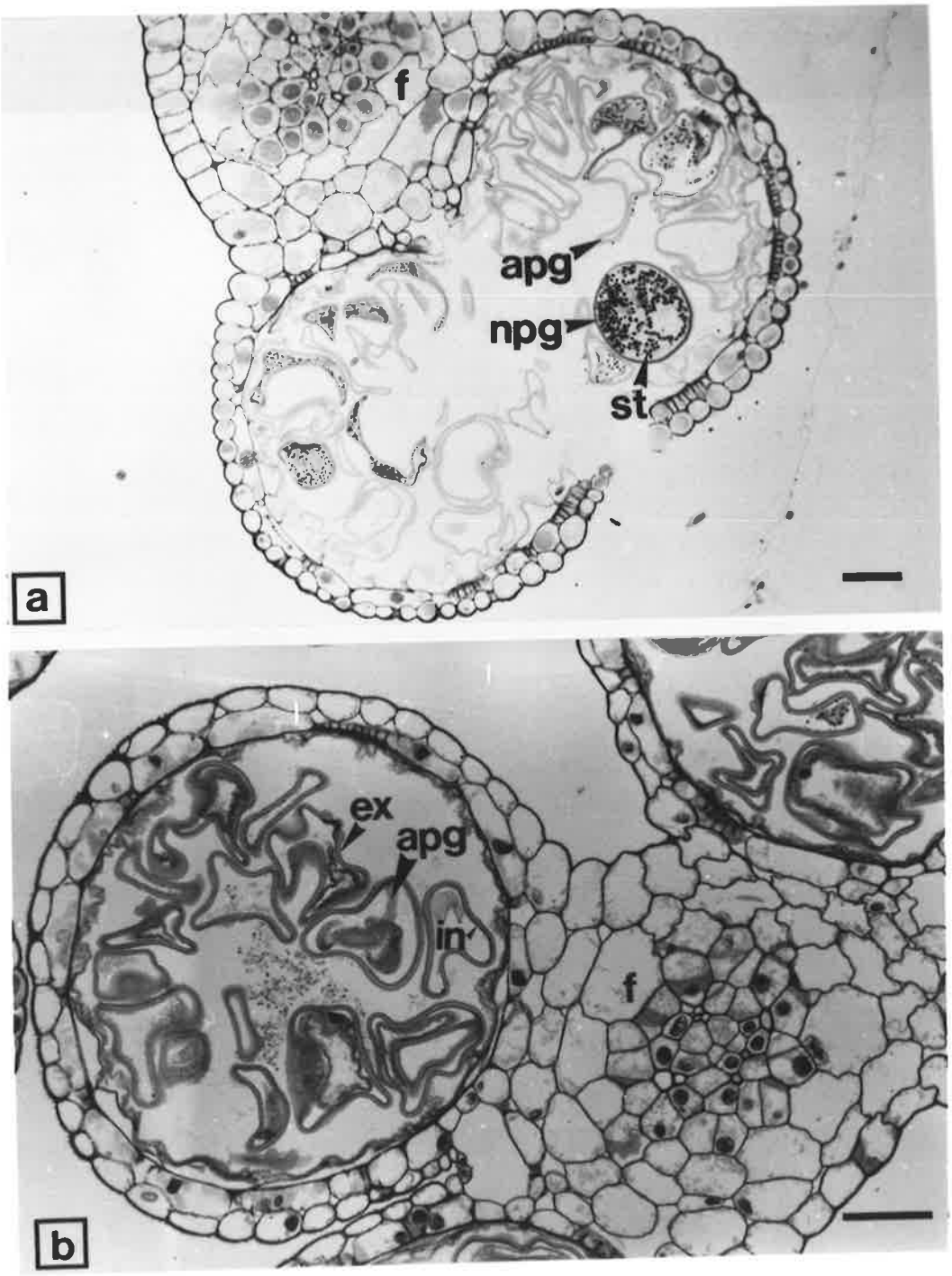


Fig. 3.4.11.

were completed at least in some pollen grains that also accumulated starch. Anthers failed to grow to normal size (Table 3.4.4.) and contained a mixture of normal and abnormal pollen grains, the latter being similar in appearance to those of type-1.

3.4.3. HEAT-STRESSED ABNORMAL ANTHERS:

Plants at the onset of meiosis in the most advanced florets of the spike were transferred to 30°C for 3 days. Anthers, harvested periodically from these plants, were subjected to microscopic examination in the same way as described in section 3.4.1.

3.4.3.1. TYPE-1:

This type of development was seen in 51.8% of the abnormal thecae (Table 3.4.3). Cytoplasm and chromosomes of meiotic PMCs stained heavily and appeared to be degenerating (Fig. 3.4.12a). Tapetal cells appeared to commence degeneration at meiosis and, migrated into the anther lumen to become interspersed with the microspores (Fig. 3.4.12a,b). All traces of tapetum were lost within about a day after the microspore release. Collapse of the outer layers of the anther wall was also frequently observed (Fig. 3.4.12c,d) although some anthers of this type had normal walls (Fig. 3.4.13a). Some pollen grains developed as far as the PGM-2 (Fig. 3.4.12c) but none accumulated starch. Anthers that aborted in this manner remained significantly smaller than the control (Table 3.4.4) and were completely sterile (see section 3.2.2.2 for evidence). Aborted pollen grains had a normal exine but no, or inconspicuous, intine and they lacked cytoplasm (Fig. 3.4.12d). Some thecae had no identifiable pollen grains but were full of orbicules (Fig. 3.4.13b). The staining characteristics of the various components of abnormal anthers were as shown in table 3.4.2.

3.4.3.2. TYPE-2:

Type-2 abnormality appeared late during the pollen development in 48.2% of abortive thecae (Table 3.4.3). Pollen development was normal up to PGM-1 following which some or all of the pollen grains within a theca stopped developing (Fig. 3.4.14a,b). Tapetal degeneration proceeded

Fig. 3.4.12. Anthers from wheat plants subjected to heat-stress (30°C for 3 days) during the sensitive stage of development, showing abnormal development of type-1.

(a) T.S. of an anther showing abnormal structure of tapetum and PMCs at meiosis.

(b) T.S. of an anther at the young microspore stage.

(c) T.S. of an anther showing degeneration of anther wall and some pollen grains at PGM-1

(d) T.S. of a mature anther with degenerated anther-wall and pollen grains.

pmc - pollen mother cell, - ms - microspore, t - tapetum, f - filament, mn - microspore nucleus, apg - abnormal pollen grain,
aw - anther wall.

Bar represents 50µm.

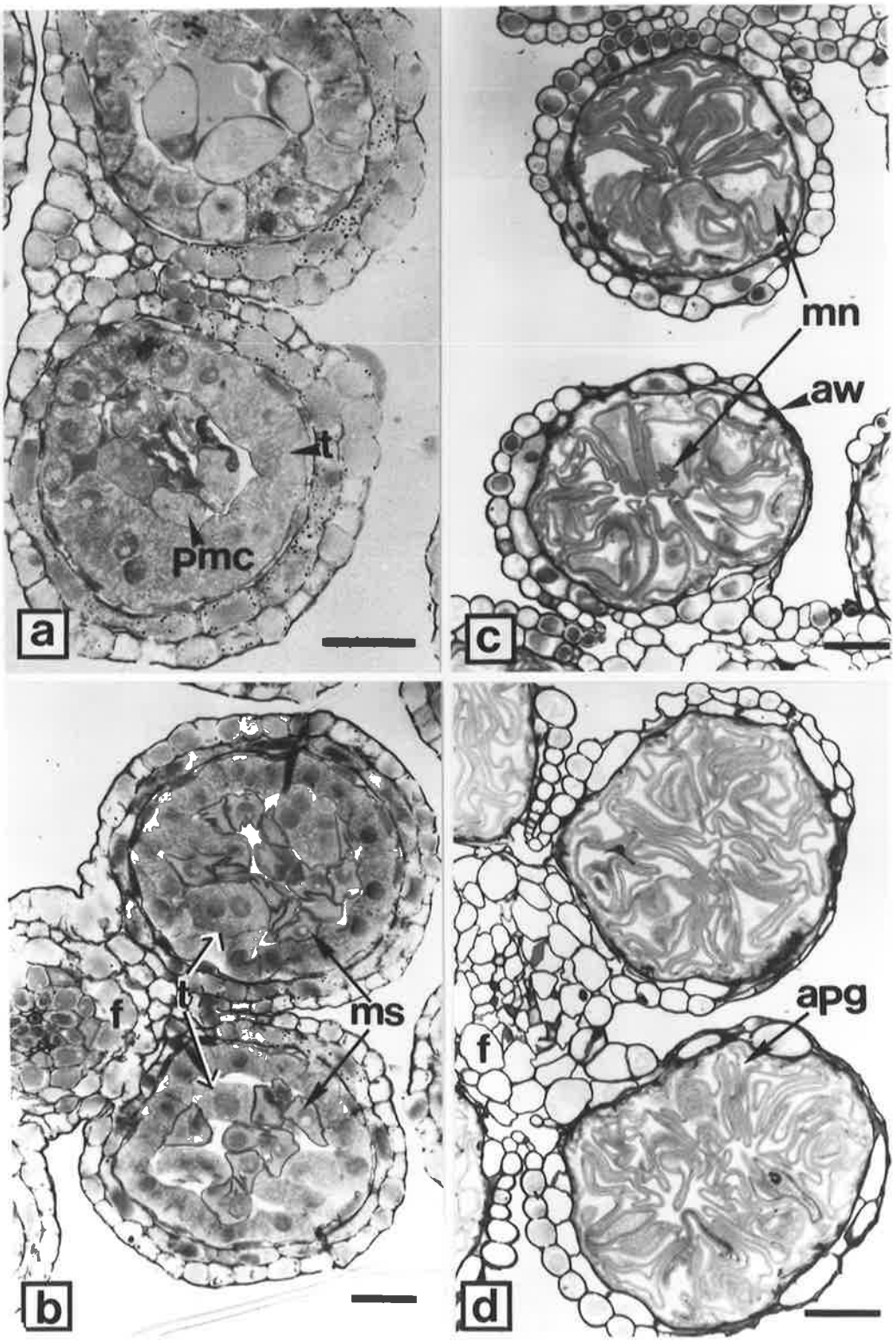


Fig. 3.4.12.

Fig. 3.4.13. Anthers from wheat plants subjected to heat-stress (30°C for 3 days) during the sensitive period of development, showing abnormal development of type-1.

(a) T.S. of a mature anther showing normal anther-wall and aborted pollen grains.

(b) T.S. of a mature anther with one theca lacking pollen grains but full of orbicules.

apg - abnormal pollen grain, f - filament, aw - anther wall, or - orbicule.

Bar represents 50μm.

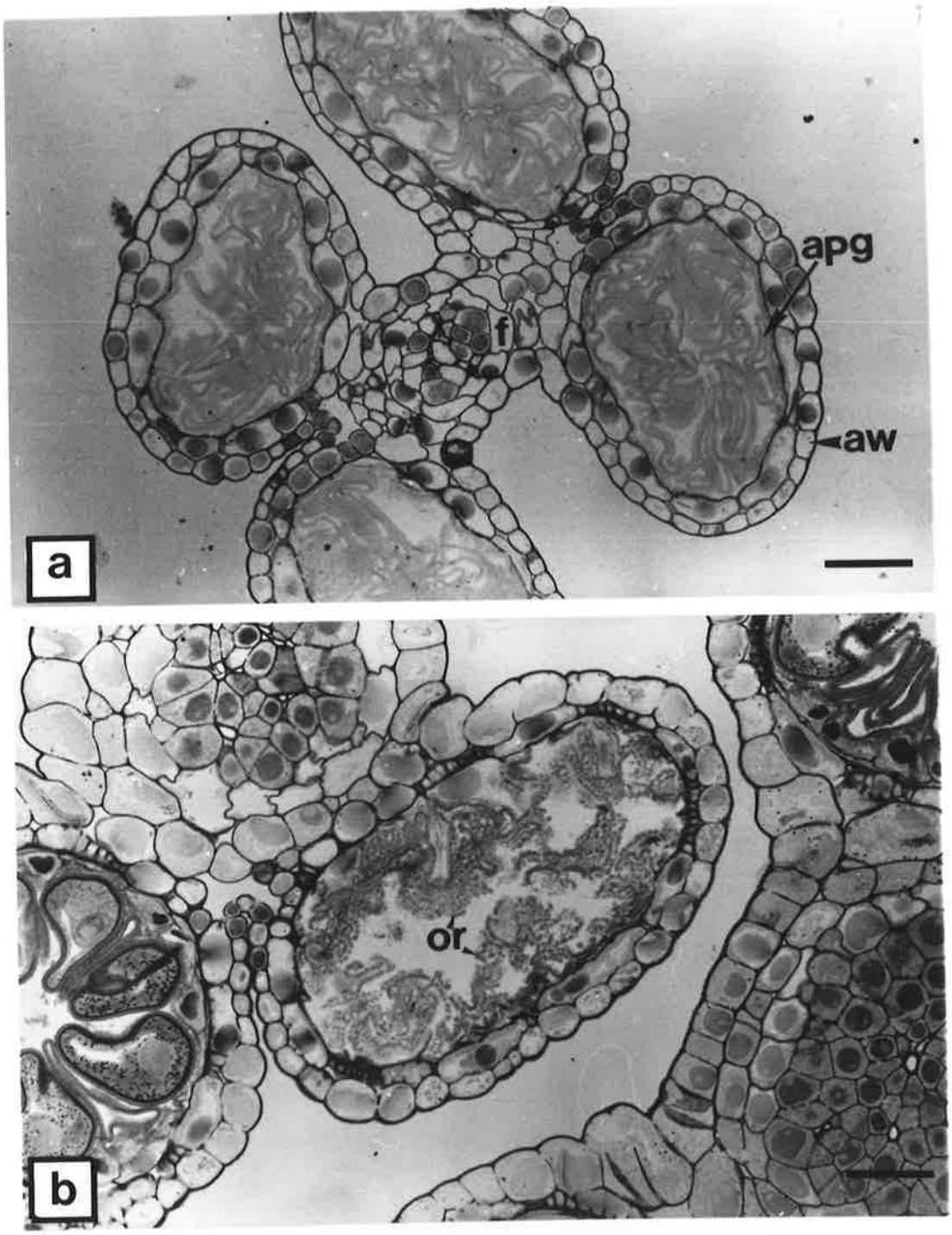


Fig. 3.4.13.

Fig. 3.4.14. Anthers from wheat plants subjected to heat-stress (30°C for 3 days) during the sensitive period of development showing abnormal development of type-2.

(a) T.S. of an anther showing beginning of pollen abortion after PGM-2.

(b) T.S. of an anther showing disoriented pollen grains

(c) T.S. of an anther showing one pollen grain at the anaphase of PGM-2 and some in the process of abortion within the same theca.

(d) T.S. of an anther showing trinucleate and aborted pollen grains within the same theca.

npg - normal pollen grain, apg - abnormal pollen grain, f - filament, gn - generative nucleus, vn - vegetative nucleus, t - tapetum,
sp - sperm, st - starch.

Bar represents 50µm.

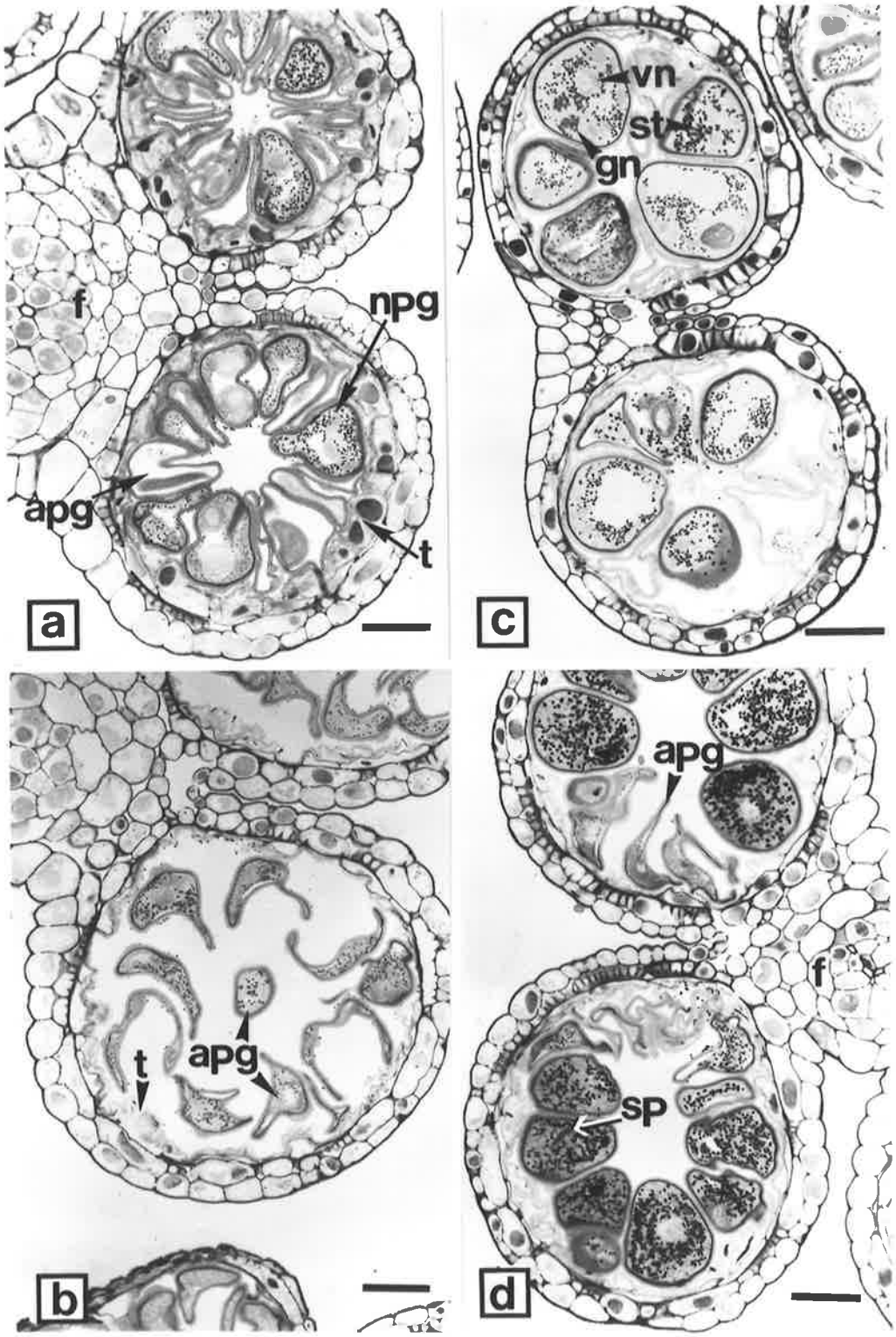


Fig. 3.4.14.

Fig. 3.4.15. Anthers from wheat plants subjected to heat-stress (30°C for 3 days) during the sensitive period of development showing abnormal development of type-2.
(a) T.S. of a mature anther showing partial sterility.
(b) T.S. of a mature anther with partial sterility showing details of the pollen grains.

pr - pore, st - starch, ex - exine, in - intine, apg - abnormal pollen grain, npg - normal pollen grain, f - filament.

Bar represents 50μm.

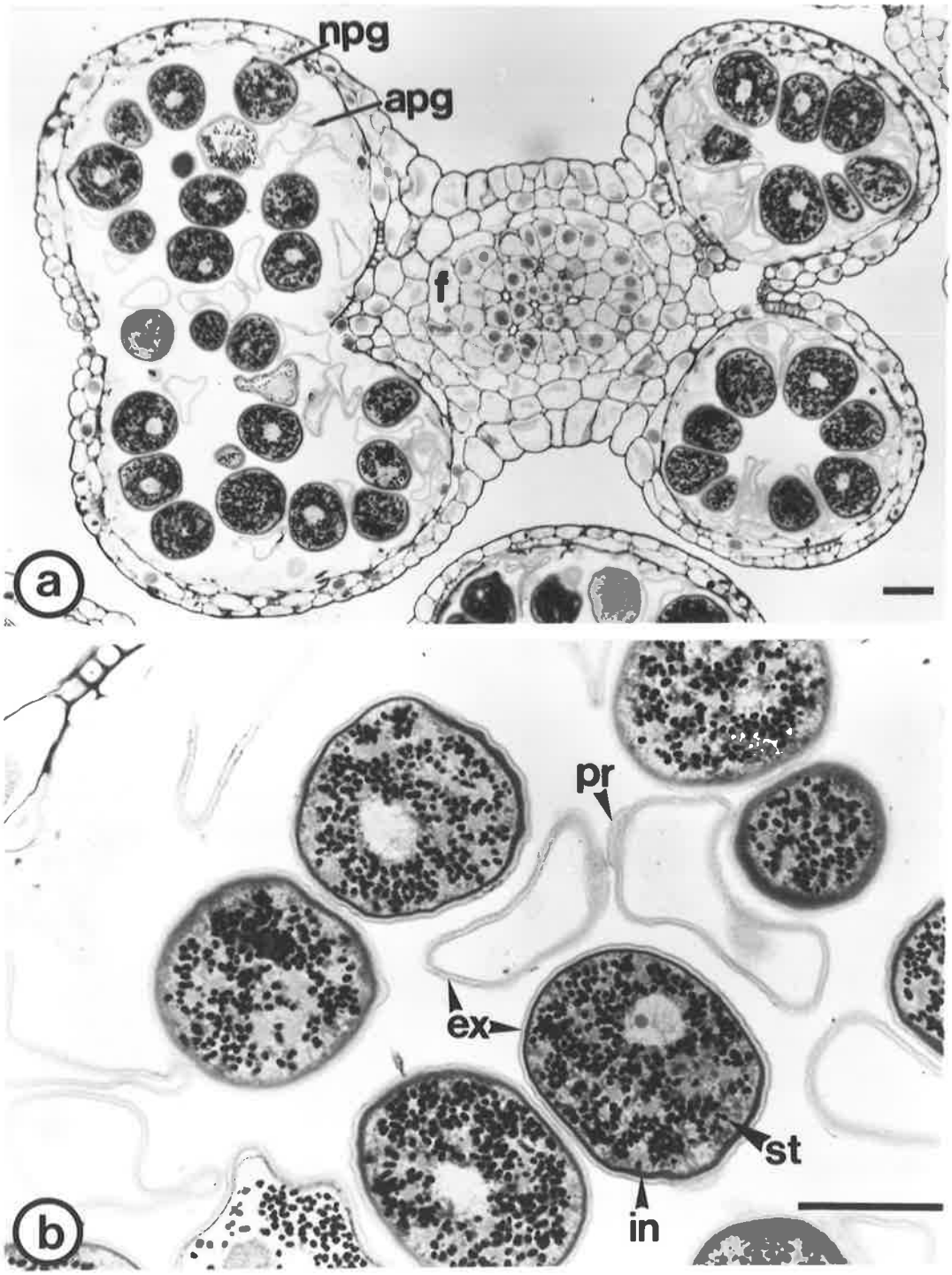


Fig. 3.4.15.

normally. Pollen grains that were apparently normal underwent PGM-2 to produce sperms (Fig. 3.4.14c,d) while others within the same theca did not. Mature anthers of this type grew to the normal size (Table 3.4.4) and had total or partial pollen sterility (Fig. 3.4.15a; see section 3.2.2.2 for evidence). The normal pollen grains were trinucleate, contained starch and had normal exine and intine. The sterile pollen grains lacked starch, were irregularly shaped, and had no or very faint intine. Exine development in sterile pollen grains was apparently normal (Fig. 3.4.15b).

3.4.4. DISCUSSION:

Development of normal anthers and pollen grains was in general agreement with the earlier descriptions of Batygina (1962), Goss (1968) and Bennett *et al.*, (1973b). Bennett *et al.*, (1973b) reported that micropsores become spherical soon after their release from the tetrads. In the present investigation, however, the developing microspores remained irregular in shape until about the PGM-2. The earlier observations were based cn microspores extruded from the anthers by squashing, which may have caused them to round off following the removal of spatial constraint imposed by the limited volume of the anther lumen. Observation of sectioned anthers in this study showed that the microspores had very little space in which to expand until anther enlargement and tapetal degeneration during later part of pollen development.

The tapetum of wheat is of the secretory type (Echlin, 1971; Bennett *et al.*, 1973b). During pollen development the pore was always observed in contact with the tapetum which suggests that the former may serve as a gateway for the importation of materials from the tapetum into the pollen grain. Such a role of the pore was suggested by Batygina(1962) and the tapetum is generally considered to have a nutritive function (Echlin, 1971). Just before the beginning of starch accumulation in the pollen grains, starch grains disappeared from the epidermis and endothecium and granular carbohydrate accumulated in the anther lumen. It is possible that starch accumulation in the pollen grains results from remobilization of reserves from other parts of the anther.

Exine development started at the same time as the orbicules appeared

on the inner walls of the tapetal cells. The orbicules and the exine had similar staining characterestics which may indicate a possible role of orbicules in exine deposition. The issue has been debated for several years but the evidence for such a role of the orbicules is still obscure (Heslop-Harrison and Dickinson, 1969; Echlin, 1971).

A majority of the anthers that aborted in response to water stress exhibited degeneration of the vascular system. This is similar to the pattern of degeneration of anthers in male-sterile lines of wheat (Joppa *et al.*, 1966). Degeneration of the vascular system is likely to curtail the assimilate supply to the developing pollen grains. Abortion of pollen grains usually followed their dislocation from the tapetum. A significant number of the abortive anthers had apparently normal vascular bundles. It is possible that the disruption of vascular supply in such anthers may be too subtle to be detected by present microscopy. It is also possible that the degeneration of the vascular system may be a result rather than the cause of anther abortion. This possibility is further supported by the fact that exine development was completed normally in the abortive pollen grains.

Filament degeneration in water-stressed anthers is unlikely to be the result of desiccation since spike Ψ did not change (section 3.1.1.2). It is, however, conceivable that although the overall Ψ of the spike remained unchanged by water deficit, the Ψ of various floral organs (e.g. filament) may have dropped.

Skazkin and Zavadskaya (1957) observed abnormalities in chromosome pairing during meiosis in water-stressed plants. It is possible that a similar lesion may have occurred in the present instance, leading to the pollen sterility.

Abnormalities in heat-stressed anthers followed a largely different course from those affected by water stress. In approximately half of the heat-affected anthers, abnormalities appeared to be associated with meiosis and tapetal behaviour. Although the PMCs divided to form microspores, the state of the PMC-nuclei at meiosis suggested that some abnormalities may have accompanied the division. Such abnormalities, though not serious enough to prevent nuclear division, could interfere

with the mechanism controlling pollen development. Rye plants grown at 25°C during reproductive development showed obvious meiotic abnormalities, such as failure of daughter cells to separate after the first division leading to pollen sterility (Bennett *et al.*, 1972). Any abnormalities in the reduction division, if they occurred at all in wheat, were not as conspicuous.

Although about half of heat- and water-stressed anthers (Type-2 in both cases) followed a very similar course of degeneration, the remainder (Type-1) aborted in very different ways. This, together with the differences in the effects on female fertility (section 3.2. and 3.3), suggests that the effects of water- and heat-stress on sporogenesis may differ qualitatively.

There were two modes of degeneration of anthers in response to both water-stress and heat and in each case one of the modes produced more severe effects than the other. This may be due to a proportion of the florets being stressed at the stage of maximum sensitivity while the remainder were at less sensitive stages, bordering the critical stage.

3.5. POSSIBLE ROLE OF ABSCISIC ACID IN THE INDUCTION OF STERILITY BY STRESS

Effects of water stress on grain set and sporogenesis in wheat, discussed in the preceding sections, were not associated with any decline in spike Ψ (section 3.1.1.2.) despite a substantial decrease in leaf Ψ. This suggests that the induction of male sterility in response to water stress is not due to desiccation of the sporogenous tissue but to some indirect consequences of lowered water potential elsewhere in the plant, possibly in the leaf. One prominent response to the drop in leaf turgor consequent upon water deficit is the rapid accumulation of the hormone, abscisic acid (ABA), which is thought to be synthesized mainly in leaf tissue and translocated to other organs (Aspinall, 1980; Walton, 1980). Application of exogenous ABA can reduce grain set and pollen viability in wheat (Morgan, 1980). It is, therefore, conceivable that the effects of water stress on male fertility may be mediated through this hormone. This, together with the possibility of ABA-involvement in the heat-induced abnormalities in sporogenesis, is examined here.

3.5.1. ENDOGENOUS ABA CONCENTRATION AND GRAIN SET IN RESPONSE TO WATER STRESS AND ABA APPLICATION:

In the first experiment, groups of plants were subjected to water stress or supplied with ABA solution at concentrations of 2, 20 or 100mgl^{-1} during the stage of development sensitive to water deficit (at the onset of meiosis in PMCs). Leaf Ψ fell to a minimum of -23.5±1.3 bar in the water stress treatment and caused a significant reduction in the number of grains per spike (Table 3.5.1). This episode of water deficit that caused a 50% reduction in per cent grain set also led to a four-fold increase in spikelet ABA concentration (Fig. 3.5.1). Supplying ABA through a wick increased the hormone concentration recovered from the spikelets at all three concentrations applied (Fig. 3.5.1). The lowest concentration of applied ABA (2mg l^{-1}) had no effect on grain set despite the fact that the ABA concentration measured in spikelets was twice as high as that in water-stressed plants. Grain set was drastically reduced by applying ABA at a concentration of 20mg l^{-1} but the

Table 3.5.1. Effect of water stress and abscisic acid application during the sensitive stage of development

	Water Stress	Control	ABA concentration applied (mg l^{-}) 2	20	100
Leaf Ψ (bar)	23.5^{B}	8.6^{A}	-	-	-
Grains/spike	16.8^{B}	30.6^{A}	30.2^{A}	4.8^{C}	0^{C}
Unemerged Spikes (out of 5)	0	0	0	2	4

Within a row, values bearing different letters are significantly different (P = 0.05). Data in the bottom row were not subjected to statistical analysis.

Table 3.5.2. Effect of water stress and abscisic acid application during the sensitive stage of development

	Water Stress	Control	ABA concentration applied (mg l^{-}) 0.5	1	2	4	8
Leaf Ψ (bar)	22.6^{B}	8.8^{A}	-	-	-	-	-
Grains/spike	16.3^{BC}	25.1^{A}	25.0^{A}	22.2^{AB}	20.0^{ABC}	19.0^{ABC}	14.7^{C}
Unemerged Spikes (out of 9)	0	0	0	0	0	0	1

Within a row, values not marked with the same letters are significantly different (P = 0.05). Data in the bottom row were not subjected to statistical analysis.

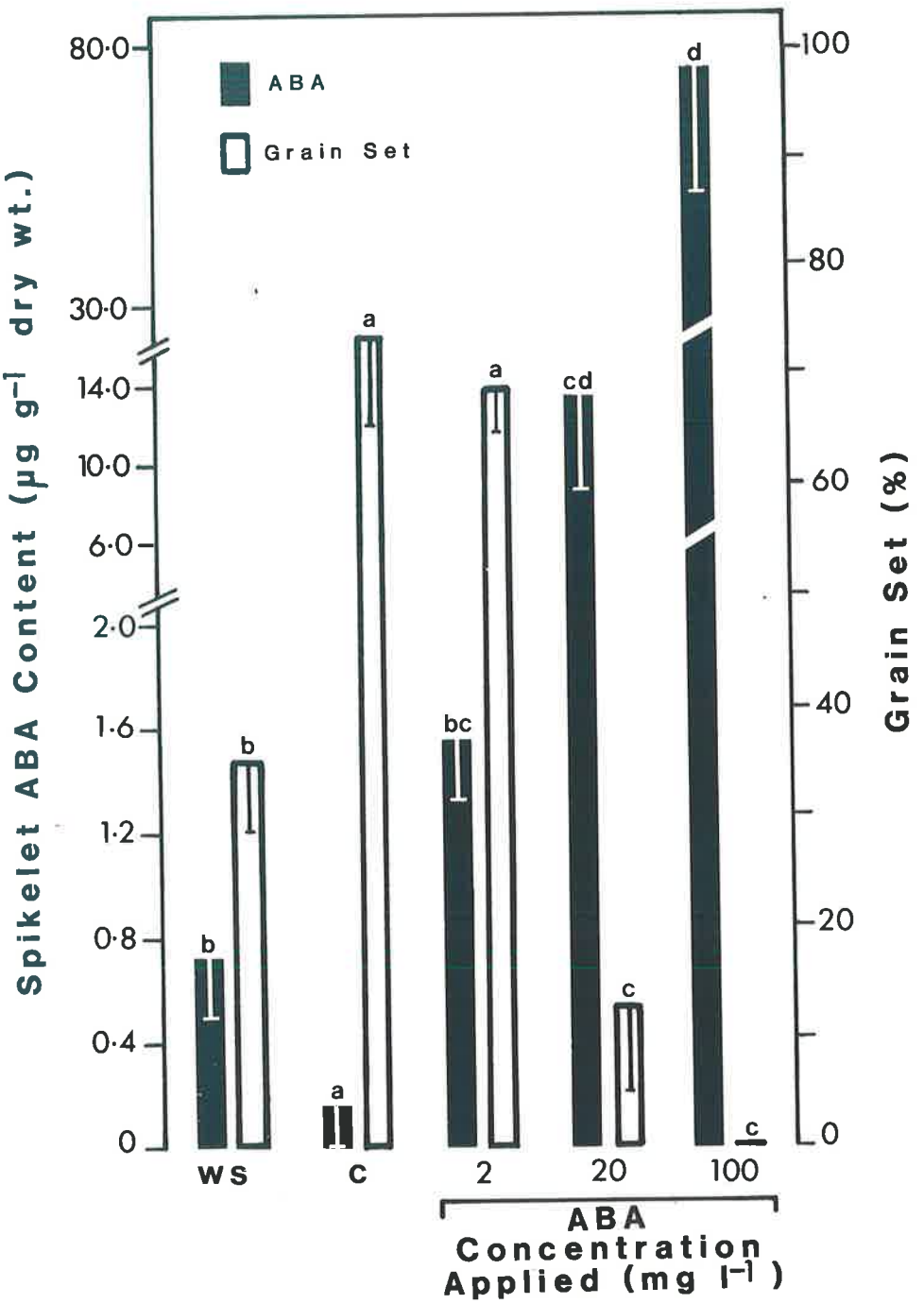


Fig. 3.5.1. Effect of water stress and abscisic acid application during the sensitive stage of development, on grain set and the concentration of abscisic acid in the spikelets. Bars (means of 3-5 replicates, S.E.s indicated) not marked with the same letter are significantly different (P = 0.05). WS - Water Stress, C - Control.

accompanying increase in the concentration in the spikelets was approximately nineteen times greater than that resulting from water stress. The application of a 100mg l^{-1} solution brought about a complete loss of grain set associated with an extremely high concentration of ABA in the spikelets. When the two higher concentrations were applied, a proportion of the treated plants showed a complete inhibition of stem elongation and an eventual death of spikes. This response occurred in a larger proportion of plants treated with the higher concentration than with the lower and was not seen in either water-stressed plants or those supplied with 2mg l^{-1} ABA (Table 3.5.1).

The results of this experiment suggested that the application of an ABA solution of a concentration between 2 and 20 mg l^{-1} might be expected to produce an inhibition of grain set similar to that caused by water stress. Accordingly, the concentrations of ABA applied in the second experiment were selected within and below that range. As in the previous experiment, water stress (to a water potential of 22.6±1.4 bar; Table 3.5.2) resulted in a significant increase in the concentration of ABA within the spikelets and lowered the percentage grain set (Fig 3.5.2) and the number of grains per spike (Table 3.5.2). A series of ABA solutions with concentrations between 0.5 and 8mgl^{-1} were applied to non-stressed plants. The number of grains per spike (Table 3.5.2) and percent grain set (Fig 3.5.2) appeared to decrease with each increase in applied ABA concentration, but only the highest concentration (8mg l^{-1}) caused a statistically significant reduction in grain set. The reduction in grain set at this concentration was comparable with that caused by water stress. The ABA concentration in spikelets increased with each increase in the concentration of applied ABA and was four and a half times higher when an 8mg l^{-1} ABA solution was supplied compared with plants subjected to water stress. Furthermore, the spike of one out of the nine plants to which this highest concentration of ABA was applied ceased elongation and eventually died (Table 3.5.2). It must be pointed out, however, that at an external supply of 2mg l^{-1} of ABA in this experiment, neither the spikelet ABA content nor grain set were significantly different from those in water-stressed plants, although grain set here was intermediate between that of control and of stressed plants.

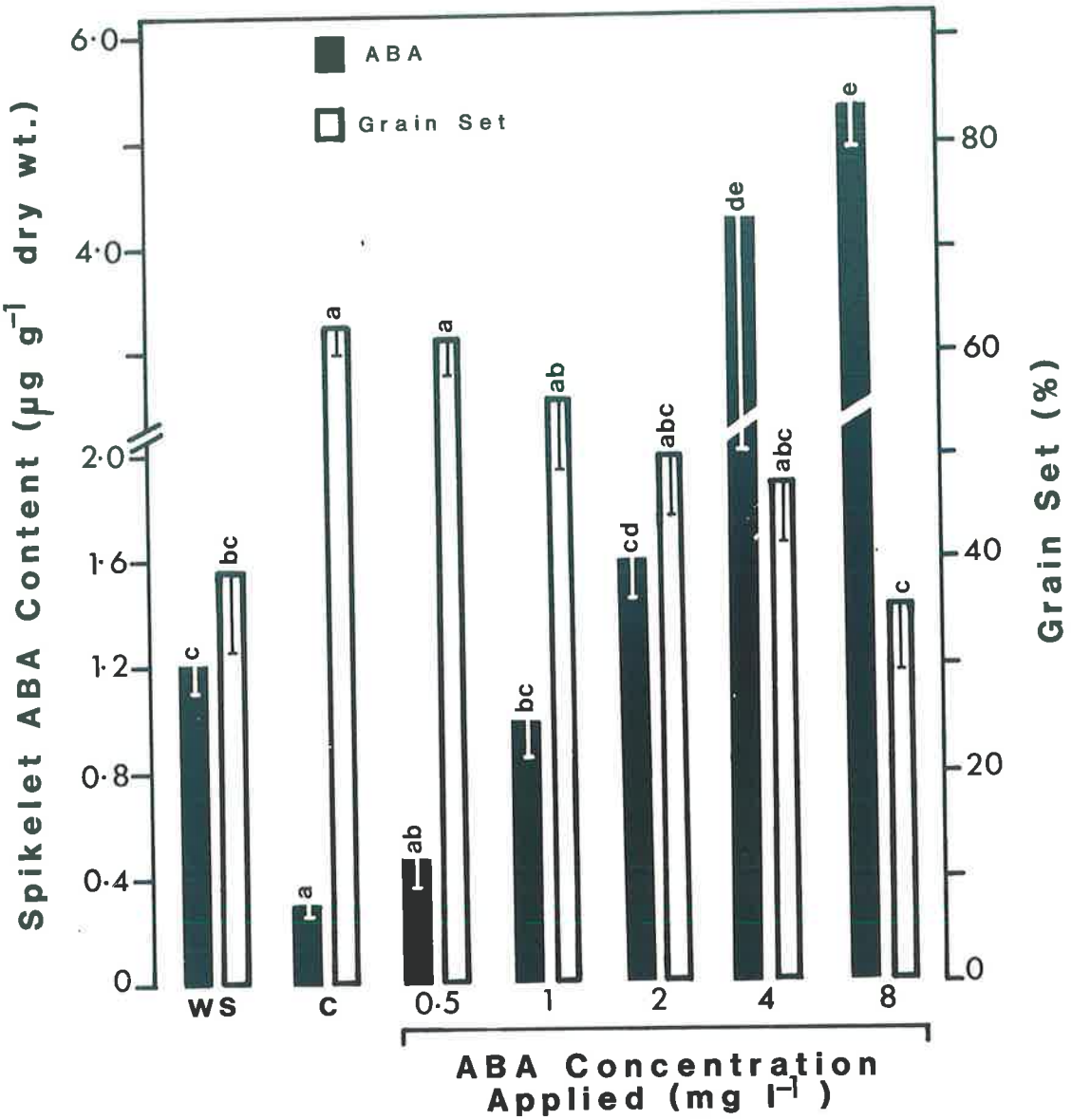


Fig. 3.5.2. Effect of water stress and abscisic acid application during the sensitive stage of development, on grain set and the concentration of abscisic acid in the spikelets. Bars (means of 3 replicates, each having 3 plants, S.E.s indicated) not marked with the same letter are significantly different (P = 0.05). WS - Water Stress, C - Control.

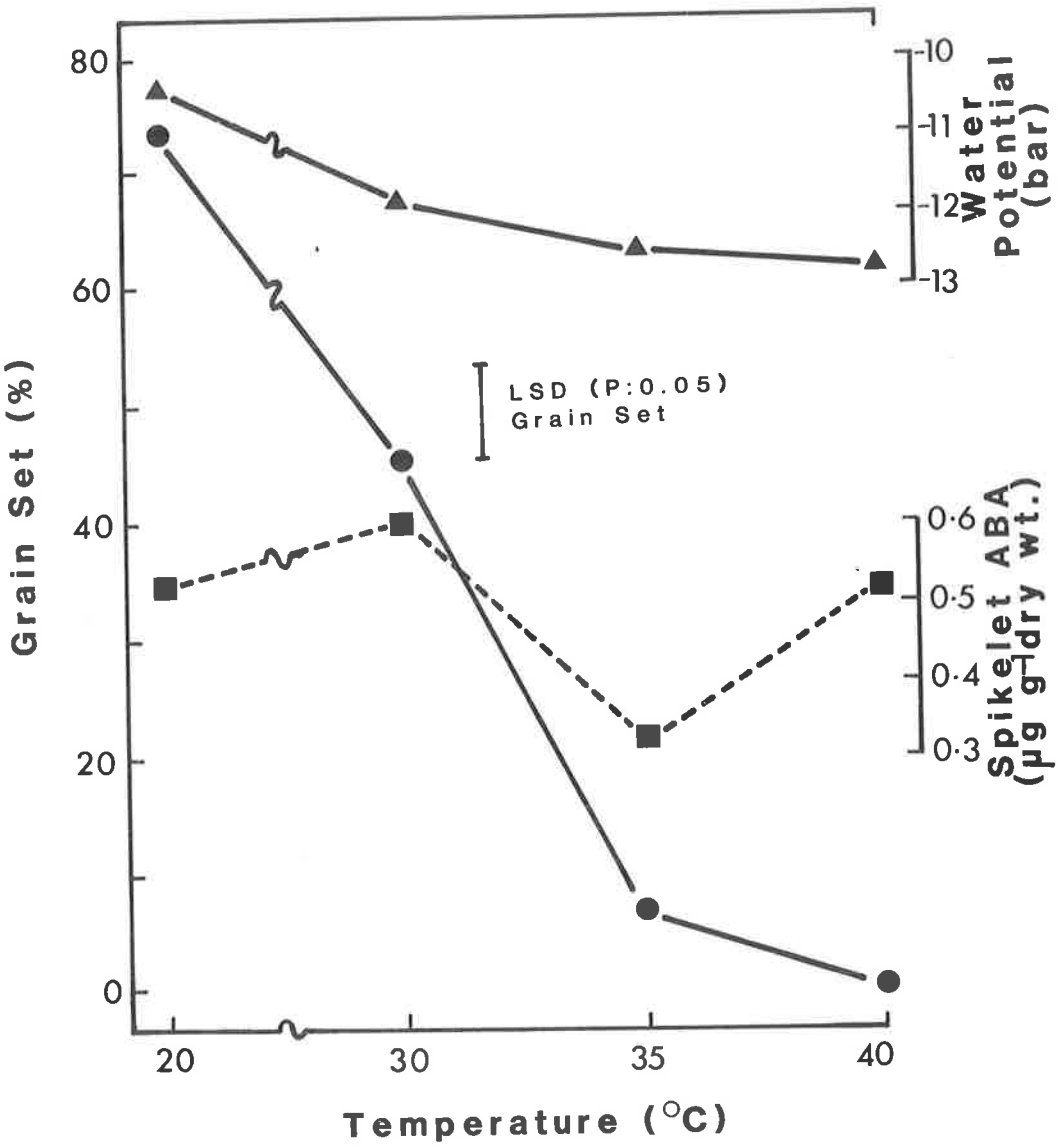


Fig. 3.5.3. Effect of exposure to various temperatures for 24 h during the sensitive stage of development on leaf water potential (▲), grain set (●) and abscisic acid concentration in the spikelets (■). All means derived from 3 replicates, each having 3 plants. Abscisic acid and water potential values did not vary significantly with temperature.

Table 3.5.3. Effect of a temperature of 30°C imposed for 72h during the sensitive stage of development on abscisic acid concentration in the flag leaf and the spikelets

	Abscisic acid (ng g^{-1} dry wt.)			
	24h		72h	
	20°C	30°C	20°C	30°C
Flag Leaf	209 ± 39	309 ± 77	155 ± 35	88 ± 5
Spikelets	436 ± 140	452 ± 60	275 ± 46	419 ± 80

Treatment means (each derived from 4 replicates) were not significantly different at any time (P = 0.05)

Fig. 3.5.4. Effect of abscisic acid (8mg l^{-1}) application at various stages of development

		Time of application			
		3 days before meiosis	onset of meiosis	During pollen Development	Onset of anthesis
Grain per Spike	Control	26.8±0.5	29.0±0.7	30.2±1.8	33.7±1.7
	ABA-treated	17.3±2.9*	18.2±1.3*	29.7±1.5	31.0±0.6
Grain set (%)	Control	67.9±2.1	68.9±	65.8±3.4	73.5±4.5
	ABA-treated	40.7±6.9*	44.6±2.7*	67.8±2.5	69.9±2.1

* Significantly lower than control (P = 0.05).
Each treatment mean is based on 6 replicates.

Application of the hormone three days before or at the onset of meiosis reduced both the number of grains per spike and percentage grain set, but application at either of the later stages of development was without effect (Table 3.5.4). This suggests that the stages of development at or near meiosis were most susceptible to inhibition by applied ABA.

3.5.4. EFFECT OF ABA APPLICATION ON FEMALE FERTILITY:

The level of female fertility in response to ABA (8mg l^{-1}) applied to well-watered plants at meiosis was determined by cross-pollinating treated plants with fertile pollen from untreated, well-watered plants. Two of the four sets of plants were perfused with ABA solution while the remaining two were supplied with a similar volume of distilled water. All except the primary and secondary florets of eight spikelets in the centre of the spike were removed two days before anthesis. The spikes from one set from each treatment were covered with glassine bags and allowed to self-pollinate, whereas those from the remaining two sets were emasculated and covered. These emasculated florets were hand-pollinated two days later with pollen from a separate pool of well-watered plants and again covered.

Results presented in table 3.5.5. show that grain set among the spikes that were allowed to self pollinate following treatment with ABA (IV) was significantly lower than that among the self-pollinated, untreated plants (III). When the two comparable sets of emasculated plants were cross-pollinated with fertile pollen, however, grain set in ABA-treated female parents (II) was similar to that in untreated female parents (I). Grain set in the cross-pollinated control plants was similar to that in the self-pollinated plants. These results suggest that ABA application did not affect female fertility, but considerably reduced male fertility.

3.5.5. EFFECT OF ABA APPLICATION ON MALE FERTILITY:

Male fertility in ABA-treated plants was also estimated by direct examination of anthers and by assessing the viability of pollen by staining with TTC. The results are given in table 3.5.6. ABA application

Table 3.5.5. Comparative grain set among control and ABA (8mg l^{-1}) treated (at the sensitive stage) wheat plants following self-pollination or cross-pollination with fertile pollen from control plants.

Treatment	Control male X Control female (I)	Control male X ABA-treated female (II)	Control self-pollinated (III)	ABA-treated self-pollinated (IV)
Grain set(%)	75.8^{A}	73.1^{A}	83.3^{A}	45.2^{B}

Values (means of 8-10 plants) bearing different letters are significantly different (P = 0.01)

Table 3.5.6. Effect of abscisic acid (8mg l^{-1}) application during the sensitive stage of development on anther morphology and pollen viability.

	Control	ABA-treated
Florets with abnormal anthers (%)	6.4	28.0*
Pollen viability in normal anthers (%)	91.4	89.0

* Significantly different from control (P = 0.05)

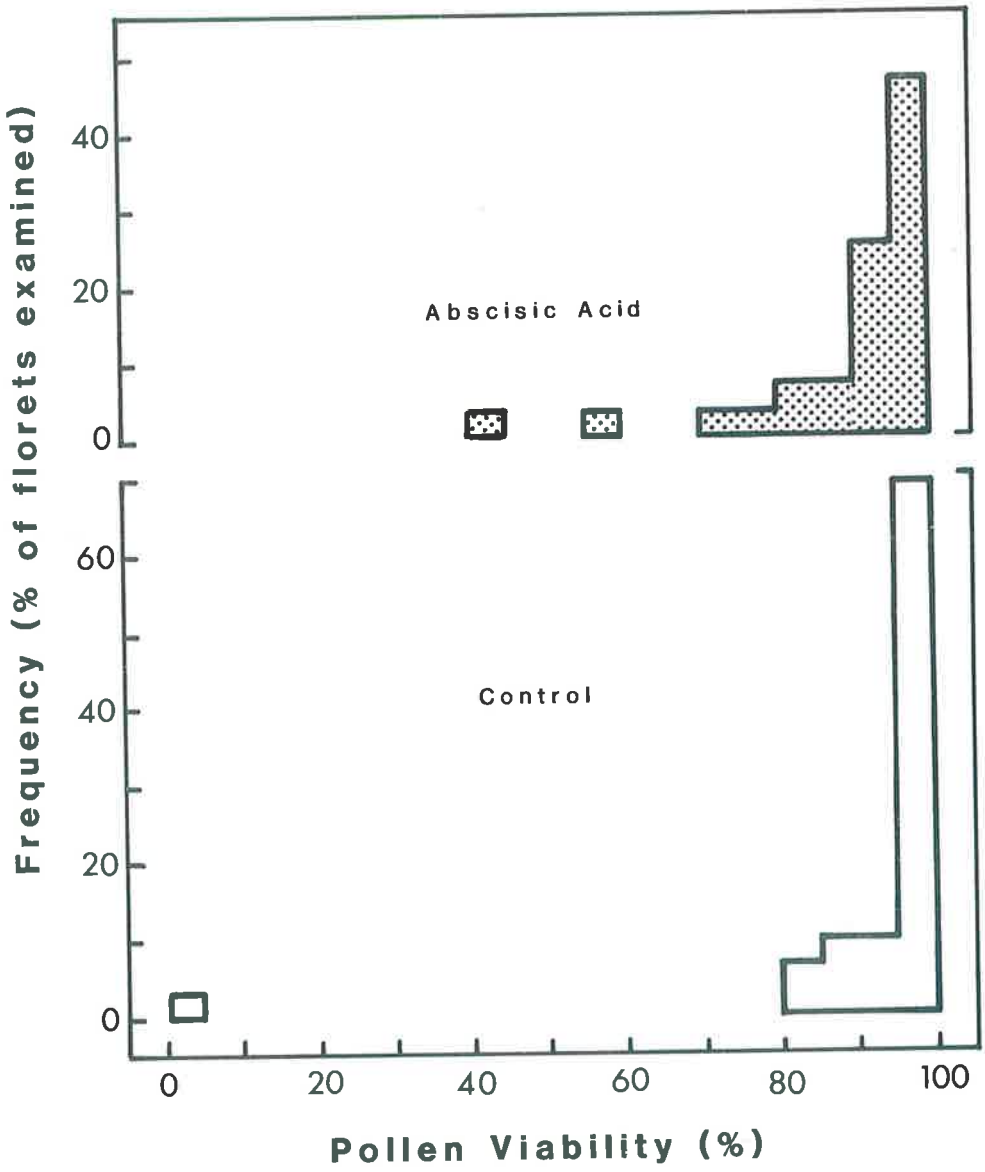


Fig. 3.5.4. Effect of abscisic acid (8 $mg.l^{-1}$) application during the sensitive stage of development on pollen viability in apparently normal anthers.

increased the percentage of abnormal anthers, similar to those affected by stress and shown in Fig 3.2.1 (Section 3.2.1.2). These anthers were small and shrivelled and generally failed to extrude and dehisce at anthesis. In control plants such anthers were found almost exclusively in the florets situated at the extremities of the spike, whereas among ABA-treated spikes they were evenly distributed. None of the pollen grains in these anthers stained red with TTC and were accordingly classified as non-viable. These pollen grians, resembling those in Fig. 3.2.2b (Section 3.2.1.2), were shrivelled and possessed little cytoplasm. In contrast, however, the application of ABA had no significant affect on the viability of pollen in anthers that dehisced normally (Table 3.5.6., Fig. 3.5.4.).

3.5.6. ANTHER- AND POLLEN-DEVELOPMENT IN RESPONSE TO ABA APPLICATION:

Plants were perfused with ABA solution (8mg l^{-1}) for 24h, at the onset of meiosis in the most advanced floret of the spike and anthers were sampled sequentially and examined microscopically following sectioning and staining as described in section 3.4.1. for control anthers.

Two types of abnormal development were observed in the anthers that aborted following ABA application.

3.5.6.1. TYPE -1:

Of the abortive anthers 75.9% showed type-1 development (Table 3.5.7). Abnormalities became apparent during meiosis, when PMCs showed signs of disorientation and tapetal degeneration started prematurely (Fig 3.5.5a). Tapetal cells remained in their peripheral position but a portion of their contents, that stained darkly, was released into the anther lumen by the time of microspore release (Fig. 3.5.5b). Often only a proportion of the thecae within an anther were abnormal while others developed normally, or aborted later. In most of these cases, the more severely affected thecae appeared to be arrested at, or close to, meiosis (Fig. 3.5.5c). This type of development led to anthers which were smaller in diameter than control anthers (Table 3.5.8) and mostly contained pollen that was completely abnormal but occasionally had a proportion of

Table 3.5.7. A summary of abnormal anther- and pollen-development in response to water-stress*, heat-stress*, (30°C for 3 days) and abscisic acid application (8mg/l) at the sensitive stage of development

Type of Development	Proportion of abnormal thecae (%)	Abnormalities	Extent of Sterility
Water Stress:			
Type 1	58.4	Abnormalities appeared near PGM-1. Filament and vascular bundle degenerated. Microspores lost contact with tapetum and no starch accumulation occurred	Complete
Type 2	41.6	Abnormalities appeared near PGM-1. Filament or vascular bundle did not degenerate. Microspores lost contact with tapetum and no starch accumulation occurred.	Partial or Complete
Heat Stress:			
Type 1	51.8	Meiosis was abnormal. Tapetum degenerated prematurely.	Complete
Type 2	48.2	Abnormalities appeared after PGM-1. Tapetal degeneration and filament were normal. Microspores lost contact with tapetum and no starch accumulation occurred.	Partial or Complete
Abscisic acid:			
Type 1	75.9	Meiosis and tapetal degeneration were abnormal.	Complete (mostly)
		or Partial	
Type 2	24.1	Abnormalities appear after PGM-1. Tapetal degeneration was normal. Microspores lost contact with tapetum and no starch accumulation occurred.	Partial or Complete

* Information repeated from table 3.4.3.

Fig. 3.5.5. Anthers from wheat plants treated with abscisic acid ($8mgl^{-1}$) solution during the sensitive stage, showing abnormal development of type-1.

(a) T.S. of an anther at meiosis

(b) T.S. of an anther at young microspore stage

(c) T.S. of an anther showing the develpment of one theca lagging behind that of the other

(d) T.S. of a mature anther showing partial sterility.

pmc - pollen mother cell, t - tapetum, f - filament, ms - microspore, apg - abnormal pollen grain, npg - normal pollen grain, st - starch, vb - vascular bundle, tr - tapetal residue.

Bar represents 50μm.

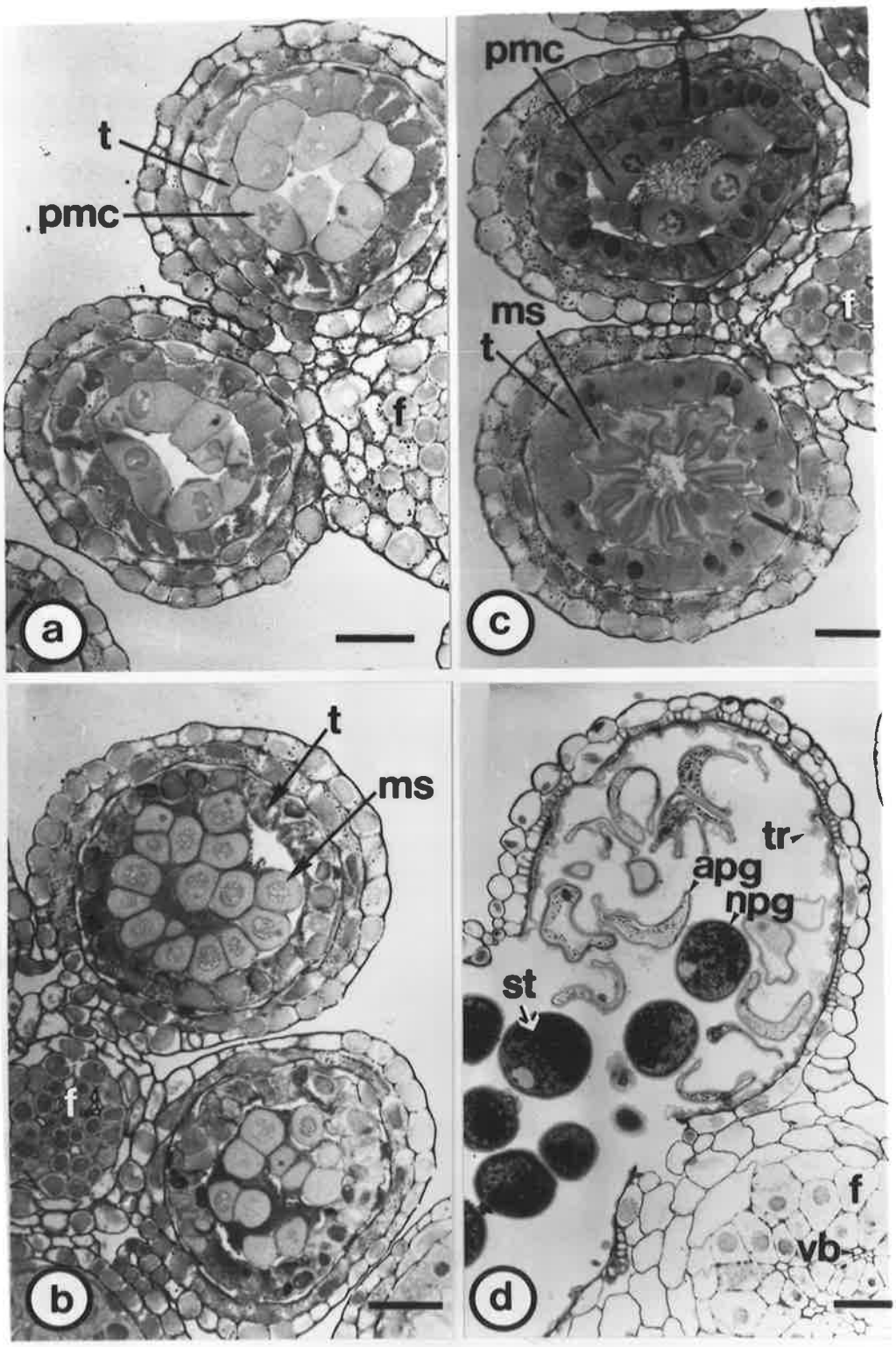


Fig. 3.5.5.

apparently normal pollen grains (Fig. 3.5.5d). The abnormal pollen grains were, possibly also sterile (Table 3.5.6). No abnormalities in the filament or its vascular system were observed. The apparently normal pollen grains were trinucleate, rich in starch and had all the usual wall-layers. The aborted pollen grains, had no or very little starch and a few had one darkly-staining nucleus. The exine was normal but the intine was either absent or very thin and faintly staining. The staining characteristics of pollen grains similar to these, are given in table 3.4.2 (Section 3.4.1).

Table 3.5.8. Mean external diameter of mature normal anthers and those rendered sterile by the application of abscisic acid (8mg/l) during the sensitive stage of development.

	Control	Abscisic Acid	
		Type 1	Type 2
Diameter (μm)	333 ± 6.1^{A}	209 ± 10.6^{B}	301 ± 11.4^{A}

3.5.6.2. TYPE-2:

In this type of abortion, represented in 24.1% of abnormal anthers, pollen development and tapetal degeneration proceeded normally up to PGM-1 following which the pollen grains became disoriented and frequently lost contact with the tapetum (Table 3.5.7., Fig. 3.5.6a). Wall formation in most of the pollen grains proceeded normally to maturity but they accumulated only small amounts of starch (Fig. 3.5.6b,d). Thecae showing this type of development were often found on anthers which had other thecae developing normally or showing type-1 abortion (Fig. 3.5.6c). This type of development usually produced anthers with a mixture of normal and abnormal pollen grains but anthers with all the pollen grains abnormal were also observed occasionally (Fig. 3.5.6d). These anthers grew to normal size (Table 3.5.8). The degeneration of the tapetum was normal and no abnormalities in the filament were observed.

Fig. 3.5.6. Anthers from wheat plants treated with abscisic acid ($8mgl^{-1}$) solution during the sensitive stage, showing abnormal development of type-2.

(a) T.S. of an anther showing deterioration of pollen grains at the beginning of abnormalities

(b) T.S. of an anther showing pollen grains with some starch and dark nuclei.

(c) T.S. of an anther showing type-1 and type-2 degeneration in different thecae. Type-1 theca has no microspores and is filled with orbicules

(d) T.S. of a mature anther showing type-2 abortion.

t - tapetum, vn - vegetative nucleus, gn - generative nucleus, apg - abnormal pollen grain, or - orbicules, f - filament, n - nucleus, dp - disoriented pollen grains.

Bar represents 50μm.

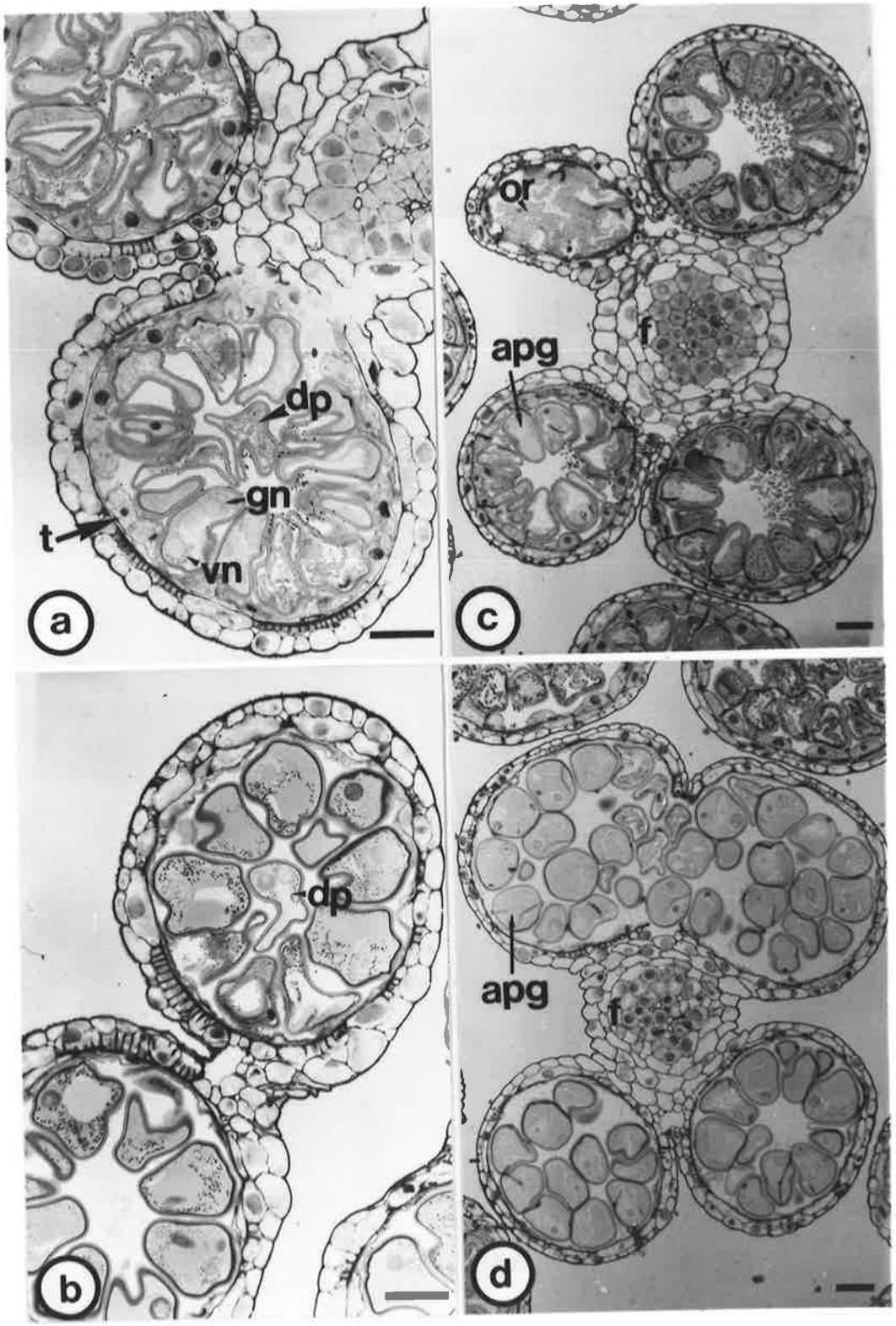


Fig. 3.5.6.

3.5.7. DISCUSSION:

The results of these experiments confirm the observations of Morgan (1980) that the application of ABA to wheat plants during the phase of reduction division leads to pollen sterility. As both water deficit and heat also lead to pollen sterility, it is important to ascertain whether such environmental perturbations also cause increases in plant ABA content and whether the stress-induced sterility is mediated through the hormone.

Water deficit is well known to induce accumulation of ABA in a wide variety of plants, including wheat (Aspinall, 1980; Walton, 1980); an increase in the ABA content of the spikelets during water deficit was apparent in the present study (Fig. 3.5.1 and 3.5.2). This being so, it is pertinent to enquire whether the same magnitude of pollen sterility can be evoked by water stress and application of ABA, when both treatments result in equivalent endogenous concentrations of the hormone in the spikelets. Morgan's (1980) data do not provide such a comparison, but the present results have demonstrated that a reduction in grain set of some 20-50% was associated with a spikelet ABA content of approximately 1μg g^{-1} dry weight when the plants were subjected to water deficit (Fig. 3.5.1 and 3.5.2). Attainment of a similar reduction in grain set by the application of ABA to non-stressed plants required an internal concentration greater than 1.6μg g^{-1} dry weight in one experiment (Fig 3.5.1) and between 1.6 and 5.0μg g^{-1} dry weight in the other (Fig. 3.5.2). Nevertheless the concentrations of ABA in plants with similar degree of sterility induced in these different ways were of the same order. It is possible that the abscisic acid is distributed differently in the two cases. More apoplastic hormone may be present in the plants fed ABA through a wick transecting the peduncle, whereas water-stress-induced ABA is reported to accumulate both in symplast as well as apoplast (Ackerson, 1982). If this is the case, and granting the assumption that the symplastic concentration of hormone is the crucial factor in the response, then the evidence from these experiments supports the proposition that male sterility induced by water deficity is mediated through endogenous ABA.

This hypothesis is further supported by the coincidence of sensitivity to both water deficit and applied ABA during the period of PMC meiosis and tetrad break-up (Table 3.5.4 and Section 3.1.1.1). The fact that ABA

applied shortly before meiosis caused some pollen sterility, whereas water stress at that time did not, may be due to persistence of ABA, perhaps in the apoplast, when a comparatively large quantity was applied. Certainly, the absence of any effect of applied ABA after meiosis corresponds with the lack of response to water deficit at that time. Radley (1980) reported a reduction in grain set when ABA was applied at, or just before, anthesis, but the hormone was applied at concentrations far beyond the usual physiological range. Such responses, together with the complete suppression of ear development found at higher rates of application in the present study, should be regarded as artefacts caused by excessive hormone concentrations within the tissues.

A further similarity between the effects of water deficit and of applied ABA is the fact that male fertility was reduced, whereas female fertility was unaffected (Table 3.5.5. and Section 3.2.1). Complete sterility was not achieved, except at concentrations which also inhibited spike growth. In contrast to water deficit, ABA did not reduce pollen fertility in anthers which were of normal appearance (Table 3.5.6; Fig. 3.5.4).

The results of microscopic observations of anther and pollen development in ABA-treated plants, however, pose a major contradiction to the hypothesis that water-stress-induced male sterility is mediated through ABA. In about 76% of abortive anthers on ABA-treated plants the abnormalities appeared during meiosis resulting in abnormal and premature degeneration of the tapetum coupled with the abortion of microspores (Fig. 3.5.5; Table 3.5.7). Although a small proportion of anthers on these plants did abort in a manner somewhat similar to the type-2 abortion in water-stressed anthers, the majority of ABA-affected anthers followed a course of degeneration different from the sequence of events in water-stressed anthers. Anatomical abnormalities in water-stressed anthers did not appear until about the time of PGM-1 and frequently pollen abortion was accompanied by the degeneration of the vascular system (Table 3.5.7 and section 3.4.2). Tapetal degeneration in this case was normal. It appears, therefore, that although both water stress and ABA result in the production of sterile anthers that look similar at maturity, the mechanism causing anther abortion is likely to be different in the two situations. The possibility of these differences being due to the

differences in hormone concentration (Fig. 3.5.2) can, however, not be ruled out.

The situation regarding the role of ABA in the induction of sterility by heat, is however, clearer. Heat affected anthers were similar in appearance to those affected by ABA and there were similarities in the anther development under the two conditions (Table 3.5.7). Nevertheless, even temperatures which produced complete sterility had no effect on spikelet or flag leaf ABA content (Fig. 3.5.3; Table 3.5.3). Furthermore, heat-stress affected female fertility (Section 3.2.2. and 3.3) while ABA did not (Table 3.5.5). Hence, the response to high temperature during meiosis appears not to be mediated through endogenous abscisic acid.

A comparison of the effects of various types of environmental stress on the ultimate structure of anthers and pollen grains could lead to erroneous interpretations. In the present investigation, for example, the anthers rendered sterile by water stress, heat or ABA-application were similar in general morphology, and the sensitive stage of development was common. Nonethless, the developmental events that led to sterility were different (Table 3.5.7), and hence the mechanisms inducing pollen sterility under these three conditions may be unrelated.

3.6. EFFECTS OF ETHEPHON AND ETHYLENE ON FERTILITY

Experiments were carried out to test whether ethylene acts as a sterilent under stress conditions. The experiments were exploratory to see whether there were enough similarities between the effects of treatment with ethylene and water- and heat-stress to warrant a more detailed investigation.

Effects on fertility of treatment with ethephon (2-chloroethylphosphonic acid), that is known to break down in plant tissue to release ethylene (Cooke and Randal, 1968; Edgerton and Blanpied, 1968), as well treatment with ethylene gas were investigated.

3.6.1. GRAIN SET:

Investigations on the effects of water deficit and heat had earlier identified a brief sensitive period during the PMC-meiosis and tetrad break-up (Sections 3.1.1.1 and 3.1.2.1). Various concentrations of ethephon, between 0 and 800ppm were sprayed on plants at the beginning of meiosis in the most advanced floret of a spike. Number of grains per spike and per cent grain set were recorded. Concentrations up to 100 ppm had no injurious effect on either of these parameters and may, in fact, have had a stimulatory (statistically not significant) influence. (Fig 3.6.1). However, application of ethephon at the rates of 200, 400 and 800 ppm significantly reduced both the number of grains per spike and percent grain set. The maximum effect was achieved with the highest concentration.

In a second experiment, plants were exposed to air containing ethylene at concentrations of 95.8, 191.4 and 420.1 ppm (flowing at 10 litre/min) for 48h, beginning at the onset of meiosis. Each of these concentrations of ethylene were applied in separate experiments and on every occasion control plants exposed to ethylene-free air were included. Due to the seasonal variations in the size and of grain number in spikes of control plants the data are presented as percentage of control values (Fig 3.6.2a,b).

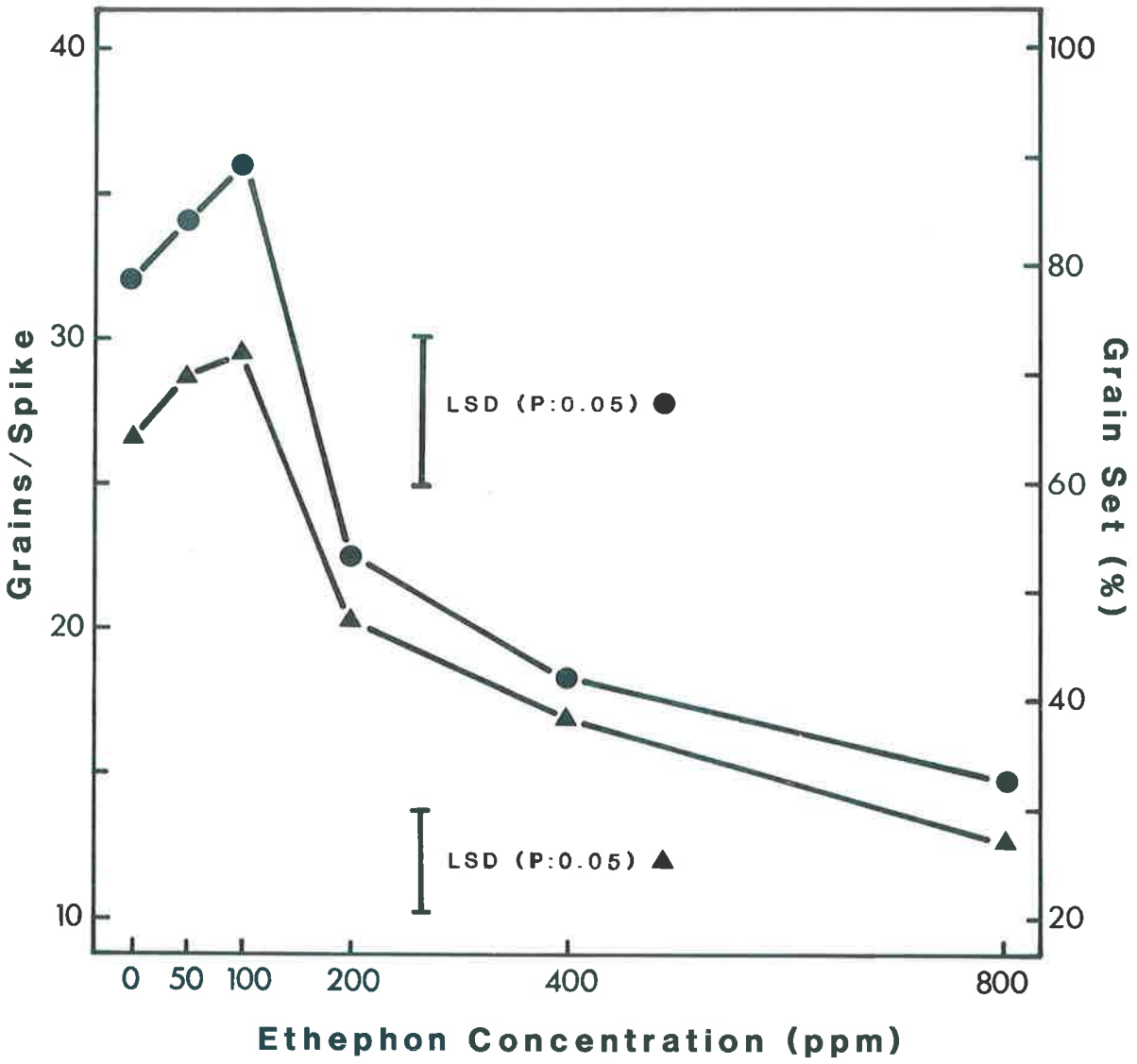


Fig. 3.6.1. Effect of ethephon application during the sensitive stage of development on the number of grains per spike (●) and per cent grain set (▲). Each treatment-mean represents 10 replicates.

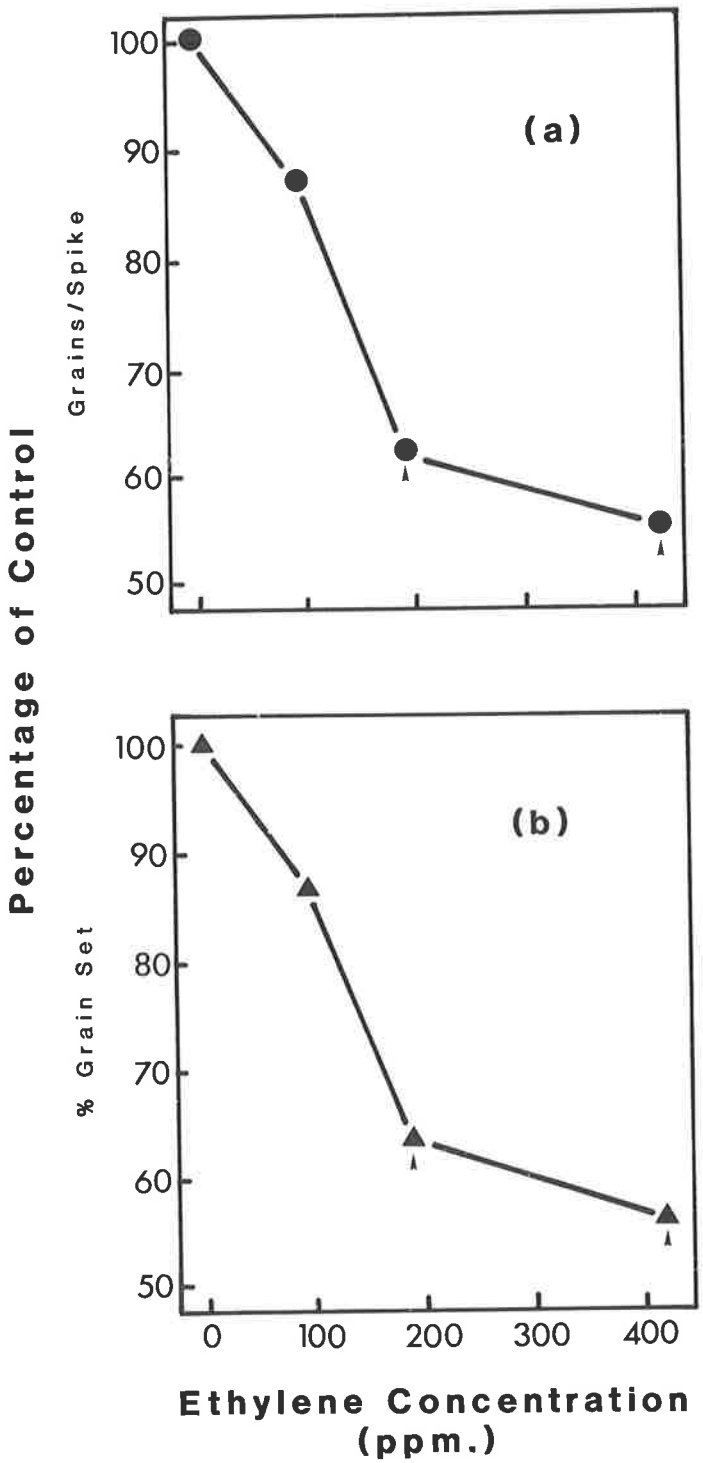


Fig. 3.6.2. Effect of exposure to various concentrations of ethylene during the sensitive stage of development on: (a) the number of grains per spike and (b) per cent grain set. Points (representing means of 4-5 replicates) marked with arrow-heads are significantly different from control.

Exposure to 95.8 ppm ethylene had a small but statistically non-significant effect on grains per spike (Fig. 3.6.2a) and per cent grain set (Fig. 3.6.2b). Both the higher concentrations, however, caused a significant reduction in these attributes.

These effects of ethephon and ethylene were spread throughout the spike and did not appear to be confined to a specific region of spike or a class of florets within a spikelet.

3.6.2. THE SENSITIVE STAGE:

Plants were sprayed with ethephon at the rate of 800 ppm or exposed to 400ppm ethylene gas at: (i) the onset of meiosis, (ii) the "boot-stage" when the tip of the spike was just touching the flag leaf and pollen development was underway, and (iii) the onset of anthesis. The results are presented in tables 3.6.1. and 3.6.2. None of these treatments when applied during the boot stage or anthesis had any significant effect on either grains per spike or per cent grain set. These treatments were effective in reducing grain set, as already described, only when applied during meiosis.

3.6.3. EFFECTS ON MALE FERTILITY:

Application of 800 ppm ethephon at the onset of meiosis in the PMCs, resulted in the production of small, somewhat shrivelled and non-dehiscent anthers (Table 3.6.3), similar in appearance to those shown in Fig 3.2.1 (Section 3.2.1.2). These anthers contained pollen grains that were shrivelled, empty and did not stain with TTC (similar to Fig 3.2.2b, Section 3.2.1.2). This treatment caused sterility by affecting anthers in 91.5% of the florets. However, there was an attendent suppression of stem elongation. At anthesis, ethephon-treated plants had significantly ($P=0.05$) shorter stems than control plants, their respective heights being 41.8 ± 1.2 and 71.8 ± 1.1 cm.

Exposure to ethylene at the rate of 400 ppm during meiosis, also caused male-abnormalities, similar to those caused by ethephon (Table

Table 3.6.1. Effect of Ethephon (800 ppm) application during various stages of development.

		Stage of Development		
		Meiosis#	"Boot-Stage" (Pollen development)	Anthesis
Grains/ Spike	Control	32.1 ± 1.7	36.8 ± 2.8	37.2 ± 1.7
	Ethephon	14.7 ± 2.7*	34.8 ± 2.1	39.2 ± 1.1
Grain Set (%)	Control	64.1 ± 2.2	73.6 ± 5.3	71.4 ± 3.7
	Ethephon	27.1 ± 4.8*	71.7 ± 5.2	71.1 ± 2.2

* Significantly different from control (P = 0.05)
Means represent 8-10 replicates.
\# Data repeated from Fig. 3.6.1.

Table 3.6.2. Effect of exposure to ethylene (400 ppm) during various stages of development.

		Stage of Development		
		Meiosis#	"Boot-Stage" (Pollen development)	Anthesis
Grains/ Spike	Control	52.0 ± 5.4	36.8 ± 6.8	36.8 ± 5.4
	Ethylene	28.6 ± 2.3*	36.0 ± 3.3	44.8 ± 2.4
Grain Set (%)	Control	75.7 ± 6.1	67.0 ± 7.1	73.3 ± 12.4
	Ethylene	42.5 ± 3.3*	66.4 ± 7.3	81.7 ± 3.7

* Significantly different from control (P = 0.05)
Means represent 4-5 replicates.
\# Data repeated from Fig. 3.6.2.

3.6.3). The number of florets in which anthers were affected was not, however, as high after treatment with ethylene as with ethephon.

Sterilization of florets in response to either treatment was not limited to any specific region within a spike.

Pollen viability in the apparently normal anthers was not recorded.

Table 3.6.3. Effect of ethephon (800 ppm) application and exposure to ethylene (400 ppm during the sensitive stage of development on anther morphology.

		Treatment	
		Ethephon	Ethylene
Florets containing abnormal anthers (%)	Control	6.2 ± 2.3	4.9 ± 3.0
	Treated	91.5 ± 4.1*	33.5 ± 4.4*

* Significantly different from control (P=0.05)

3.6.4. DISCUSSION:

Application of ethephon at concentrations of about 2000 ppm during the reproductive phase has been previously found to induce male sterility in wheat and barley (Rowell and Miller, 1971; Bennett and Hughes, 1972; Fairey and Stoskopf, 1975; Verma and Kumar, 1978). This effect of ethephon has been attributred to the ethylene that is produced upon the breakdown of ethephon following its entry into the cell and exposure to a pH favourable for such a conversion (Cooke and Randal, 1968; Edgerton and Blanpied, 1968). The present investigation confirms these earlier reports of the male-gametocidic activity of ethephon. The influence of ethylene gas on male fertility has hitherto been untested. The results presented here have shown that ethylene, like ethephon, can induce male sterility.

The sensitivity to ethephon or ethylene appears to occur at about the same time as that to water stress (section 3.1.1.1.) and heat (section 3.1.2.1). Ethephon application before meiosis has been reported to induce male sterility in wheat (Bennett and Hughes, 1971), but these effects may have been due to the persistence of ethephon in the tissue since relatively high amounts of the compound were applied. Similarly, sterilization by a post-meiotic application of ethephon (Rowell and Miller, 1971) may well have been due to the high rates at which the substance was applied, since a lower rate of application in the present investigation induced sterility when applied at meiosis but not after this stage (Table 3.6.1).

Ethylene generation from plant tissues is enhanced by a variety of environmental stresses, including water deficit and heat (Lieberman, 1979; Aspinall, 1980; Field, 1981a,b). The concentrations of ethylene required to induce a significant amount of sterility were rather high (Fig 3.6.2) compared to the amounts produced endogenously by the plants under stress (Wright, 1977; Apelbaum and Yang, 1981; Field, 1981a,b). However, the amount of exogenously supplied gas that actually enters the cell is unknown and may only be a small fraction of the ambient concentration.

Effects of ethephon and ethylene on male fertility (Table 3.6.3) appeared similar to those of water stress and heat (Section 3.2.1.2. and 3.2.2.2). Effects of ethephon and ethylene on pollen viability in normal-looking anthers and on female fertility were not assessed. However, following treatment with these chemicals, the production of small, shrivelled and non-dehiscent anthers containing sterile pollen was an effect, similar to that following water-stress and heat.

To sum-up, there appears to be enough *prima facie* evidence to consider ethylene as a possible mediator in the induction of sterility by water stress and/or heat. ABA accumulates in plant organs in response to water deficit (Section 3.5; Walton, 1980) and application of the hormone at meiosis can cause male sterility in wheat (Section 3.5; Morgan, 1980). since ABA application can also enhance ethylene evolution from various plant tissues (Cooper *et al.*, 1968; Goren *et al.*, 1979; Sagee *et al.*, 1980), it was initially envisaged that the induction of sterility by stress may be mediated through an interaction of the two hormones.

However, the later evidence of important anatomical differences between the anther development in water-stressed and ABA-treated plants has cast serious doubts about the validity of this hypothesis. The evidence, nevertheless, suggests the possibility that ethylene alone may mediate the stress-induced sterility.

Despite several similarities between the effects of water stress and exogenously applied ABA on male fertility, the courses of anatomical events leading to anther sterility in the two situations appear to be different. Hence, apparent morphological similarities between the effects of different treatments need to be treated with caution. Although ethylene induced male-sterility bears notable resemblance to that induced by water stress or heat, the causes of pollen abortion under these conditions may not be so similar as, indeed, was illustrated by the example of ABA-induced sterility. It is pertinent to mention that Bennett and Hughes (1972) found additional mitosis in pollen grains to be the cause of pollen-sterilization by ethephon application several days before the PMC meiosis. No such abnormalities were found in pollen grains rendered sterile by heat or water stress (Section 3.4). Ethephon or ethylene treatment at meiosis, however, may affect fertility in a way different to that when the chemicals are applied before meiosis.

It has been suggested that ethephon spray may be used as a treatment to induce male sterility and so substitute for hand emasculation in breeding programmes (Bennett and Hughes, 1972; Fairey and Stoskopf, 1975; Verma and Kumar, 1978). Such a treatment would, however, be of limited usefulness if it produces side-effects such as the curtailment of growth shown in one of the present experiments.

4. INTEGRATIVE DISCUSSION

This project had three basic aims: (i) to identify in wheat the stage of reproductive development that is sensitive to water deficit and heat, (ii) to clarify the nature of injury caused to the microspores and megaspores by stress, and (iii) to enquire into the physiological mechanism underlying such injury.

The maximum sensitivity to water deficit and heat appears to occur during the period from the onset of meiosis in PMCs to the break-up of tetrads to release microspores (Sections 3.1.1.1 and 3.1.2.1). The concurrent stages of female development include meiosis in the MMC and the degeneration of three redundant megaspores (Bennett *et al.*, 1973b). This identification of stress-labile stages of development may not be absolute however, because of the asynchrony between different florets of a spike and the relatively long durations of the periods of stress. It is possible that the maximum sensitivity occurs during a short and specific stage of development that is bordered by stages with diminishing sensitivity. Such a critical stage could possibly be identified by using stress-periods of shorter duration and monitoring the development of anthers in specific florets using sampling techniques similar to those described for timing the duration of meiosis by Bennett *et al.*, (1971). Such an approach is more likely to succeed when applied to the study of the effects of heat than to those of water stress. Subjecting plants to short periods of water-stress using polyethylene glycol (m.wt. 4000) was attempted. The technique, however, was unsuccessful as the osmoticum had irreversible toxic effects and the plants died despite a complete removal of the chemical from the rooting medium 24h after the application (Results not reported here).

When the grain set was reduced by stress, some increase in the weight of the surviving grains followed and this tended to partially compensate for the loss of yield (Sections 3.1.1.3 and 3.1.2.3). There is some in-built capacity in wheat for yield-compensation during almost every developmental phase; any negative effects of fluctuations in a yield component on yield are compensated to some extent by those components which are subsequently determined. For example poor germination is

compensated by increased tillering (Evans *et al.*, 1975; Evans and Wardlaw, 1976). Yield losses resulting from sterility are similarly compensated for, to a variable degree, by increased grain growth later during the life cycle (Lupton and Ali, 1966; Bingham, 1969; Pinthus and Millet, 1977; Fischer and HilleRisLambers, 1978). This compensation of yield is only partial and there is generally a net reduction in yield when the grain set is reduced significantly.

The stress-induced reduction in grain set was due to an induction of abnormalities in sporogenesis. Both water stress and heat caused male sterility whereas only the latter affected female fertility (Section 3.2). It is possible that more severe water stress than that applied in this investigation would induce female sterility, as was found in oats (Skazkin and Lukomskaya, 1962). However, there are reasons for suspecting that the effects of water stress and heat on sporogenesis may differ qualitatively. Apart from the differences in their effects on female fertility, (Sections 3.2. and 3.3) these stresses also appeared to induce male sterility in different ways (Section 3.4). Water-deficit-induced abortion of pollen grains, that occurred close to PGM-1, was associated with and possibly caused by the degeneration of the filament and its vascular system in at least half of the anthers. The heat-induced abnormalities, on the other hand, became apparent during meiosis and involved abnormal and premature degeneration of the tapetum. Thus the course of events leading up to pollen sterility under water- and heat-stress conditions was different although both resulted in mature anthers and pollen grains similar in appearance and defectiveness. The sterile pollen grains tended not to accumulate starch in the cytoplasm and their intine remained thin.

Heat-induced abnormalities in the ovule comprised suppression of development or degeneration of nucellus and embryo sac cells (Section 3.4). The abnormal behaviour of pollen tubes in heat-affected pistils may reflect a lack of attraction from the sterile embryo sacs. This possibility could be examined further by observing pollen tube growth and ovary anatomy in the same pistil to correlate severity of damage to the amount of pollen tube growth. Alternatively pollen-tube growth could be observed *in vitro* (e.g. on agar blocks) in response to extracts of sterile and fertile ovules applied as attractants.

Water stress resulted in a substantial drop in the leaf RWC and Ψ but did not affect spike Ψ (Section 3.1.1.2). Heat stress had no effect on leaf RWC, leaf Ψ or spike Ψ (Section 3.1.2.2). Consequently, the effects of these stress environments do not appear to be a result of the desiccation of sporogenous tissue. It is, nevertheless, conceivable that the Ψ in some floral organs could change, even though the stress did not alter the overall spike Ψ.

Speculations about the mechanisms of these results in physiological and biochemical terms might include:- Disruption by stress of chromosomal pairing and separation during meiosis, nucleic acid and protein synthesis, chromosomal repair mechanisms, enzyme activity, membrane integrity and transport processes related to anther development.

The possibility of hormones as mediators between the stress and the resultant sterility was examined by supplying exogenous ABA, ethephon or ethylene and comparing their effects with those of water deficit and heat. The alternative hypotheses that were postulated are summarised in Fig. 4.1.

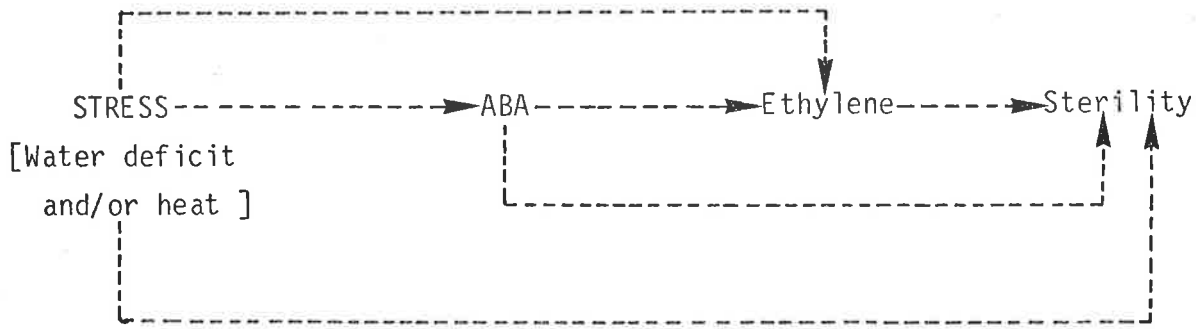


Fig. 4.1. Possible hypothetical pathways for the induction of sterility by stress.

The results have suggested that any mechanism involving ABA in the heat-induced sterility is unlikely since high temperature did not cause any increase in the concentration of endogenous ABA (Section 3.5). On the other hand, water-stress-induced sterility may be mediated through ABA since:

(i) Water stress induced accumulation of ABA and caused male sterility.

(ii) Application of ABA induced male sterility and increased endogenous ABA concentration to a level similar (but higher) to that induced by water stress

(iii) Neither water deficit nor ABA application caused female sterility

(iv) Similar stages of reproductive development were sensitive to both water deficit and applied ABA.

Despite these similarities, the sequence of anatomical events leading up to male sterility in water-stressed anthers differed from that in ABA-affected anthers. ABA-affected anthers started showing abnormalities in microspore development and tapetal degeneration early during meiosis, as against the late abortion of water-stressed anthers that involved degeneration of the filament-vascular system but no abnormalities in the tapetum. These findings suggest negation of the hypothesis that endogenous ABA mediates the induction of sterility under water-stress conditions. Also questionable is the wisdom of drawing firm conclusions from the apparent similarities in the structure of mature anthers and pollen grains resulting from various treatments. Very different courses of development may lead to mature anthers with apparently similar defects. The possibility remains, however, that the differences between the development of water-stressed and ABA-affected anthers may have been due to differences in endogenous concentration or compartmentation of ABA.

Both water-stress and heat can cause increase in ethylene emanation from various plant tissues (McMichael *et al.*, 1972; Adato and Gazit, 1974; Ben-Yehoshua and Aloni, 1974; Hall *et al.*, 1977; Wright, 1977; Field, 1981a,b). Spraying with ethephon or exposing plants to ethylene-supplemented air induced male sterility similar to that induced by water deficit and heat (Section 3.6). The developmental stage sensitive to ethephon or ethylene coincided with the stage which is

sensitive to water deficit or heat. The results of the present investigation and similar findings by other workers (Rowell and Miller, 1971; Bennett and Hughes, 1972; Fairey and Stoskopf, 1975; Verma and Kumar, 1978), suggest the desirability of further work on the possibility of ethylene acting as a mediator in the stress induced sterility. The present results on male sterility induced by exogenously applied ABA suggest that the investigation of a similar role for ethylene should not be based solely on superficial similarities in anther and pollen structure following different treatments. The use of inhibitors of ethylene biosynthesis may be a useful tool in this regard. Since ABA can influence ethylene evolution from plant tissue (Cooper *et al.*, 1968; Mayak and Dilley, 1975; Goren *et al.*, 1979; Sagee *et al.*, 1980), it is possible that the sterility induced by the application of ABA may be due to an increased level of ethylene in the tissue.

It is also possible that the similarities in the responses of stress and exogenously applied hormones may be only superficial and that the stress-induced sterilization of spores may not be hormonally mediated at all.

Based on the experiences of this investigation, the hypotheses that were initially formulated can be restructured as shown in Fig. 4.2.

Finally, it is pertinent to consider whether the responses described here are likely to occur in field-grown wheat. Approximately 40% of the total land area of the world lies within the arid and semi-arid zones where the annual net dry matter production ranges from 25 to 400 and 250-1000g m^{-2} respectively; this compares with values of up to 3000g m^{2} for vegatation with abundant water supply (Meigs, 1953; Fischer and Turner, 1978). Wheat is a crop of subhumid as well as semi-arid areas (Zohary *et al.*, 1969). In most regions of the world, heat and water-stress affect wheat crops by varying degree, often increasing as the crops develop. The risk of damage due to these stresses in most wheat growing areas, particularly in Australia, is greater during the grain filling phase (Davidson and Birch, 1978); however onset of these conditions only a few weeks earlier could seriously jeopardise the chances of a good harvest by effects on sporogenesis. In fact serious loss of grain set was noticed in wheat growing at Port Wakefield, South Australia

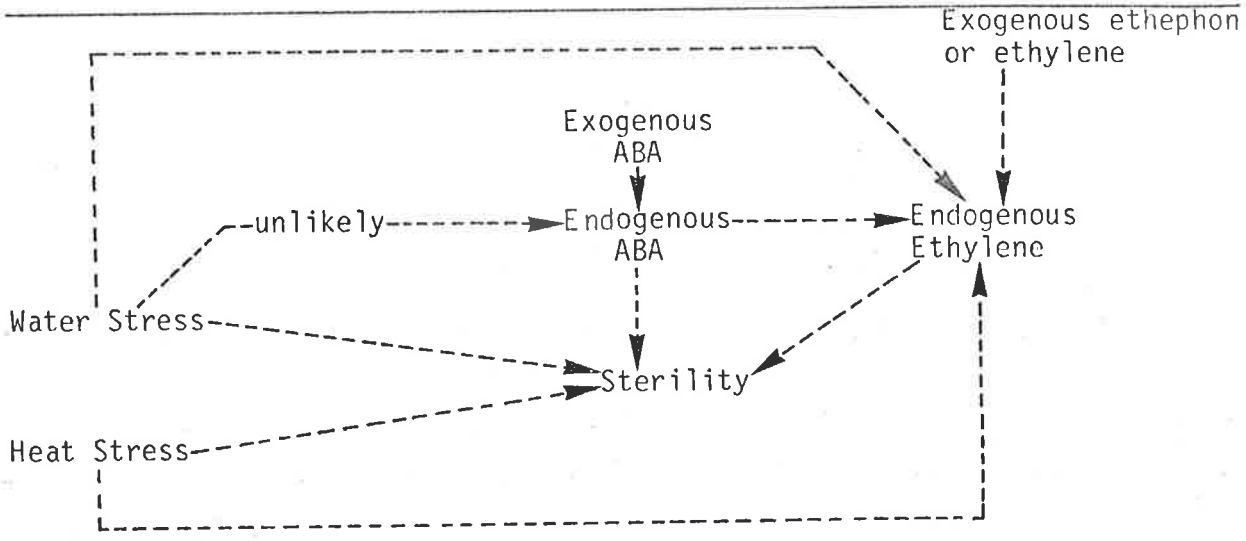


Fig. 4.2. A hypothetical model suggesting various possible mechanisms of sterility by stress, derived from the initial hypotheses proposed in table 4.1' in the light of the present results.

during 1979-80, attributable to the drought during the period of reproductive development (personal observation). Although 72h of continuous exposure to a temperature of 30°C is an unlikely event at this stage of development in field crops, a similar period at 30°C day and 20°C night is possible, particularly in the more severe environments in which wheat is grown. As such temperatures are frequently accompanied by water deficit (Hall *et al.*, 1979) it is likely that in variable environments the effects of both stresses account for some of the seasonal variability in yield. The extent of this factor is not known. The grain set obtained with covered ears, as in the present experiments, may not be an accurate estimate of the response in the field. Also, plants in the field may have a chance of conditioning against stress because of the manner in which the environmental factors fluctuate; conditioning may prevent the serious yield losses as found under controlled environments. Cross-pollination under field conditions might be another mitigating influence; effects of stress-induced partial pollen sterility on yield could possibly be offset by cross-pollination by pollen from unaffected florets (Hoshikawa, 1961; De Vries, 1971). Such compensatory ability is however impossible in the event of female sterility. For this reason, high temperature during the late reproductive development of wheat is likely to be a more serious threat to yield than is water deficit.

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APPENDIX

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