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GENETIC VARIATION FOR FRUIT DEVELOPMENT OF PISUM SATIVUM L., WITH SPECIAL REFERENCE TO THE EFFECTS OF THE RUGOSUS LOCUS

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GENETIC VARIATION FOR FRUIT DEVELOPMENT OF *PISUM SATIVUM* L., WITH SPECIAL REFERENCE TO THE EFFECTS OF THE *RUGOSUS* LOCUS

by

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To

Mum and Dad

Christine Smith. Plymouth Polytechnic / John Innes Institute. 1986

Genetic variation for fruit development of Pisum sativum L., with special reference to the rugosus locus.

Abstract

This study has compared various aspects of pea fruit development, using two green-podded lines near-isogenic except for the r locus and a yellow-podded mutant having the genotype gpgp.

The gp gene is associated with reduced chlorophyll in the pod wall which then appears yellow. The r gene is associated with a wrinkled appearance of mature dried seed and cotyledonary starch grains which are small and fissured.

An ultrastructural survey of tissues from pods, testas and cotyledons showed there to be no effect of the r locus on chloroplast structure. The structure of amyloplasts however, appeared to be affected by the r locus; starch grains in the cotyledons having a rugged outline in the wrinkled type.

Chloroplasts from the inner tissues of the yellow pod were similar to those in leaves, green pods, green-podded testas and cotyledons. Chloroplasts in the yellow pod mesocarp and in the testa of yellow pods had dilated thylakoids, less starch and more lipid than the green-podded types.

Growth analysis of the near-isogenic lines showed there to be no difference between the round (RR) and wrinkled (rr) lines in pod and testa growth, but embryo growth differed. The wrinkled embryo contained more water during development but had a lower final dry weight than the round embryos.

Water potential (an indication of the osmotic regulation) was lower in the wrinkled embryos than in the round embryos, except very early in development when it was higher in the wrinkled embryos.

In vitro culture of embryos showed that optimum growth was obtained in liquid media containing 10% sucrose as a carbon source. Replacing sucrose with mannitol determined that it was the sucrose which was important, not its resulting water potential.

These gp and r gene effects and their relevance to future breeding programmes are discussed with other biochemical studies on similar genotypes.

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CONTENTS

PRELUDE

"When I use a word," said Humpty Dumpty,
"it means just what I chose it to mean-
neither more nor less."

Through the Looking-glass

On 18 April 1905, William Bateson FRS wrote to Professor Adam Sedgewick at Cambridge to recognise a new science and found a Chair:

"...the best title would, I think, be 'The Quick Professorship of the Study of Heredity'. No single word in common use quite gives this meaning. Such a word is badly wanted, and if it were desirable to coin one, 'Genetics' might do. Either expression clearly includes variation and cognate behaviour."

He thus recognised the potential for understanding how organisms develop by studying the relationship between parents and offspring and the science of genetics was founded.

By using genetic mutants it is possible to compare the norm with deviations from the norm within species and determine the specific effects of different genes. In particular much can be learned by defining the differences between two generations, in effect a study of the inheritance of genes. Although the use of mutants has been used widely in the animal kingdom, particularly in biomedical research (Green, 1981) and developmental studies (Baker, 1978; Steward and Hunt, 1982), there is a paucity of information on mutants in the plant kingdom.

Mutants may be considered as natural "experiments" used to ask and answer questions of growth and development. Heritable deviations from normal plant structure and function may be used as a means of understanding the normal condition rather than be dismissed as useless anomalies or as signs of degeneration. Mutants with similar action occur in different species and it may therefore be profitable to look for common control mechanisms. They may affect organelles, biochemical pathways or physiological

events and they may be manifested in a variety of ways; the departures from the normal types may act at the beginning or end of a process, or may be local or general according to the time and or site of gene action. Such deviations thus provide a powerful tool for investigating regulation and development.

The most basic problem of development is to provide an accurate detailed description of the morphological, cytological and temporal sequence of events which occur during this process. The most useful mutants are those with discrete, qualitative effects which may be manipulated individually and are hence analysable in Mendelian terms. Such qualitative mutants in the plant kingdom have been characterised in only a few species, most of which are annual, diploid economic plants (Marx, 1983). For example the broad range of variation in maize has been exceptionally well described (Coe and Neuffer, 1977; Neuffer, Jones and Zuber, 1968). Despite the magnitude and intricacy of mutant systems, they remain a largely untapped source for determining the mechanisms of development and regulation, perhaps the most renowned example of which is the discovery of contolling (transposable) elements in maize by McClintock (1956).

One species which is genetically diverse and which has had its mutants described (Blixt, 1972) is the garden pea, Pisum sativum L. This is an economically important crop, legumes being the second most important protein source and providing approximately 10 % of the worlds' supply of protein (Matthews and Arthur, 1985), with 50 million tonnes of pulses produced each year. In 1984, of 57,000 ha of peas grown, 13,300 ha were for direct

human consumption (Knott, 1985).

Much work is currently being undertaken to improve the quality and quantity of protein in legumes for human consumption and particular attention has been given to Pisum sativum as it grows well in temperate climates. From the diverse genetic background of the pea, many mutants are known which affect all aspects of plant development, from the size of the plant to the number of leaflets to the colour of the seed coat. Mutants often exhibit other features important to development, as they reveal how apparently unrelated characters may be related. Different genes may combine or interact to affect a function or character in addition to a single gene having multiple effects (pleiotropy). However, genetic variability and its manifestation is often completely swamped by unpredictable environmental variables which act either within the agricultural environment or within the plant itself (Matthews and Arthur, 1985). The mechanism of gene action is thus very complex.

A method of overcoming most of these difficulties, evaluating the allelic effects without the confounding influence of other genotypic differences, is by the use of isogenic lines which differ only at a single gene locus. If these are then grown in controlled (identical) environments it should be possible to determine specific gene effects and subsequently put them to use. The only true isogenic lines are those which arise from a spontaneous mutation at a single gene locus, and their production is therefore haphazard. However, it is possible to produce lines which are near-isogenic except for a single defined locus by a process of continuous back-crossing and selection.

In the light of the economic importance of peas as a food source, their genetic diversity and the potential of genetic mutants for determining regulation of plant growth, this thesis describes the effects of two of the many known mutations in the pea in an attempt to understand better some of the mechanisms involved in pea fruit and seed development. Both genes used were first described by Mendel (1865) but were not given gene symbols until 1917 (White). They are the gp gene which converts pod colour from green to yellow and the r gene which is responsible for the wrinkled shape of the dried seed.

The gpgp line is an example of a single gene mutation on chromosome 5 and plants described in this thesis are derived from a mutation observed in 1923 by Pellew and Sverdrup at the John Innes Institute. No isolines are available but comparisons may be made between normal green-podded types and the mutant.

The rr line is an example of a single gene mutation on chromosome 7. Plants described in this thesis are near-isolines developed by Dr C.L. Hedley at the John Innes Institute over the past 8 years and thus allow more valid comparisons to be made.

This thesis is divided into two sections; The first describes and compares the structure and anatomy during development of fruit of the green-podded GpGp RR and GpGp rr genotypes and the yellow-podded gpgp RR genotype. In particular their chloroplast formation and distribution was examined in order to determine effects of the r and the gp genes on these characters. The second section deals exclusively with the two lines near-isogenic except for the r-locus. Here a detailed study of growth analysis in vivo and a preliminary study of growth in vitro were undertaken in order to determine more specifically the gene action at the r-locus.

SECTION 1

HISTOLOGY AND ULTRASTRUCTURE OF PEA FRUIT

"What is the use of a book," thought Alice,
"without pictures..."

Alice in Wonderland

INTRODUCTION

Embryo development is a complex process, a result of its genetic constitution as influenced by environmental conditions in addition to effects of the seed-coat and pod-wall. It is therefore important to understand something of the structure and development of the whole fruit if one is to understand embryo development.

The origins and genetic constitution of the different parts of the fruit have been described previously (D.C. Cooper, 1938; G.O. Cooper, 1938; Reeve, 1948; Marinos, 1970). The pericarp or pod wall develops from the carpel and bears the seeds alternately (Plate 2.2B) along its two dorsal veins (D.C. Cooper, 1938; G.O. Cooper, 1938; Esau, 1965). The testa develops from the two integuments (Corner, 1951) and is attached to the pod by a short stalk, the funiculus (Plate 2.2B). Following maturation and abscission a scar remains on the testa, the hilum (Plate 2.6). Close to the funiculus is a small hole, the micropyle, resulting from the incomplete fusion of the two integuments.

Peas are self-fertile and are usually self-pollinated (D.C. Cooper, 1938). Pollination occurs 24-36 hours before open-flower stage (Plate 1.1), fertilisation having occurred and zygotic division being underway by the time the flower is fully open (D.C. Cooper, 1938). The pollen tubes grow along the ventral suture of the ovary, one of which enters the embryo-sac through the micropyle and releases two male gametes.

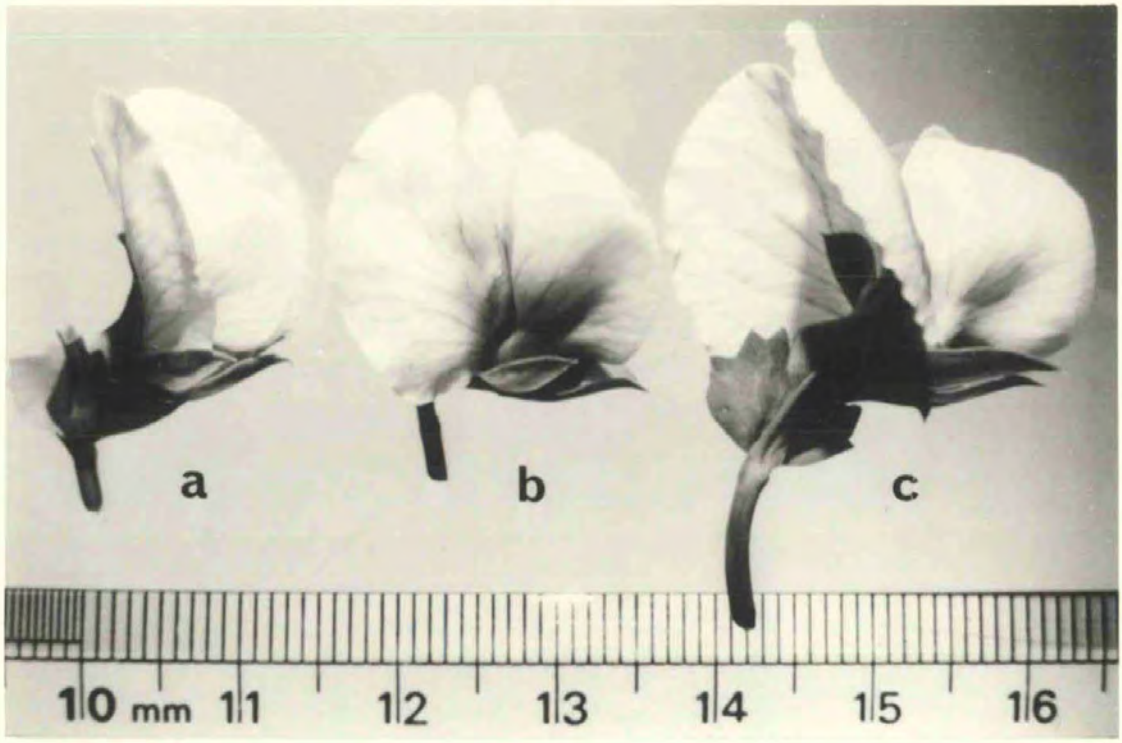
One gamete fuses with the egg-cell to form a diploid zygote which divides transversely to form a pro-embryo of two equal-sized cells (D.C. Cooper, 1938). The basal cell (nearest the micropyle)

PLATE 1.1

Characteristic flowers of the Pisum sativum L.

genotype BC/R (RR GpGp)

a) just prior to anthesis
b) at anthesis, when they would be date-tagged
c) just post anthesis


a
b
c
10 mm 11 12 13 14 15 16

divides longitudinally to form the suspensor while the apical cell divides to form an apical embryo mother-cell plus a middle cell which undergoes one more division.

No further divisions occur in the suspensor or middle cells, but their nuclei divide as they expand resulting in multi-nucleate suspensor and middle cells as the embryo divides normally. Expansion of the suspensor pushes the embryo down to the apex of the embryo sac opposite the micropyle where it continues to develop and differentiate into the cotyledons, epicotyl and hypocotyl. The suspensor subsequently disintegrates and therefore can play no role in transport.

The second male gamete fuses with the two polar cells to form a triploid endosperm (D.C. Cooper, 1938). This is both cellular and liquid and is absorbed during development by the cotyledons, peas being non-endospermic seeds (D.C. Cooper, 1938; Smith, 1973). The boundary wall of the embryo sac is the greatly expanded and modified wall of the megaspore cell (Marinos, 1970).

Normal wild-type (GpGp) pods contain chlorophyll (an effect of the dominant Gp gene) and appear green (Plate 1.2A). Pods may appear purple (Plate 1.2B), when the chlorophyll is masked by the presence of anthocyanin just beneath the epidermis. This purple colouration occurs when two dominant pod genes, Pu and Pur, are present with the master gene A (Lamprecht, 1938).

The outer epidermis of the pericarp (Plate 1.3B) is similar to that of a leaf upper epidermis (Plate 1.3A) and consists of a single layer of cells bearing stomata, suggesting active transpiration and gas exchange. The stomatal density of the pericarp is less than that found on leaves (Table 1.1; Flinn, 1969), but they have been found to

PLATE 1.2

Variation in appearance of fruits of *Pisum sativum* L.

A. Example of the normal green pod of the BC/R genotype (*GpGp*, *RR*)

B. Example of the mutant purple pod of the JI 60 genotype (*PurPur*, *PuPu*, *AA*, *GpGp*)

Magnification: x 0.75


A

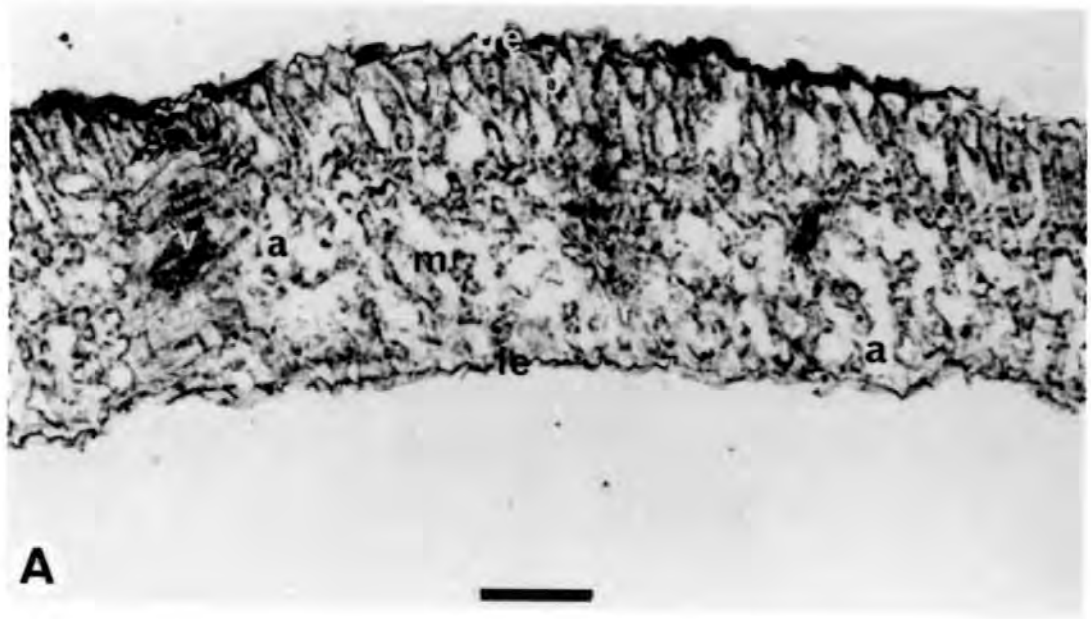

B

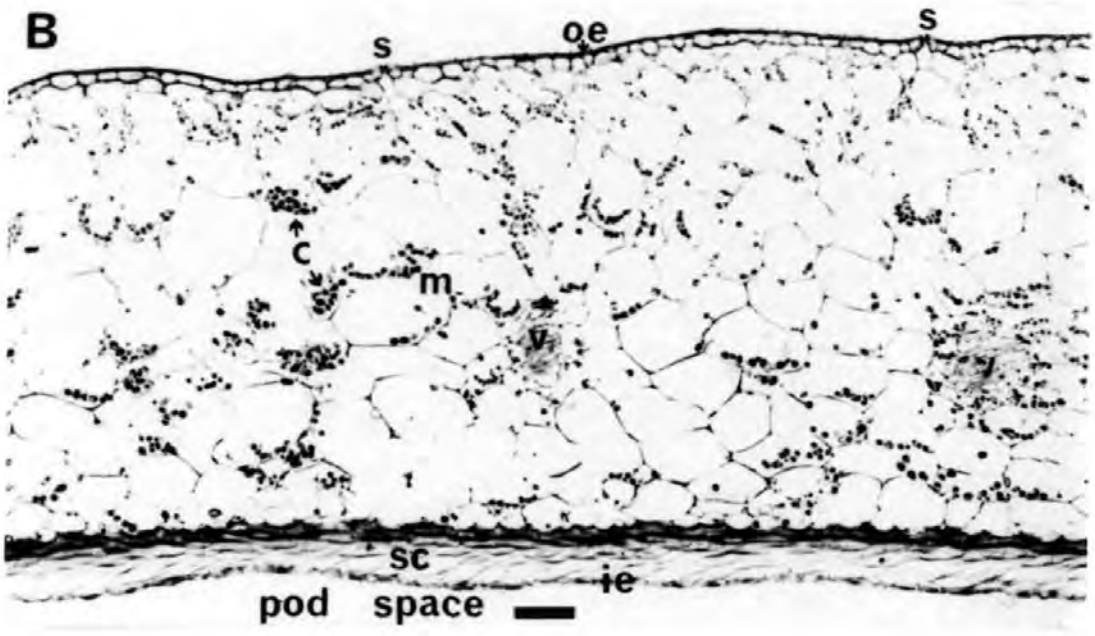
PLATE 1.3

Comparison of light micrographs of transverse sections through a fully-expanded leaf (A) and its subtending pod (B) of *Pisum sativum* L. from the BC/R genotype (*GpGp*, *RR*, *PP*, *VV*)

a	air spaces
c	chloroplasts
le	lower epidermis (leaf, adaxial)
ue	upper epidermis (leaf, abaxial)
ie	inner epidermis (pod)
oe	outer epidermis (pod)
m	spongy mesophyll (leaf)
	mesocarp (pod)
p	palisade mesophyll
s	stomata
sc	sclerenchyma
v	vascular tissue

bar line 0.1mm


a
mr
ie
a
A


B
s
oe
s
c
m
v
sc
ie
pod space

function similarly to those in leaves (Donkin, Price, Martin, Smith and Hull, 1983).

The epidermis is covered by a waxy cuticle which minimises water loss by evaporation and may reduce infection by pathogens (Reeve, 1948). The presence of wax is determined at the wp locus, wax being absent when the gene is present in its double recessive form (Blixt, 1977). There are very few air spaces in pods, unlike in leaves and these are concentrated in the outer layer close to the stomata (Plate 1.3B). The most extensive layer is the mesocarp, positioned beneath the epidermis. The thickness of this layer varies according to genotype, but is particularly pronounced in pods which are double recessive at the n locus (Blixt, 1977).

Extensive vascular strands are present throughout the mesocarp (Plate 1.3B). These are connected to the single ventral and two dorsal vascular strands which join the parent plant at the peduncle, although the vascular systems in each pod-half are independent of each other (Müntz, Rudolf, Schlesier and Silhengst, 1978).

Chloroplasts are found in the mesocarp of normal green pods and are particularly abundant around the vascular bundles. However, in yellow (gpgp) pods (Plate 1.4) chloroplasts fail to develop in this layer (White, 1917).

Beneath the mesocarp is a layer of sclerenchyma, which is only fully developed when the genes P and V are present together (Plate 1.3B). If either one of these genes is present as double recessive, partial sclerenchyma results and it is absent altogether when both genes are present as double recessive (Plate 1.5). In wild-type pods the sclerenchyma is part of the explosive seed-dispersal mechanism (Fahn and Zohary, 1955; Esau 1965), but this characteristic has been

PLATE 1.4

Variation in appearance of fruits of Pisum sativum L.

Example of the yellow pod of the mutant JI 13 genotype (gpgp)

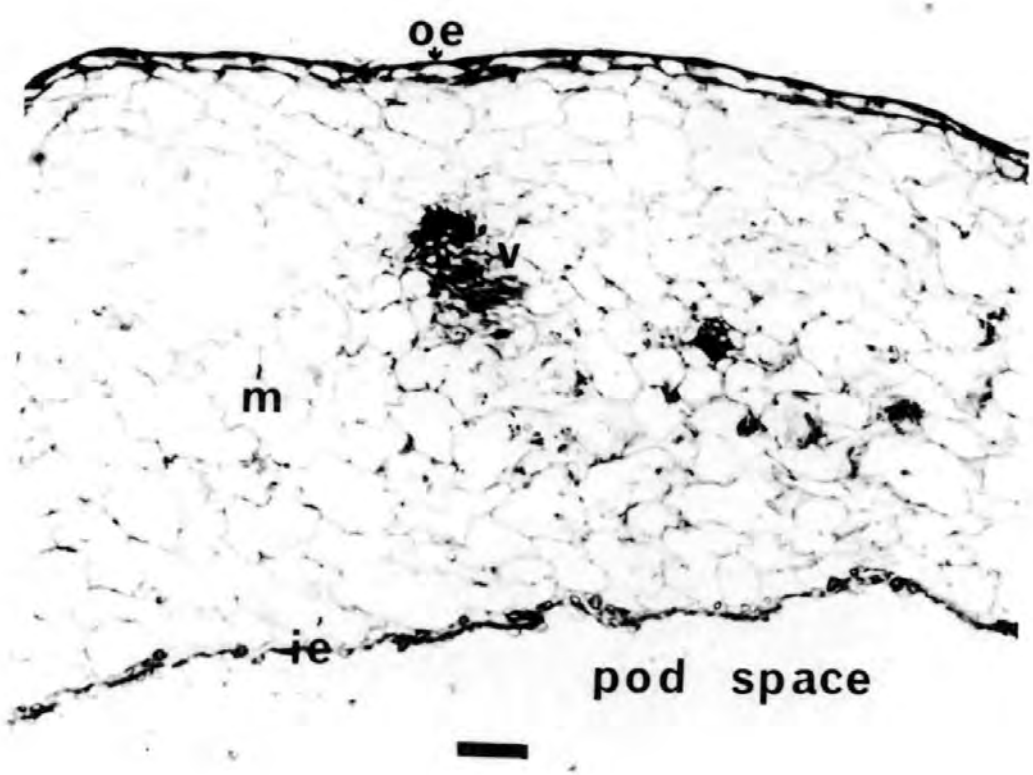
Magnification: x 0.75

PLATE 1.5

Light micrograph of a transverse section through the pod wall of a mature green parchmentless pod of *Pisum sativum* L. of the JI 467 genotype (*GpGp*, *pp*, *vv*)

ie inner epidermis
oe outer epidermis
m mesocarp
v vascular tissue

bar line 0.1mm


oe
v
m
ie
pod space

eliminated in commercial varieties. It has also been suggested (Atkins, Kuo, Pate, Flinn and Steele, 1977) that this layer may play a role in the transfer of photosynthates from different parts of the pod.

Beneath the sclerenchyma is an epidermal layer 1-3 cells thick lining the pod space (Plate 1.3B). Some of the cells of this layer may produce hairs which extend into the pod space. This inner epidermal layer plus the sclerenchyma constitute the endocarp. Chloroplasts are abundant in the inner epidermis and light penetration to this layer could be sufficient to support photosynthetic fixation of the high levels of CO_2 found within the pod-space (Harvey, Hedley and Keely, 1976; Atkins et al., 1977; Price, Hayward and Smith, 1983). However, these radiation levels will vary according to pod wall pigmentation and thus affect pod physiology. It is also possible that some non-photosynthetic assimilation may occur.

Starch is present throughout the pod tissue (Plates 1.3B), but it tends to accumulate around the vascular bundles and is virtually absent from the inner epidermis (Flinn 1969).

Although considerable variation occurs in the structure and appearance of the mature dried seed, most of this is not expressed early in development.

Prior to cellular differentiation (Plate 1.6A), the outer cells of the testa are square and regular with no secondary thickening. All these cells are nucleate and beneath them are irregularly-shaped cells containing chloroplasts (Plate 1.6A). There is a thin cuticle covering the outermost cells which is present throughout development. Later in development the outer cells become elongated and thickened

PLATE 1.6

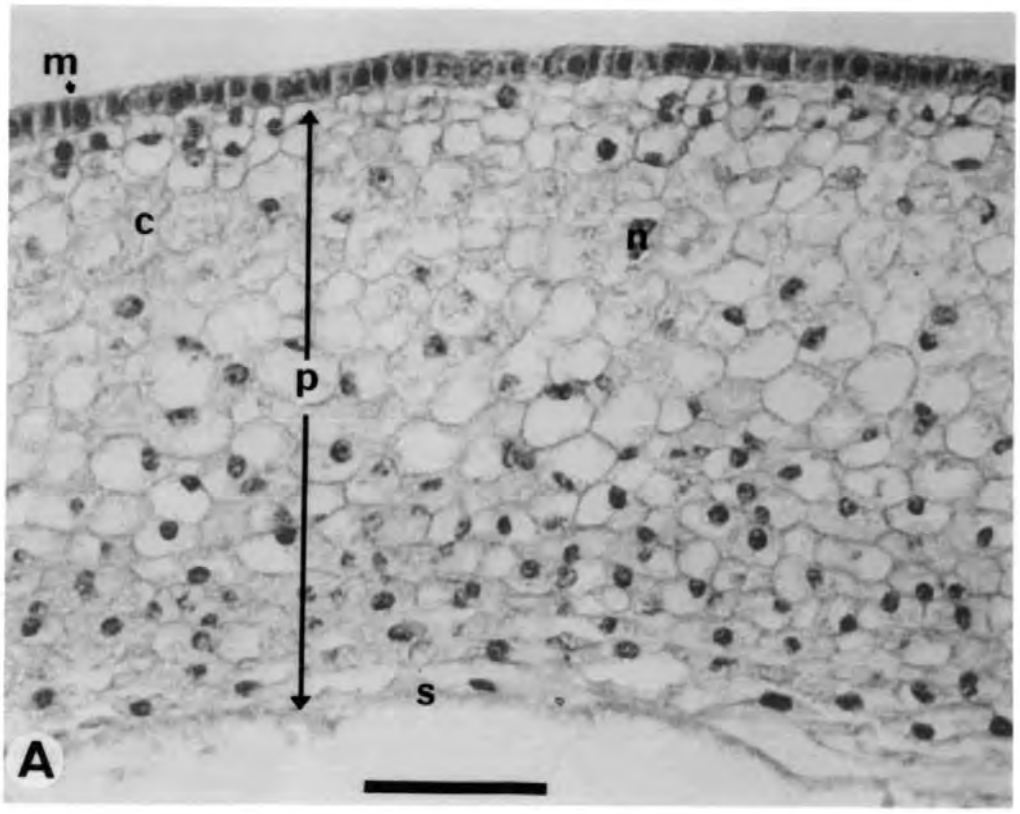
Comparison of light micrographs of testas of normal green-podded, round-seeded fruit of the BC/R genotype (GpGp, RR) of Pisum sativum L. at two developmental stages

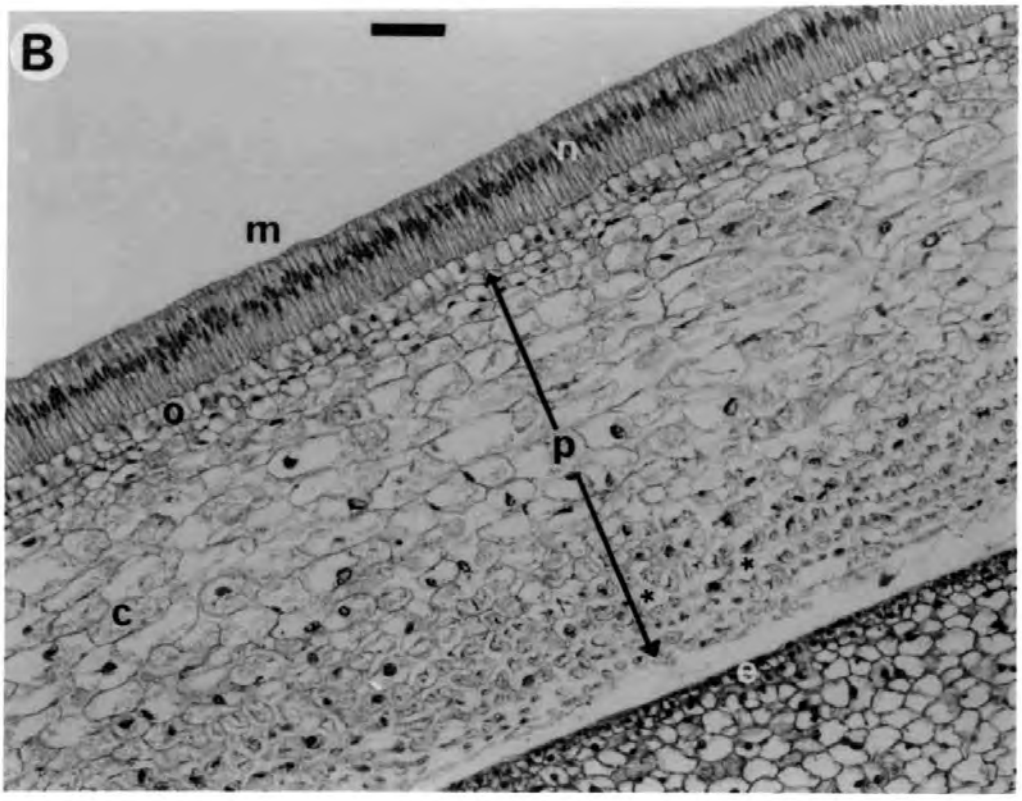
A. Prior to secondary thickening of the macrosclereids

B. After secondary thickening of the macrosclereids

c chloroplasts
e epidermis of cotyledons
m (A) outer cell layer of tissue with nuclei. (This will differentiate into the macrosclereid layer)
m (B) macrosclereids
n nuclei
o osteosclereids
p inner cell layer. (This will become the parenchyma)
s embryo sac
* inter-cellular spaces

bar line 0.1mm


m
c
p
s
A


B
m
o
p
c
e

(Plate 1.6B) and have been ascribed various descriptive names:- malpighian cells, prism cells, palisade cells, macrosclereids (Reeve, 1948). Because of their histological classification (Foster, 1942), these cells will be referred to in this thesis as macrosclereids. Underlying these macrosclereids is a single layer of cells, the osteo-sclereids (I-cells or hour-glass cells). During development these become constricted around their centre, resulting in inter-cellular spaces (Plate 1.7). Beneath this osteo-sclereid layer is parenchymatous tissue several cells thick. These cells tend to be spherical which results in many inter-cellular spaces (Plate 1.6B and 1.7). These spaces become diminished later in development due to compression by the expanding cotyledons. Early in development parenchyma cells contain chloroplasts and starch, but the starch disappears before the maturation phase (Reeve, 1948; Stafford, 1978).

In addition to its protective role during and after maturation, the testa is the route through which nutrients must flow to the developing embryo. Despite this, compared with the pericarp the vasculature of the testa is limited. It consists of a single non-branched strand running three-quarters of the way round the testa (Hardham, 1976), a section of which is illustrated in Plate 1.7.

The embryo-sac is situated beneath the parenchyma and is present throughout development, even after contact point when the liquid endosperm has been absorbed (Plate 1.7 and 1.8B). Although the seed-coat has been shown to modify imports (Raake, 1957a,b,c; Murray, 1979), the transport routes from the testa into the embryo are not fully understood. The vascular system of the cotyledons is very limited and appears to develop from areas of meristematic cells during the cell expansion phase of development (Craig, Goodchild and

PLATE 1.7

Light micrograph of a transverse section through the testa of the green-podded, round-seeded fruit of the BC/R genotype (GpGp, RR) of Pisum sativum L. at stage III in development, showing the vascular tissue

c chloroplasts
e remains of embryo-sac
i inter-cellular spaces
m macrosclereids
o osteo-sclereids
p parenchyma
v vascular tissue

bar line 0.1mm

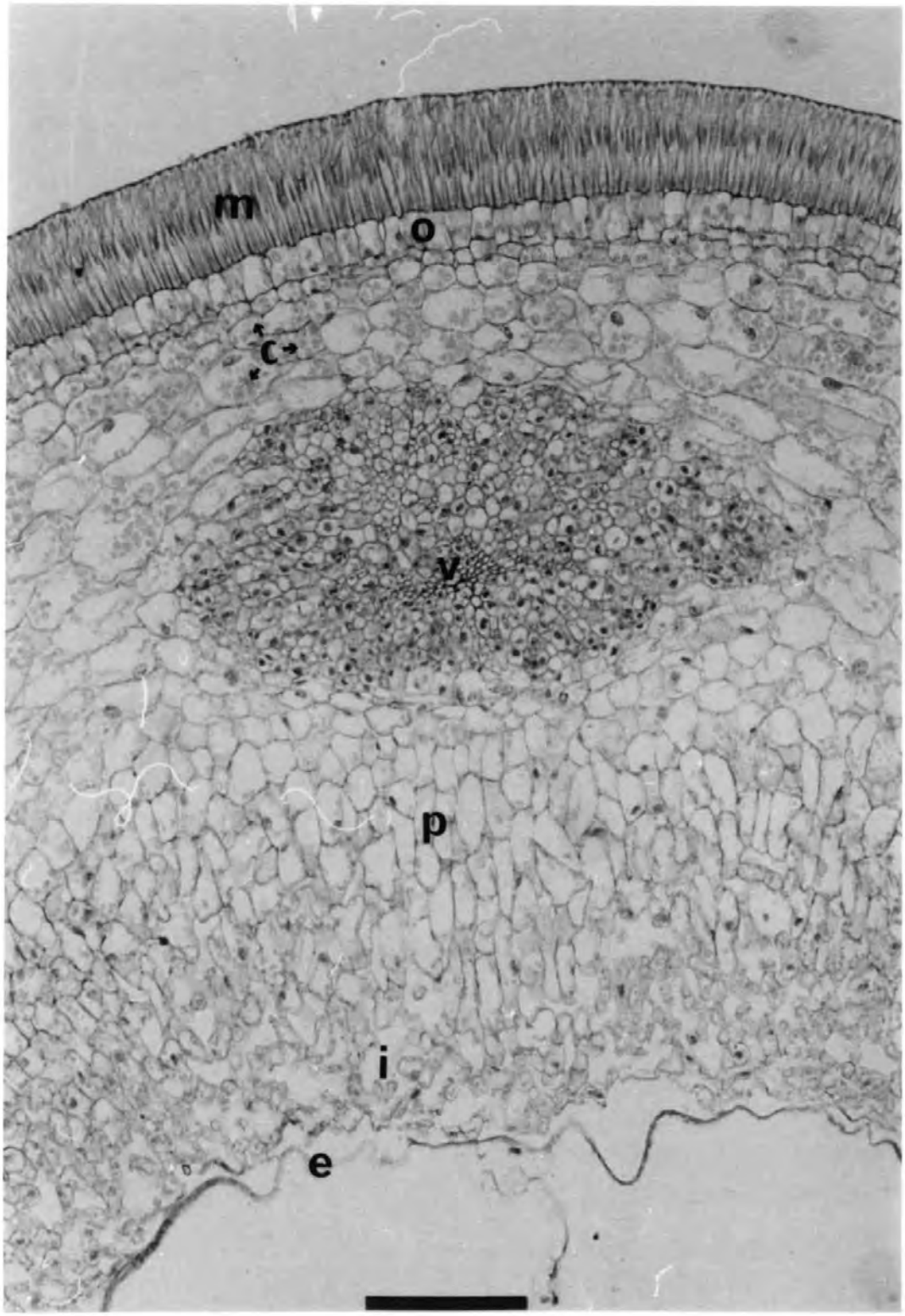

m
o
c
v
p
i
e

PLATE 1.8

Comparison of light micrographs of seed stucture of the normal green-podded, round-seeded BC/R genotype (GpGp, RR) of Pisum sativum L. at two developmental stages

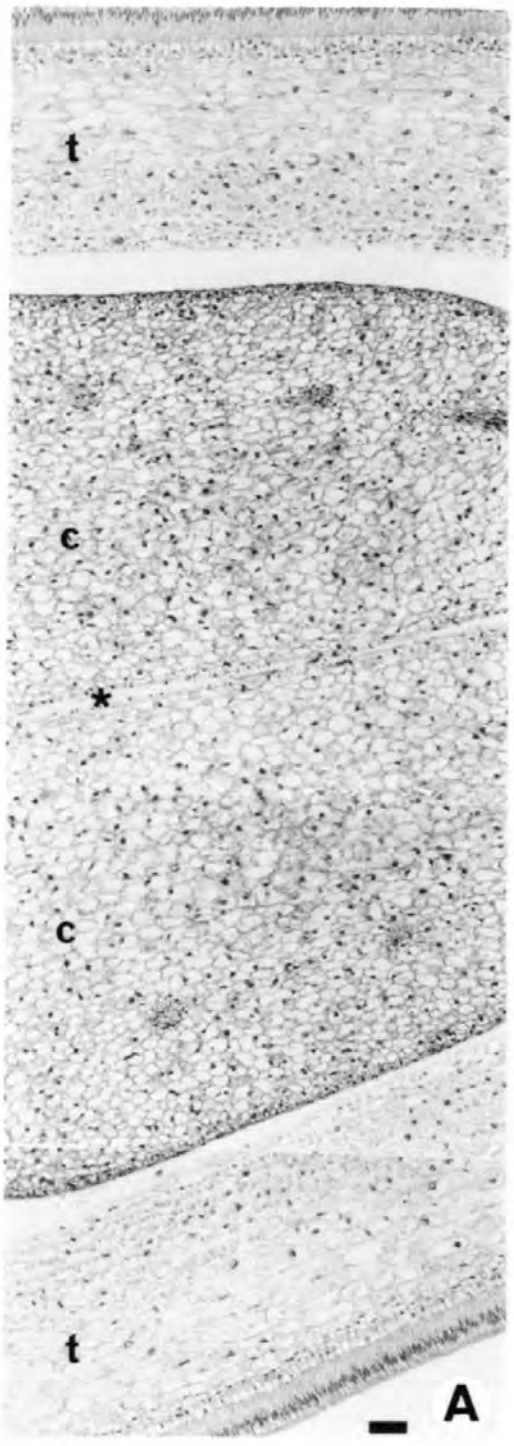
A. Transverse section through a whole seed before the liquid endosperm has been absorbed (stage II)

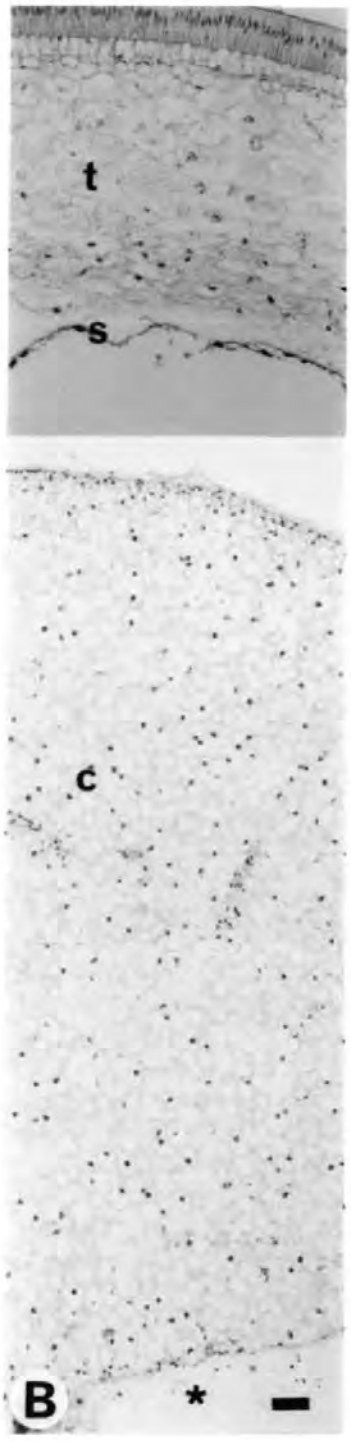
B. Transverse section through half a seed after the liquid endosperm has been absorbed (stage III)

c cotyledons
s remains of the embryo sac (also along the upper surface of the cotyledon)
t testa
* junction of the two cotyledons

Pro-vascular tissue is visible as groups of darkly stained, irregular-shaped cells

bar line 0.1mm


t
c
*
c
t
A


t
s
c
B
*

Hardham, 1979). Such areas are visible in the cotyledons in Plates 1.8A and 1.8B. It does not differentiate fully but remains in the pro-vascular stage until at least the beginning of maturation (Craig et al., 1979).

The developing embryo differentiates into an embryonic axis and two cotyledons (D.C. Cooper, 1938). The cotyledons comprise the bulk of the embryo once cell expansion is underway and early in development cotyledon cells are uniform (Plate 1.8). Embryo growth exhibits a biphasic pattern of growth (Bisson and Jones, 1932; Mckee, Robertson and Lee, 1955; Carr and Skene, 1961) with one or two lag phases (Hedley and Ambrose, 1980) depending on the environment (Bain and Mercer, 1966).

Bain and Mercer (1966) divided embryo development into four phases: cell division, cell expansion, synthesis of storage products, maturation and dormancy. However, these phases overlap, some cell division occurring alongside cell expansion and much of the protein being synthesised during the latter part of expansive growth (Millerd, Spencer and Dudman, 1974).

At the end of the period of rapid cell division the embryo occupies only a small part of the seed, which consists mostly of testa and liquid endosperm (Plate 1.9A and 1.9B). During the cell expansion phase the embryo enlarges to fill the seed cavity and all the endosperm is absorbed (Plate 1.9A and 1.9B). Some storage products begin to appear prior to this stage, as small as 2 mg fresh weight (Millerd and Spencer, 1974; Domoney, Davies and Casey, 1980) but most are laid down later in development (Bain and Mercer, 1966). During this period fat, sugar (Bisson and Jones, 1932; McKee, Nestel and Robertson, 1955), storage protein and starch (Beevers and

PLATE 1.9

Comparison of (A.) seeds and (B.) the embryos contained within them of two lines of Pisum sativum L. near-isogenic except for the r-locus, at different stages of development

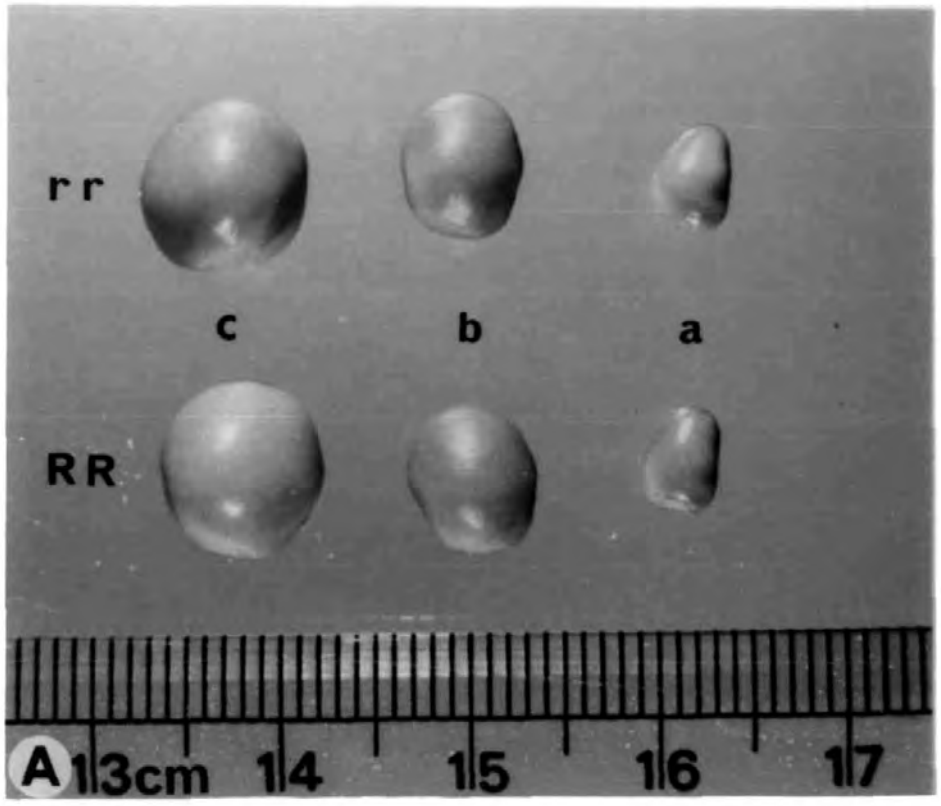
rr wrinkled-seeded near-isoline BC/r genotype (rr)

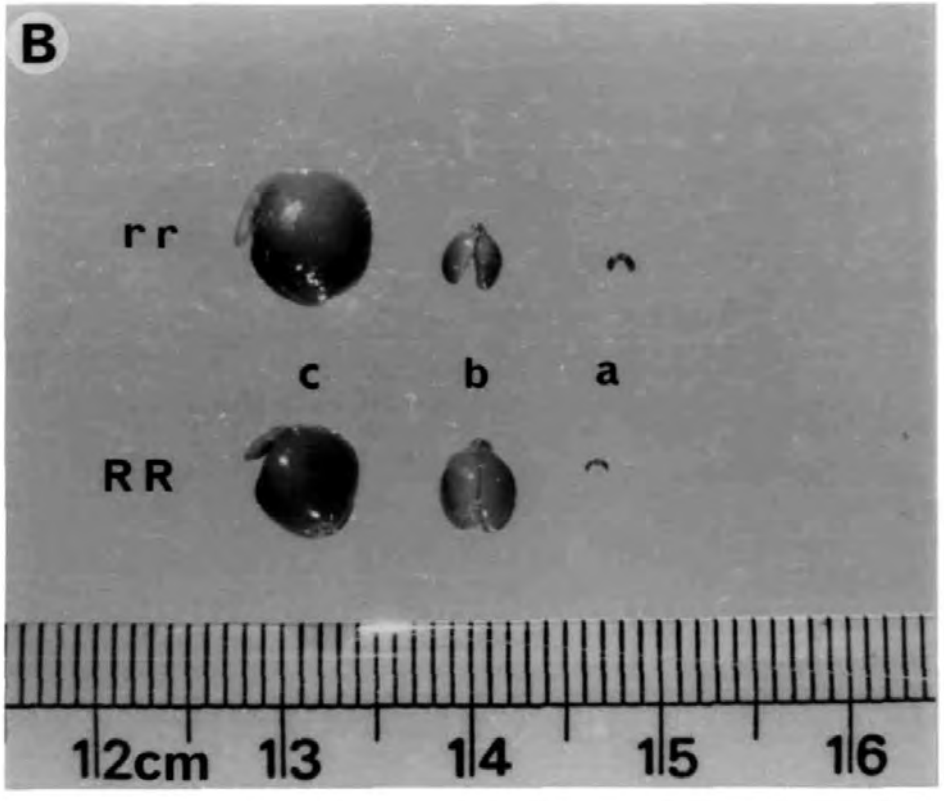
RR round-seeded near-isoline BC/R genotype (RR)

a stage II: testa expanding, embryo cells dividing, resulting in an increase in the endosperm volume

b stage III: testa growth slower, cotyledons expanding, endosperm volume decreasing

c stage IV: endosperm absorbed, cotyledons expanding


rr
c
b
a
RR
A
13cm
14
15
16
17


B
rr
c
b
a
RR
12cm
13
14
15
16

Poulson, 1972; Millerd, Spencer and Dudman, 1974; Raake, 1957a and b), accumulate.

The maturation and dormancy phase is marked by a decline in the rate of increase in fresh weight. Although the embryo dry weight continues to increase, the embryo is losing water (Turner and Turner; 1957; Murray, 1979). Starch and crude fibre continue to increase throughout, while sucrose content increases to a maximum half-way through development and then declines (Bisson and Jones, 1932; Turner and Turner, 1957; Flinn and Pate, 1968). This increase in storage products (and hence dry matter) in the embryo is accompanied by a net loss of dry matter and nitrogen from the pod (Flinn and Pate, 1968; Flinn, Atkins and Pate, 1977). Here, as in most studies of pea fruit development, a limited number of genotypes were used which were all "normal" green-podded commercial varieties. Thus the likely genetic variation in the above and following parameters will be obscured.

During development, considerable changes occur in the ultra-structure of the cotyledons due to the continued synthesis of storage products and transition into dormancy (Bain and Mercer, 1966; Craig, Goodchild and Hardham, 1979; Horowitz, 1983). In particular the plastid membranes degenerate as the starch grains expand.

Estimates of carbon-fixation by different parts of the fruit (pod, testa and embryo) are difficult since by their positions they are all inter-dependent, but several attempts have been made. The green fruit of some trees has been found to supply between 20 % (Quercus macrocarpa) and 65 % (Acer platanoides) of the carbon used by the fruit (Bazzaz, Carlson and Haper, 1979). Lupin seeds have been found to be capable of photosynthesis (Atkins and Flinn, 1978) but seeds of pea apparently are not (Flinn and Pate, 1968).

Any discrete analysis of, for example, embryo metabolism would necessitate removing it from its natural environment and would be unlikely to give true, in vivo, results. An alternative method would be to examine the structure and ultra-structure of plastids and other cellular components in these tissues. If this is considered in relation to the photoenvironment in which the plastids operate, their potential for carbon-fixation may be implied. An important facet of this approach is to make use of the genetic variation for fruit structure which exists for Pisum. One such genetic variant is the yellow pod (gp gene) described earlier. The colour of the cotyledons is not affected by this gene and remains green throughout development and in the mature dry seed, if the recessive i gene is present (Blixt, 1972).

Mature dry seed from plants having the rr genotype have a characteristic wrinkled appearance (Mendel, 1865; White, 1917). The structure of the starch grains of the wild-type RR (round-seeded) phenotype appears simple, while that of the mutant rr (wrinkled-seeded) phenotype is complex (Kooistra, 1962). This may be related to observed differences in the proportions of amylose and amylopectin within the starch, discussed in Section 2.

This chapter reports on the variation in plastid structure and distribution in the different fruit tissues during development. Other physiological parameters; chlorophyll content, transmission of radiation through pods, stomatal density and thickness of pod walls are also reported in an attempt to relate these characters to the potential of pea fruits to photosynthesise. The two GpGp (green-podded) RR (round-seeded) and rr (wrinkled-seeded) genotypes are compared with one another and with the gpgp (yellow-podded) type to determine the effects of the r and gp genes on these characters.

RESULTS

A. DESCRIPTION OF MATERIAL

General features of the 3 genotypes used are described in Table 1.1, below.

All of the vegetative parameters measured, e.g. height, stipule and leaf morphology, colour, were identical in the two near-isogenic BC lines. This gives confidence in the assumption that these two lines are isogenic for all but the r locus. They differed in only a few reproductive characters measured. Mature dried seed weight was less in the wrinkled genotype, which also exhibited the classic wrinkled seed morphology (Plate 1.10).

Despite chosing the yellow-podded genotype (JI 13) for its similarity to the above (BC) genotypes, this line shows some differences in both vegetative and reproductive features. In addition to bearing yellow pods characteristic of the gpgp genotype, these are smaller and contain fewer ovules with mean mature dried seed weight of less than that of either GpGp genotypes, although their shape is round (Plate 1.10). Mature dried seed shape of JI 13 appears slightly dented, but this is due to the di (dimple) gene, not to the r gene.

TABLE 1.1

General plant morphology of the 3 genotypes of Pisum sativum L. used in the structural survey of fruit morphology (including the two genotypes used in the growth analysis, Section 2)

Accession No. / Name:	BC/R	BC/r	JI 13
Genotype	RR GpGp	rr GpGp	RR gpgp
Height to apex	1-1.5m	1-1.5m	1-1.5m
Leaves	green	green	green
Stipules	green	green	green
Tendrils	green	green	absent
Flowers	white, in pairs	white, in pairs	white, in pairs
Pod			
colour	green[x]	green	yellow[+]
length	10-11cm	10-11cm	6cm
Ovule no./ pod	11	11	7
Immature cotyledons	green	green	green
Mature dried seed			
colour	pale green	pale green	green/brown
shape	round*	wrinkled*	round*
mean weight	350mg	300mg	220mg

x Plate 1.2A

+ Plate 1.4

* Plate 1.10

PLATE 1.10

External morphology of mature dried seed of the 3 genotypes of Pisum sativum L. used in the structural survey

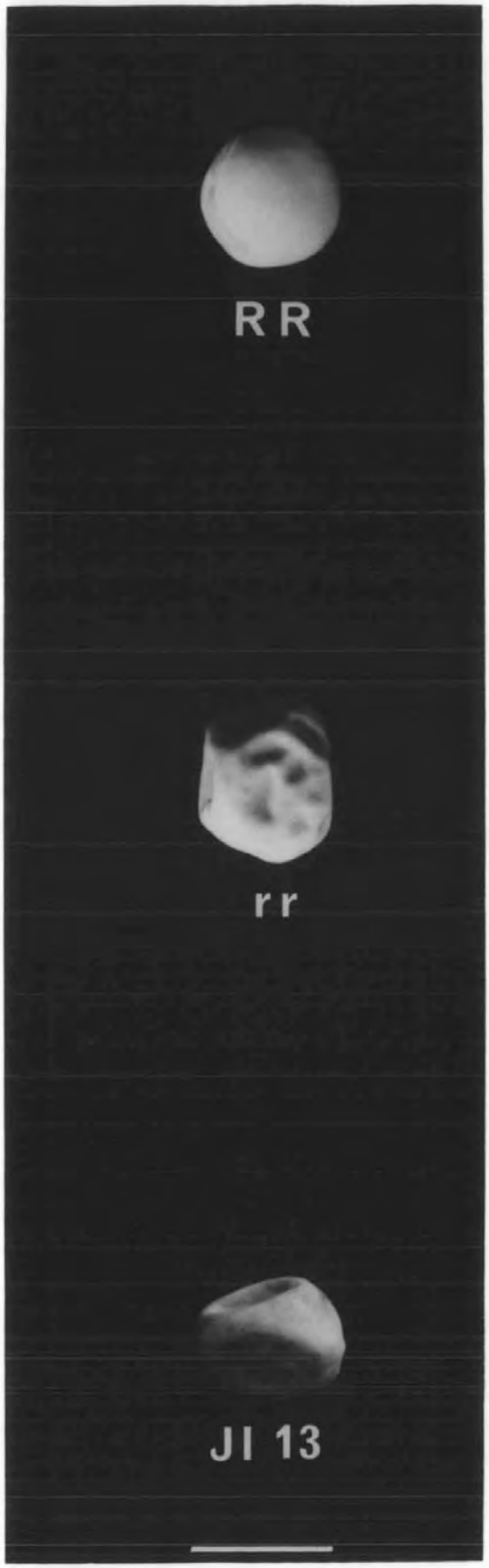
RR: round-seeded, green-podded BC/R genotype (RR, GpGp)

rr: wrinkled-seeded, green-podded BC/r genotype (rr, GpGp)

JI 13: round-seeded, yellow-podded JI 13 genotype (RR, gpgp)

seeds were side-illuminated to highlight their surface topography

bar line 8 mm


RR
rr
JI 13

B. GROWTH STAGES

From observations of BC/R, BC/r and JI 13, several distinct phases of fruit development could be identified. These are summarised in Table 1.2 below.

Due to the effects of environmental fluctuations on the development of plants, it was decided to use "stage" rather than "day post-anthesis" as a measure of fruit development. It is apparent that the pod grows in length and inflates (stages I and II) before there is a significant increase in seed size. The space resulting from the ensuing testa growth is filled with endosperm (stage II and III) which is subsequently absorbed by the rapidly growing embryo and absent by stage IV. Pod and seed continue to develop and mature in tandem although the seed accumulates dry matter even in the final stages of pod senescence (stage V).

This qualitative classification correlates closely with the growth analysis data presented in Section 2 (Figs. 2.4 and 2.5). While recognising that these observations are based on the lines used in this thesis and are thus partly subjective, they may generally be applied to other genotypes.

These identified stages were used in the subsequent investigations reported in this section and this nomenclature is used throughout.

TABLE 1.2

Classification of developmental stages of fruits of Pisum sativum L. used in the structural survey; BC/R genotype (RR, GpGp) and BC/r genotype (rr, GpGp)

Stage	Pod appearance	Pod space	Testa volume	Endosperm	Embryo state
+I	flat, expanding	absent	very small, cells dividing	absent	very small, most cells dividing
+II*	inflated	present	expanding	present, increasing	very small, all cells dividing
III*	inflated max. fresh weight	decreasing	expanding to contact-point	decreasing	cotyledon cells expanding
+IV*	early senescence	absent	max. fresh weight	absent	cotyledons cells expanding
V	senescence	absent	constant, at maximum	absent	dry weight accumulating

* seeds of these 3 stages are illustrated in Plates 1.9 A and B.

+ pods of these 3 stages are illustrated in Plate 2.3 A and B

C. ULTRASTRUCTURE

i) Pods

No evidence for differential expression of the r gene was found at the ultrastructural level in pods, no differences being observed between the BC/R and BC/r lines. They will therefore be described as a single green-podded genotype (GpGp) and compared with pods of the gpgp genotype.

This survey concentrates on particular tissues known to contain plastids, these being of most interest as discussed earlier. However, other features of interest are described as and when they occur.

All the pods described are at stage II/III unless otherwise stated.

a. Mesocarp

Chloroplasts were abundant throughout this layer in green pods (Plate 1.11) and contained limited amounts of starch. Internal membranes of these chloroplasts were extensive (Plate 1.18) and were organised into granal stacks, although these were not as well-developed as those of pea leaf mesophyll (Plate 1.17B).

Mitochondria, rough endoplasmic reticulum (R.E.R.) and ribosomes were also abundant (Plate 1.11). Transfer cells were present associated with vascular elements and plasmodesmata were evident within cell walls (Plate 1.11).

Amyloplasts were numerous, particularly in cells adjoining vascular tissue (Plate 1.12). These contained large starch grains and limited but well-defined internal membranes. The vascular tissue (Plate 1.12) has xylem elements at different developmental stages.

PLATE 1.11

Electron micrograph of a transversesection through the carpel wall of the normal green-podded, round-seeded BC/R genotype (GpGp, RR) of Pisum sativum L., at stage III in development

a amyloplast
c chloroplast
m mitochondria
n nucleus
p ← plasmodesmata
s starch grains
t transfer cell / phloem

bar line (top left-hand corner) 10 µm

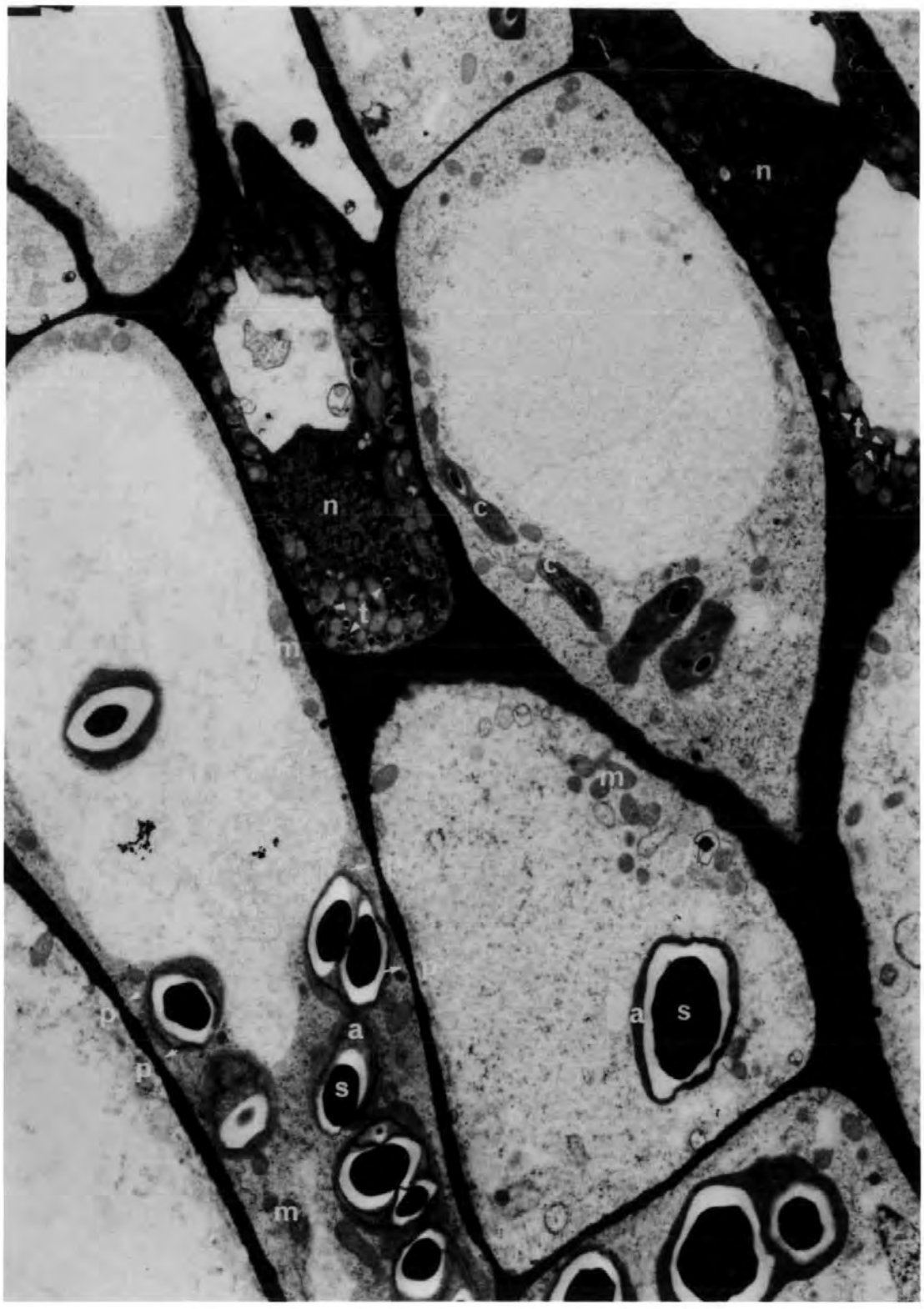
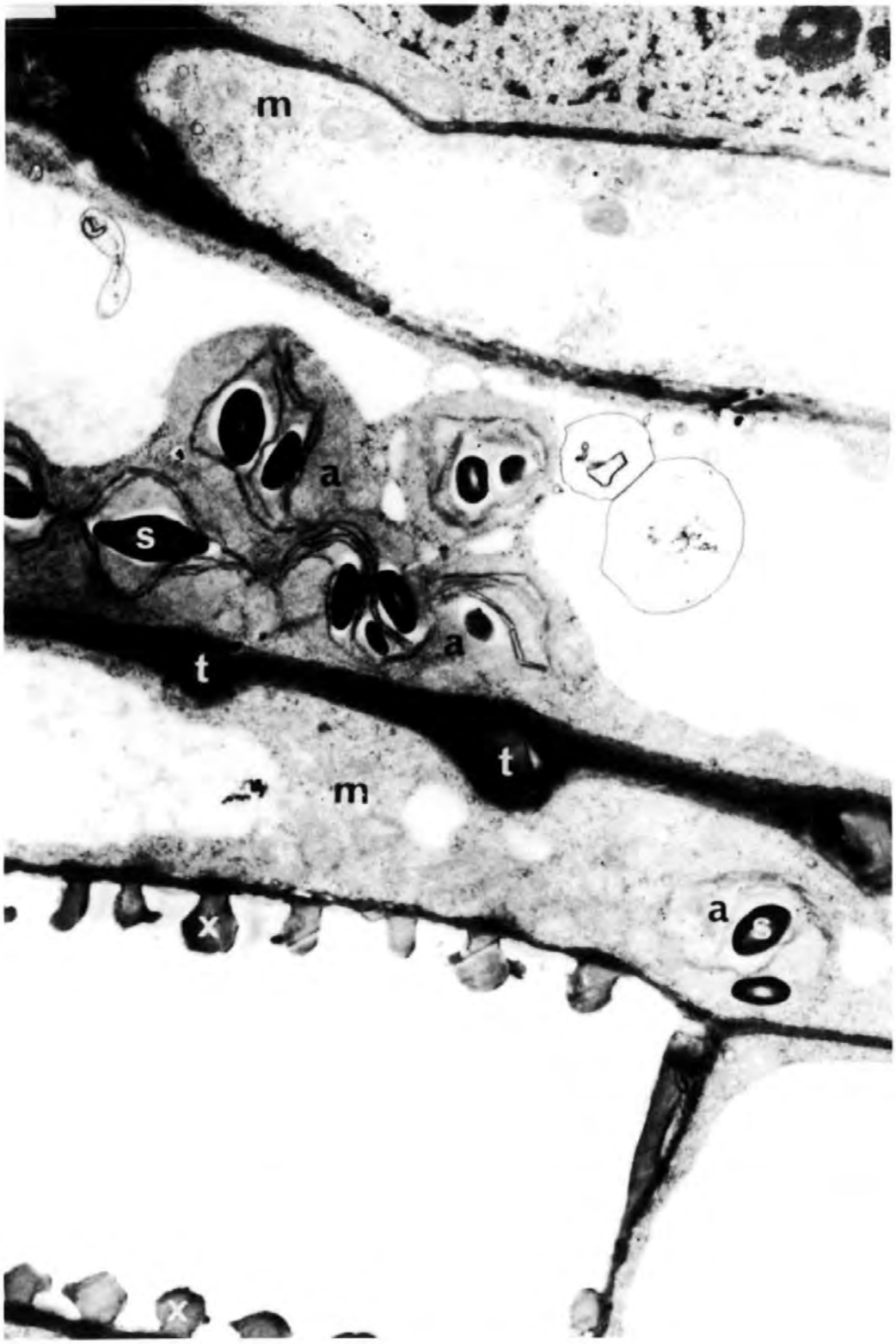

n
n
c
c
t
m
m
p
p
a
s
a
s
m

PLATE 1.12

Electron micrograph of a transverse section through the carpel wall of the normal green-podded, round-seeded BC/R genotype (GpGp, RR) of Pisum sativum L. at stage III in development

a amyloplast
m mitochondria
s starch grain
t thickening of cell wall adjacent to xylem element (x)

bar line (top left-hand corner) 10 μm


m
a
s
a
t
t
m
x
a
x

Little variation in plastid structure was found during development except just prior to senescence, when more starch appeared to be present and the internal membranes were degenerating.

In comparison plastids of the yellow pod mesocarp had internal membranes which were very dilated, with only limited granal stack formation, but which contained some starch (Plates 1.13 A, B and C). Some of these plastids nearer the outer epidermis contained randomly distributed thylakoid membranes which lacked obvious orientation (Plates 1.13 C and 1.14). Around their boundary these irregular plastids contained vesicles (peripheral reticulum). No such vesicles were found in the plastids of green pods.

In the stages examined, few differences were observed in the plastid membrane structure of yellow pods. However, lipid droplets became abundant in plastids later in development (stage III/IV) and this correlated with some membrane disintegration (Plate 1.13 C and 1.14).

Apart from plastids, other organelles such as mitochondria and R.E.R. were present throughout the tissue but did not appear to be structurally different from those of the green pod (Plates 1.12 and 1.13).

b. Endocarp

In green pods, chloroplasts were numerous around the periphery of the inner epidermal cells (Plate 1.15) at all developmental stages examined and a few were apparent in the sclerenchyma (Plate 1.15). No amyloplasts were present, though a few chloroplasts contained small amounts of starch (Plate 1.16).

Chloroplasts in pods prior to the pod-inflation phase (stage

PLATE 1.13

Electron micrographs of transverse sections of characteristic plastids from the mesocarp of fruit of the yellow-podded, round-seeded JI 13 genotype (gpgp, RR) of Pisum sativum L. at 3 different developmental stages

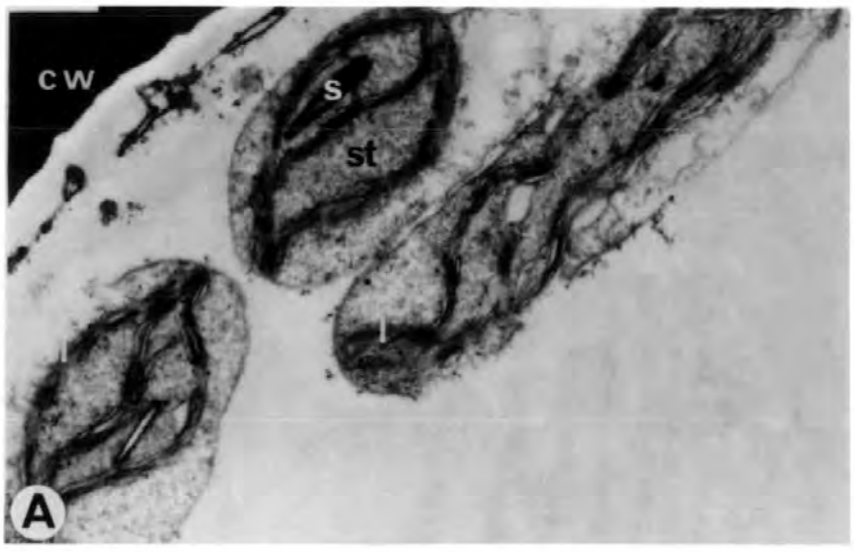
A. Stage II

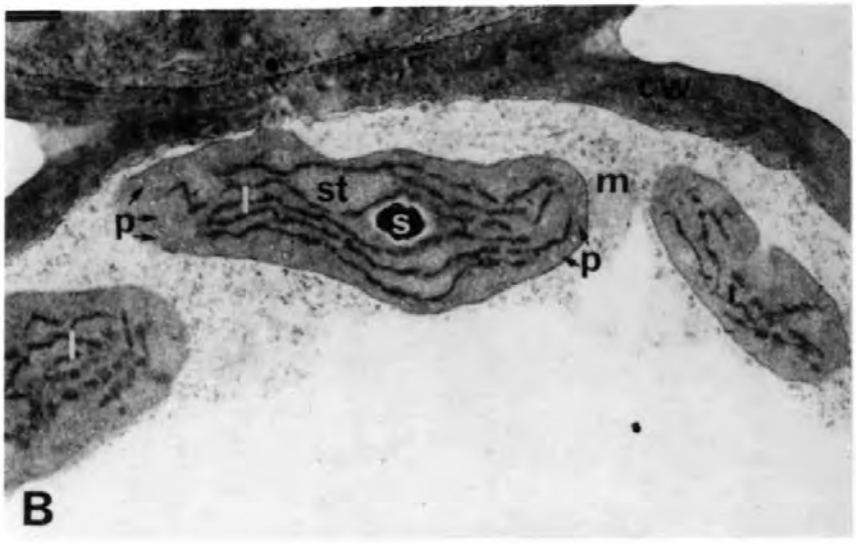
B. Stage III

C. Stage IV

cw cell wall
| lamellae
m mitochondria
p peripheral reticulum
s starch grain
st stroma

bar line 1 μm


cw
s
st
l
A


cw
m
st
s
p
p
l
l
B

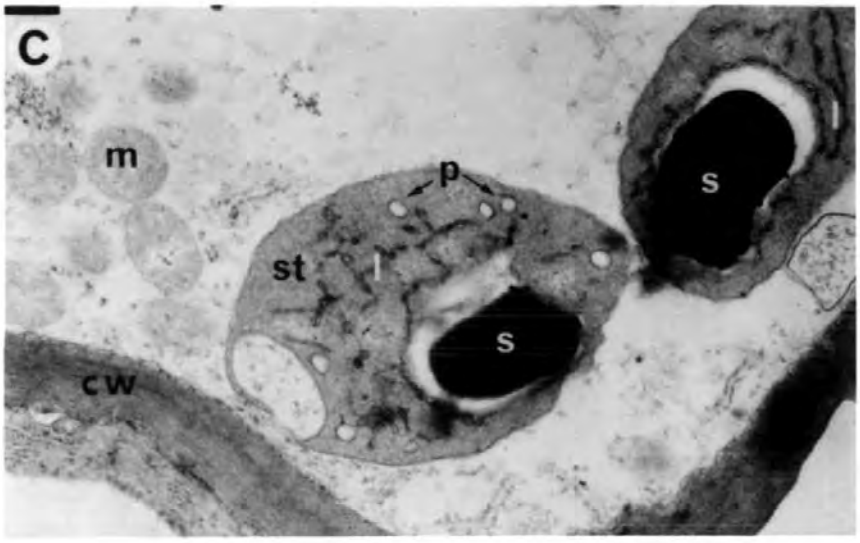

C
m
p
s
st
l
s
cw

PLATE 1.14

Electron micrograph of a transverse section of a plastid characteristic of the outer mesocarp of fruit of the yellow-podded, round-seeded JI 13 genotype (gpgp, RR) of Pisum sativum L. at stage III

→	outer plastid membrane
|	lamellae
o	lipid (osmiophilic) droplets
st	stroma

bar line 0.5 µm

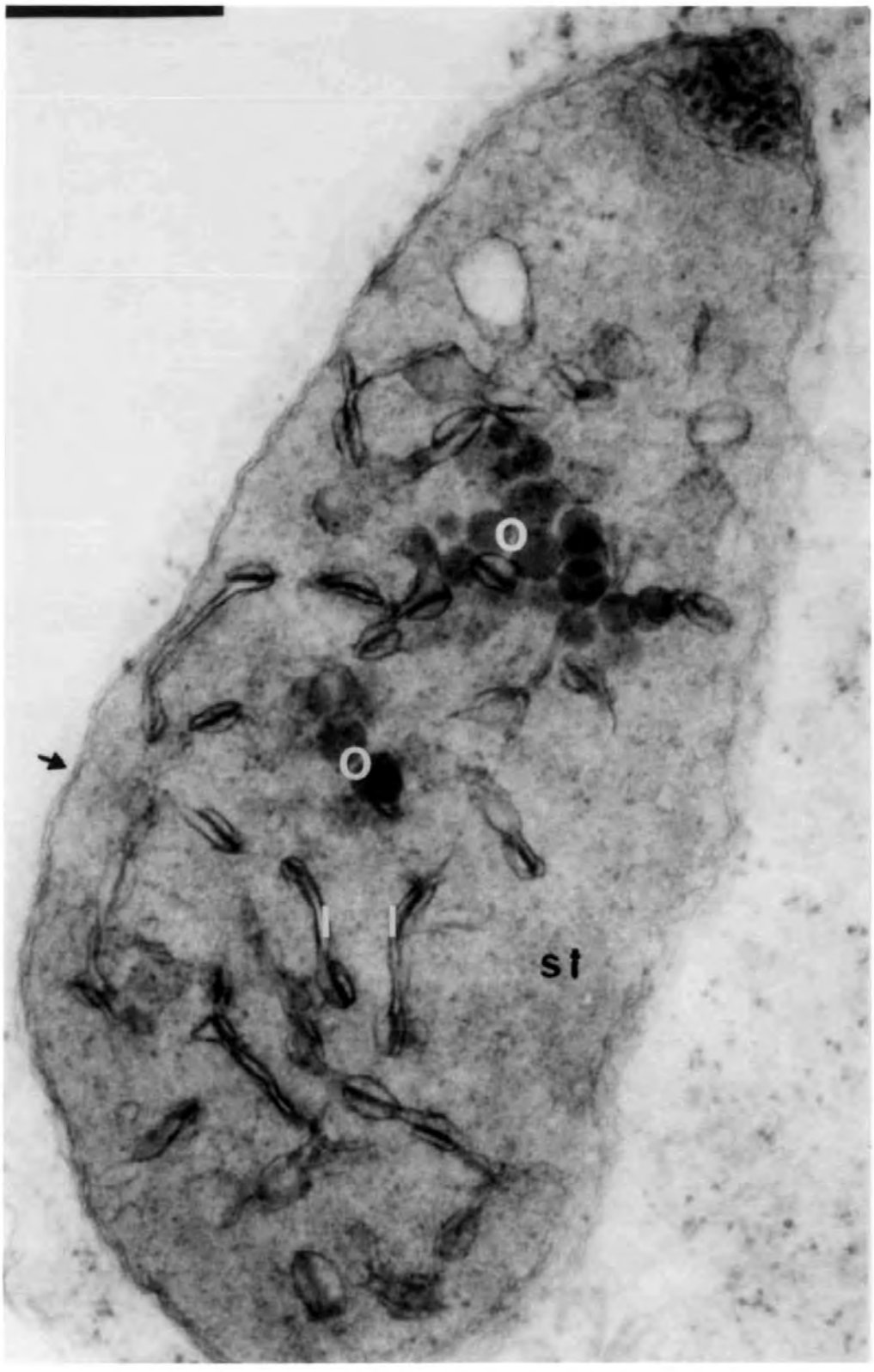

O
O
st

PLATE 1.15

Electron micrograph of a transverse section through the inner cells of the carpel wall of fruit of the normal green-podded, round-seeded BC/R genotype (GpGp, RR) of Pisum sativum L. at stage I in development

c chloroplast
i inner epidermis
n nucleus
↔ parchment / sclerenchyma layer

bar line 5 μm

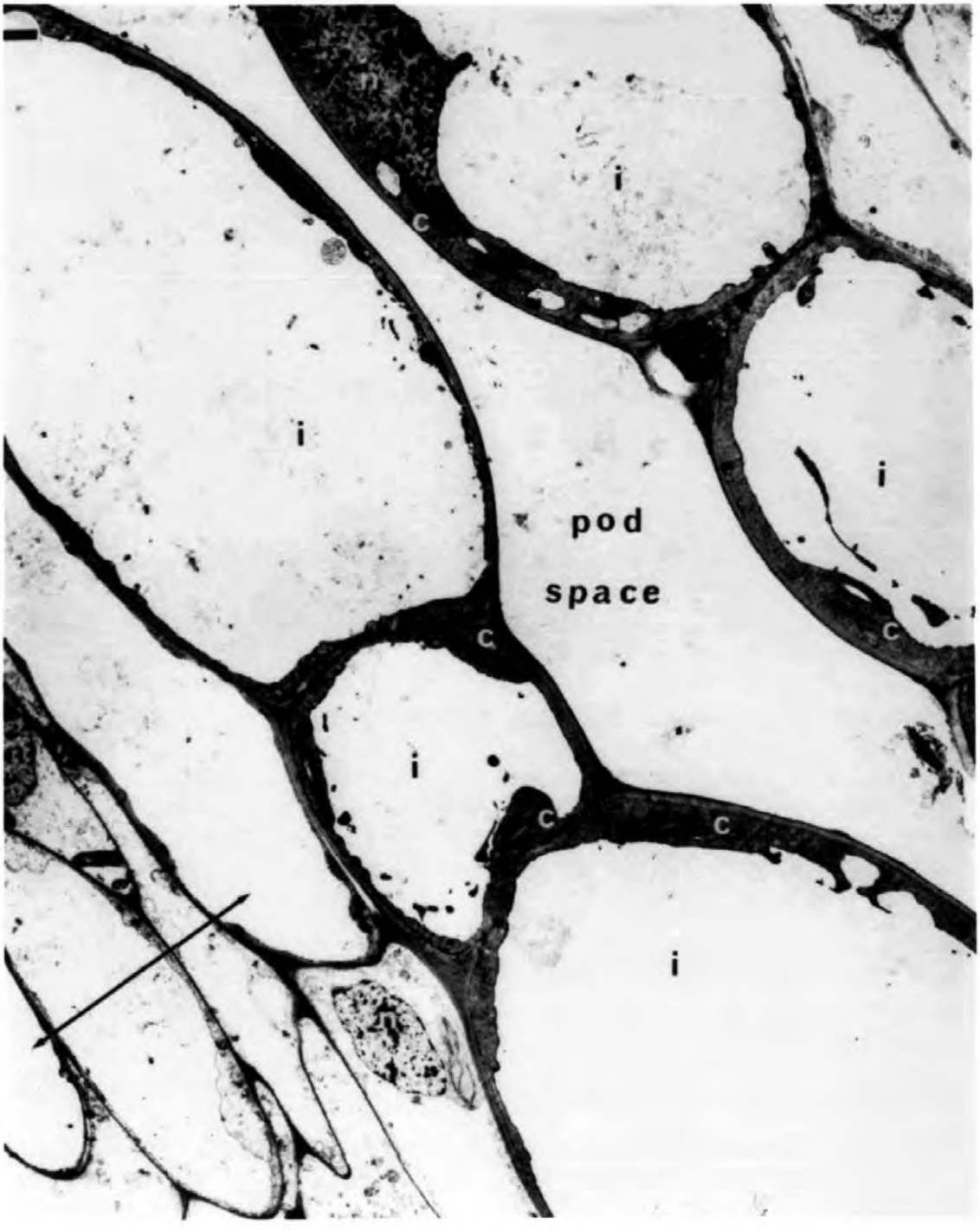
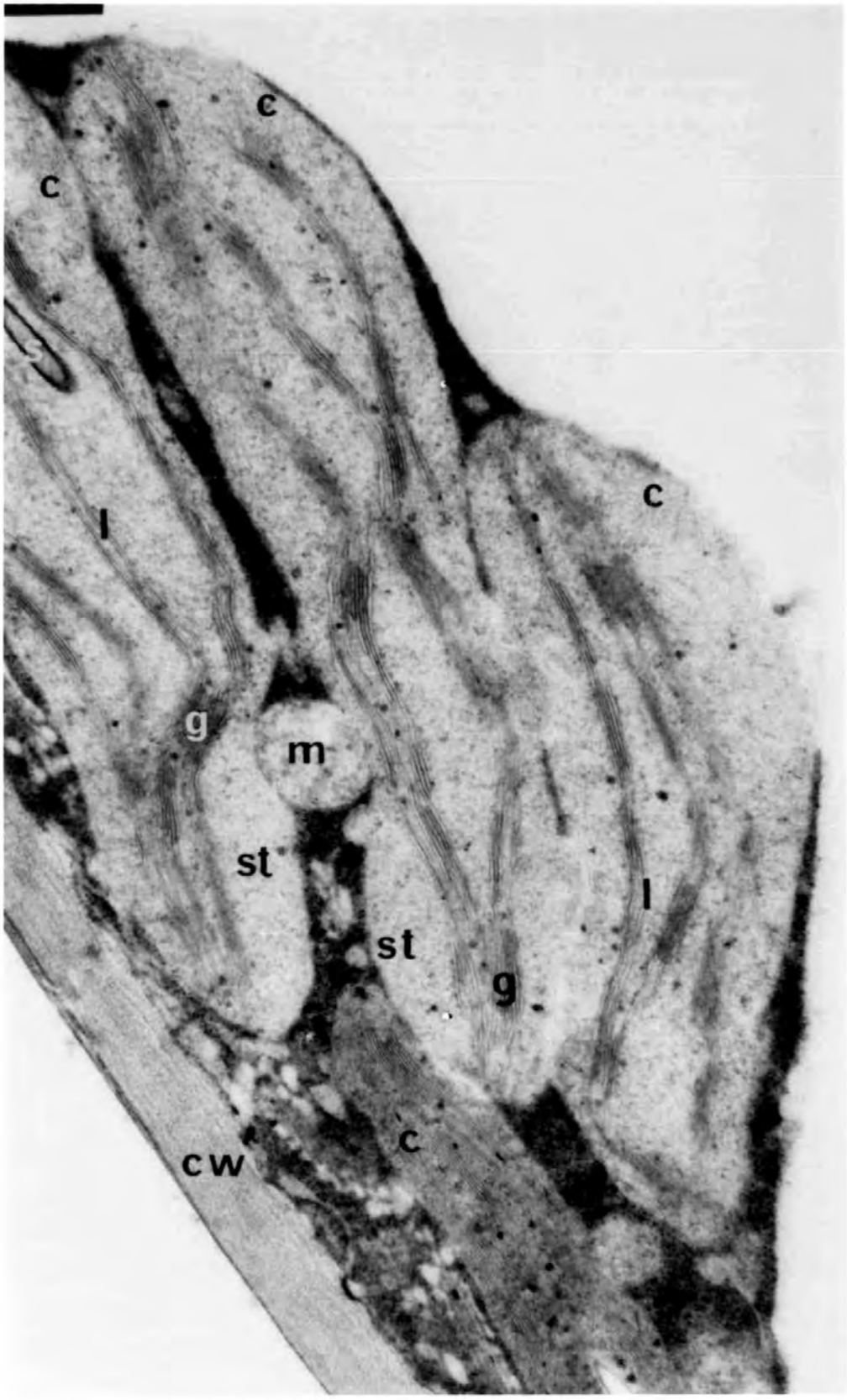

i
c
i
i
pod
space
c
c
i
i
c
c
i

PLATE 1.16

Electron micrograph of a transverse section through the inner epidermal cells of the carpel wall of the normal green-podded, round-seeded BC/R genotype (GpGp, RR) of Pisum sativum L. at stage II in development

c chloroplast
cw cell wall
g granal stack
| lamellae
m mitochondria
s starch grain
st stroma

bar line 1 μm


c
c
c
l
g
m
st
st
g
l
cw
c

I/II) had very well-ordered internal membranes, with distinct granal stacks (Plate 1.16). These granal stacks were larger in slightly older inflated pods at stages II/III (Plate 1.17A). Chloroplasts in the inner epidermis were very similar to those of leaves (Plate 1.17B), having well-structured internal membranes and granal stacks, but were more elongated.

Mitochondria were particularly abundant in the inner epidermal cells around the pod space (Plate 1.14). Plasmodesmata and R.E.R. were also present. In the stages examined, the other tissue of the endocarp, the parchment, was still developing as plastids and mitochondria were evident here.

At all stages examined, many chloroplasts in yellow pods were situated around the periphery of these cells (Plate 1.18B) and a few were present in the sclerenchyma. These chloroplasts contained well-structured thylakoid membranes (Plate 1.18B), particularly at older stages (Plate 1.18A), but little or no starch. They were quite unlike the chloroplasts of the gpgp mesocarp (Plate 1.14), but were very similar to those of GpGp mesocarp and leaves (Plates 1.17A and B). Unlike green pods, chloroplasts of the inner epidermis in the yellow pods contained lipid droplets just prior to senescence (stages III/IV).

Mitochondria and R.E.R. were present at levels comparable to those found in the green pod endocarp.

ii) Testas

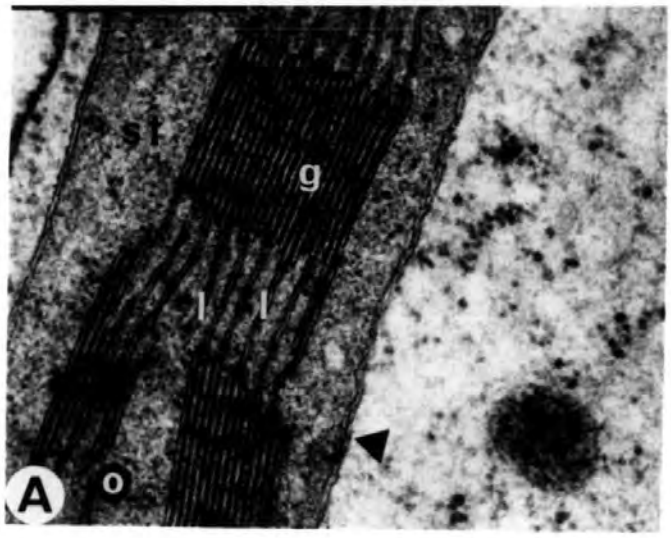
Plastids were observed in the testas of all genotypes examined. However, no structural differences were observed between plastids of round (RR) and wrinkled (rr) types. These will therefore be

PLATE 1.17

Comparison of electron micrographs of chloroplasts from the inner epidermis of the pod wall (A) and from the mesophyll of its subtending leaf (B) of the green-podded, round-seeded BC/R genotype (GpGp, RR) of Pisum sativum L. at stage III in development

◀ chloroplast membrane
g granal stack
| lamellae
o lipid (osmiophilic) droplet
st stroma

bar line 0.5μm


st
g
l
l
A
o

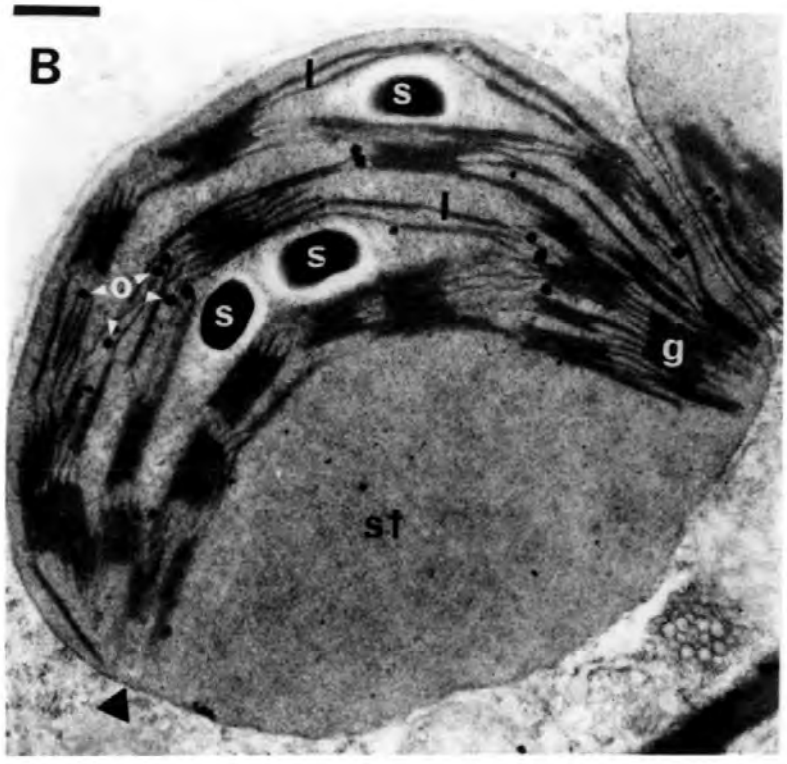

B
l
s
l
s
o
s
g
st

PLATE 1.18

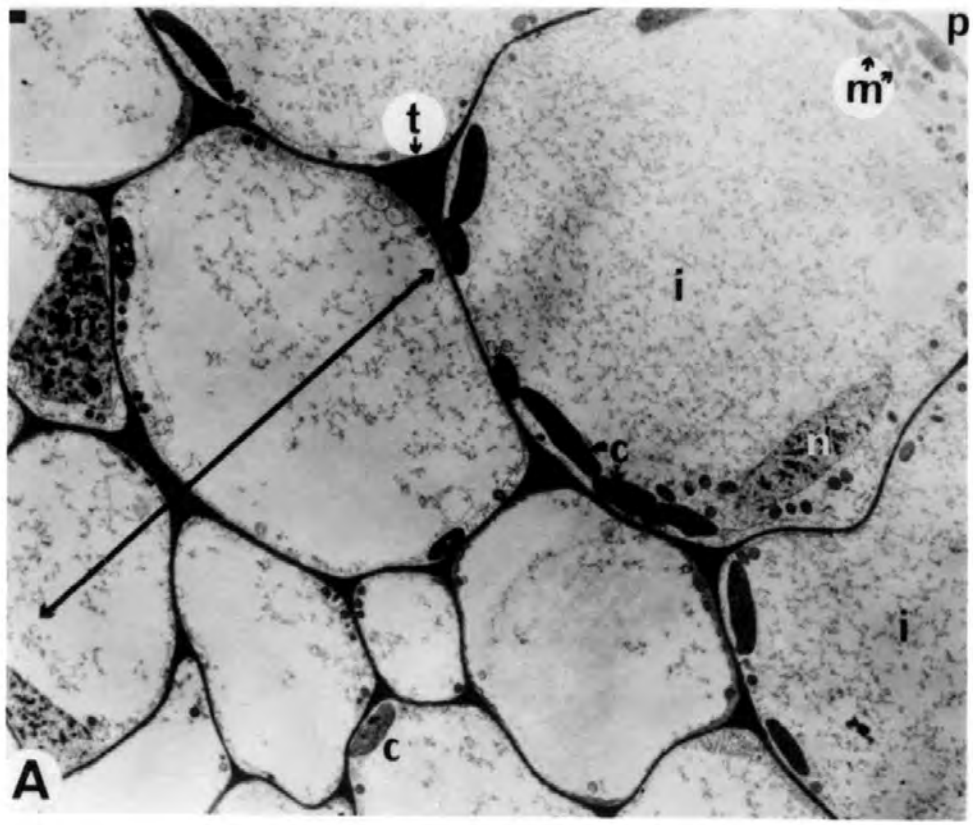
Comparison of electron micrographs of the inner cells from the pod wall of 2 genotypes of Pisum sativum L. at a single stage (III) in development

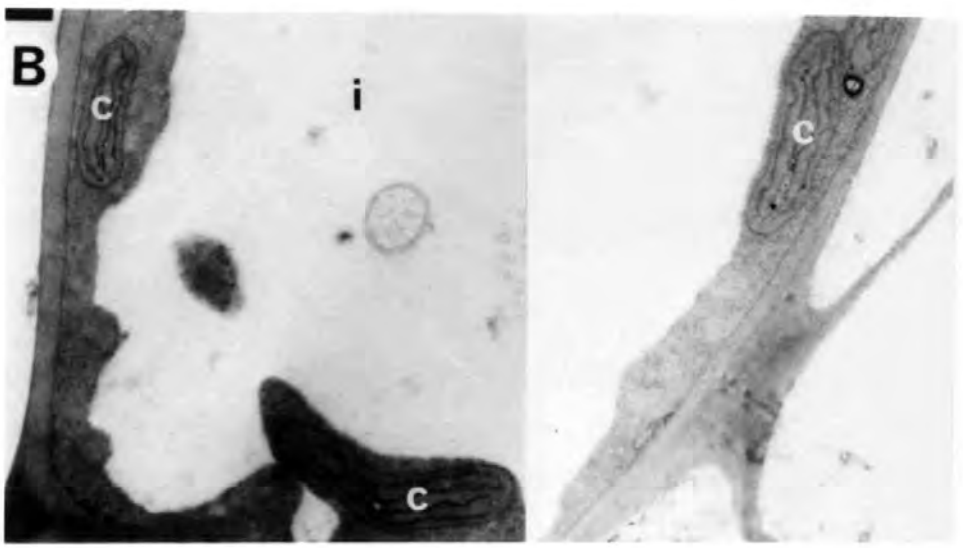
A. green-podded, round-seeded BC/R genotype (GpGp, RR)

B. yellow-podded, round-seeded JI 13 genotype (gpgp, RR)

c	chloroplast
i	inner epidermis
m	mitochondria
n	nucleus
p	pod space
t	thickening
↔	parchment / sclerenchyma layer

bar line 1 μm


p
m
t
i
c
n
i
c
A


B
c
i
c
c

described as a single GpGp (green-podded) genotype and compared with the gpgp (yellow-podded) genotype. After contact point, the secondary thickening in this tissue was such that it prevented sections being taken. Thus only the early stages, II and III, prior to and immediately after minimum endosperm were examined and will be described here.

a. Macrosclereids

Plastids had limited internal membranes but contained starch (Plates 1.19 A and B). The thylakoid membranes were organised into small granal stacks of only 4-5 partitions, quite unlike those of pods and leaves which had granal stacks of up to 25 partitions (Plate 1.17 A and B). Plastids in this layer did not increase in size during development, unlike those of the pod inner epidermis. Mitochondria were present, as were ribosomes and rough endoplasmic reticulum (Plates 1.19 and 1.20).

Although the plastids of RR and rr lines were similar, a structural difference was observed in formation of secondary thickening. Wall ingrowths were present in the macrosclereid cells of the round testa but no such growths were apparent in the similarly-aged wrinkled testa (Plate 1.21). They were found by phloroglucinol tests to be lignified, as was the rest of the secondary thickening. The walls of these cells in both genotypes became more thickened during the growth stages examined, particularly at the 'corners' of the cells (Plate 1.20A). However, there was always a small area of the outer (Plate 1.20 B) and inner (Plate 1.20C) cell surface adjacent to the pod space and osteosclereid cells respectively, which had no secondary thickening.

PLATE 1.19

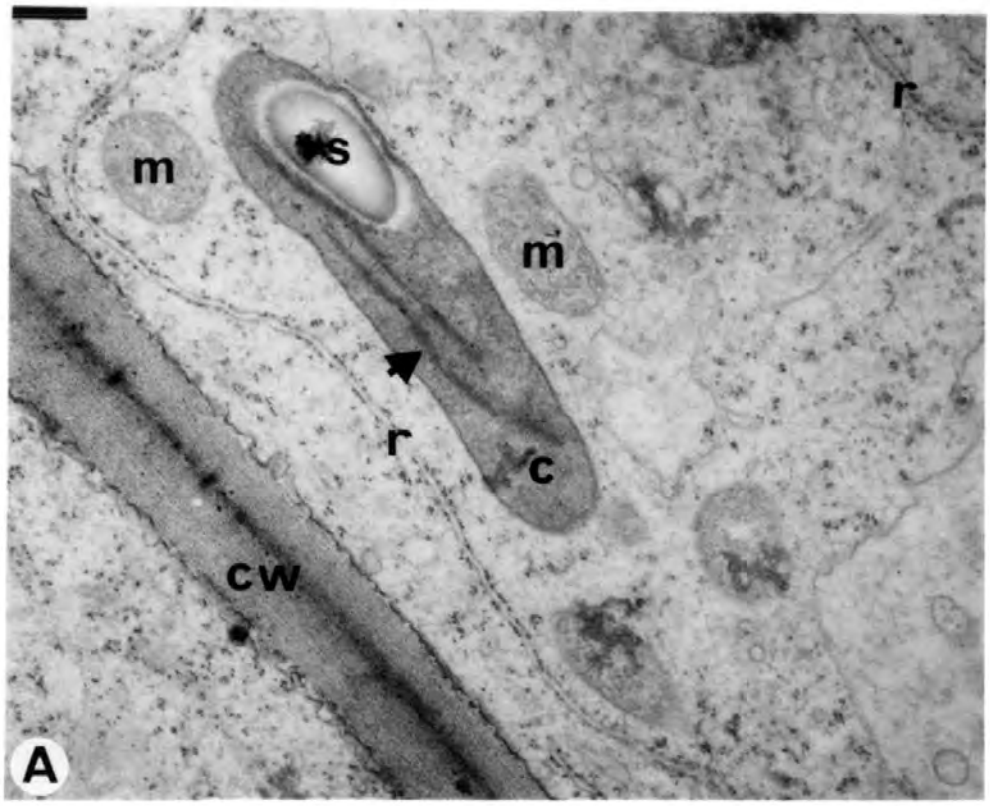
Comparison of electron micrographs of macrosclereids from testas of fruit from the green-podded, wrinkled-seeded BC/r genotype (GpGp, rr) of Pisum sativum L. at two stages of development

A. Stage II

B. Stage III

c chloroplasts
cw cell wall
m mitochondria
r rough endoplasmic reticulum, R.E.R.
s starch grains

bar line 1 μm


m
s
r
m
r
c
cw
A

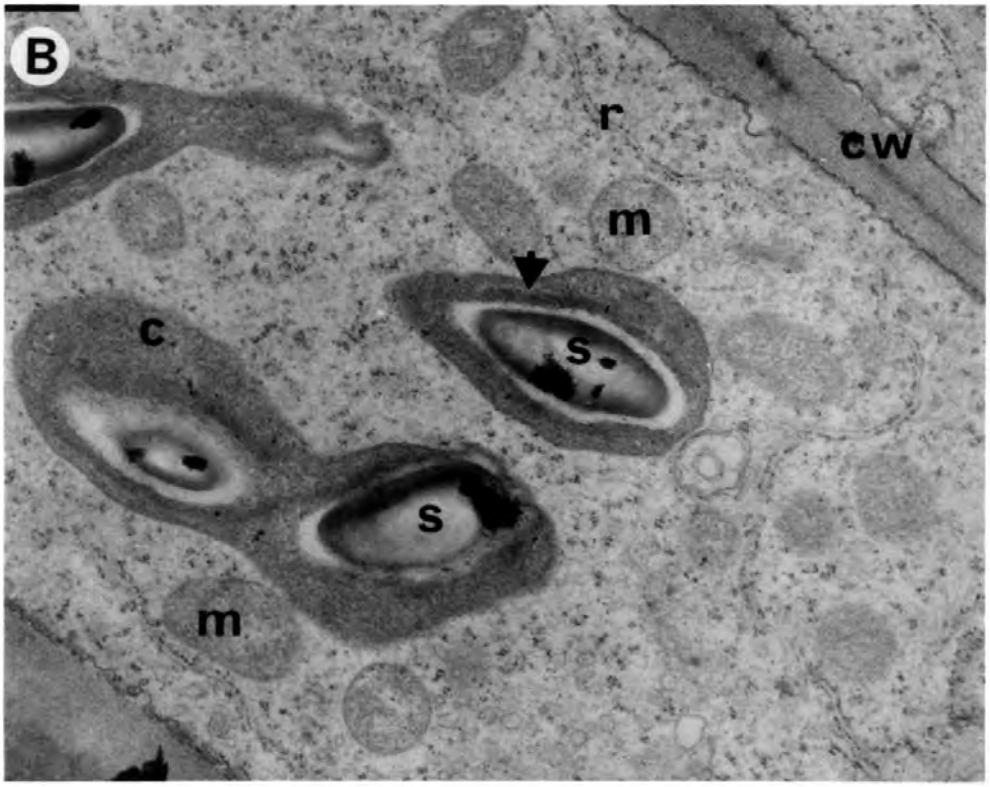

B
r
cw
m
c
s
s
m

PLATE 1.20

Electron micrographs of macrosclereids of testas from the green-podded, round-seeded BC/R genotype (GpGp, RR) of Pisum sativum L. at stage II in development

A. Outer portion

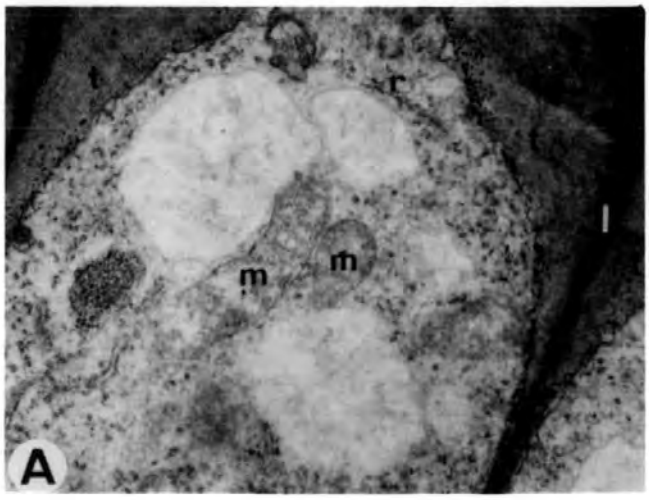
t thickening
| middle lamellum of cell wall
m mitochondria
r rough endoplasmic reticulum (R.E.R)

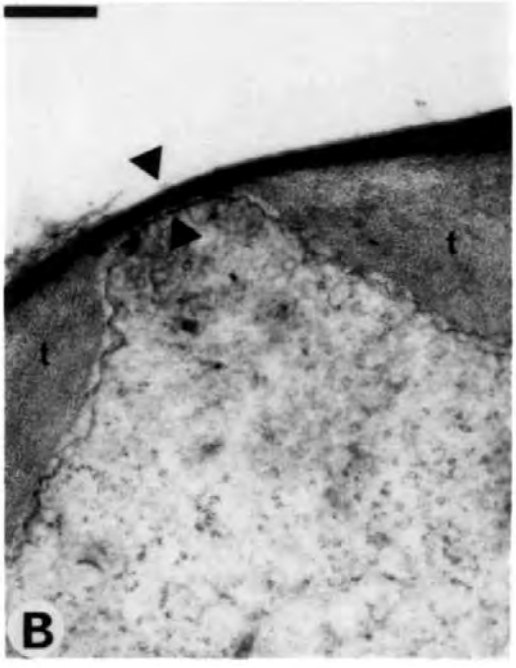
B. Outer portion, showing cuticle and absence of thickening at cell apex between arrows

C. Junction of macrosclereid cell with osteo -sclereid cell showing absence of thickening at cell apex between arrows

t thickening
m mitochondria
r R.E.R.

bar line 0.5 μm


t
r
l
m
m
A


t
t
B

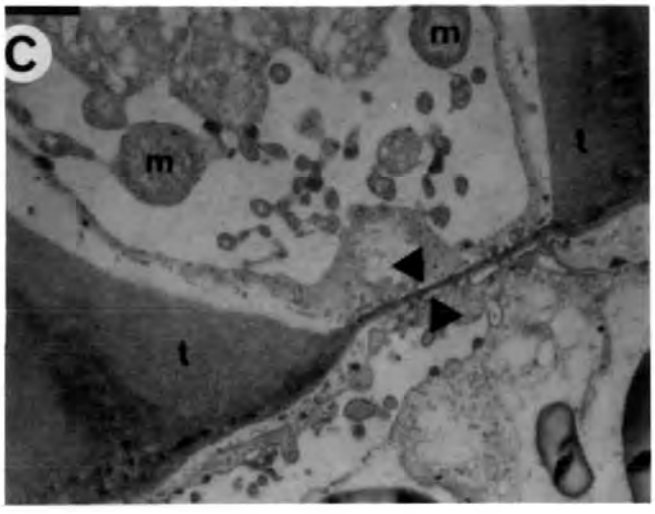

C
m
m
t
t

PLATE 1.21

Electron micrographs of transverse sections through macrosclereid cells from testas of the green-podded, round-seeded BC/R genotype (GpGp, RR) of Pisum sativum L. at 2 stages of development to show secondary thickening

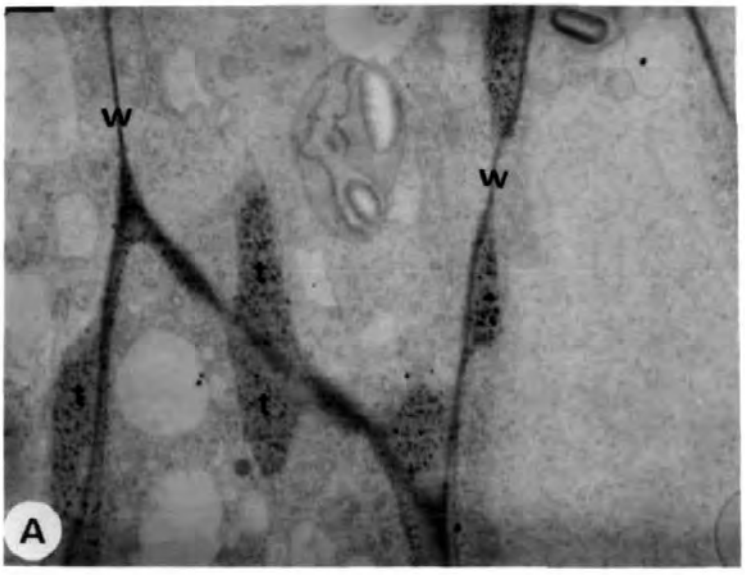
A. Stage II

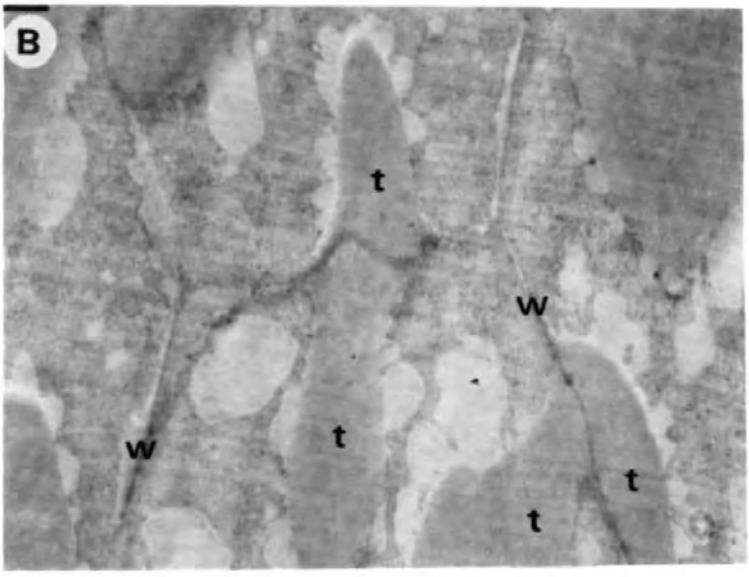
B. Stage III

t thickening

w cell wall

bar line 1 μm


w
w
t
t
t
A


B
t
w
w
t
t
t
t

There were no apparent differences in this layer between the testas of the gpgp and GpGp types in terms of plastid structure; they too had limited internal membrane structure and contained starch.

Mitochondria and R.E.R. were also present in this tissue. No wall ingrowths were observed in this layer of the gpgp RR genotype and this corresponded with the GpGp RR genotype which also lacked such structures.

b. Osteo-sclereids

Chloroplasts in this layer of the GpGp lines were well-developed, having many thylakoids built up into substantial granal stacks of up to 20 partitions (Plates 1.22). These chloroplasts resembled those of leaves (Plate 1.17 A and B respectively). During development, between contact point and just after contact point, the chloroplasts developed larger granal stacks. Although the number of single thylakoids and number of granal stacks per chloroplast was not significantly different at the two stages (II and III; Table 1.3), the number of partitions per granum had increased by 20% (Table 1.3). Similar analysis was not carried out for all tissue types due to the difficulty in obtaining micrographs of sufficient resolution of all the granal stacks in a chloroplast section.

By the onset of senescence the plastids of this layer were still highly structured and organised (Plate 1.23), unlike those of the other layers which had virtually disintegrated. Chloroplasts at this stage however contained lipid droplets and peripheral reticulum (Plate 1.23 B). Some of the chloroplasts at this stage had unusual 'diagonally-sloping' grana (Plate 1.23 C), which were not observed elsewhere.

PLATE 1.22

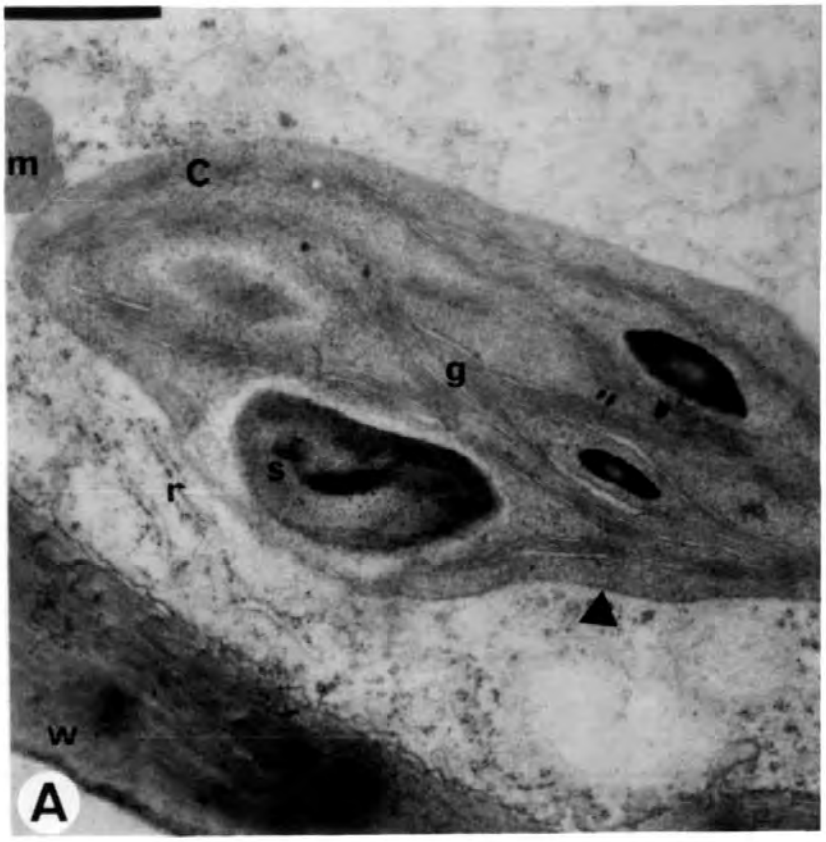
Comparison of electron micrographs of chloroplasts from osteo-sclereid cells of testas of the green-podded, wrinkled-seeded BC/r genotype (GpGp rr) of Pisum sativum L. at 2 stages of development

A. Stage II

B. Stage III

c chloroplast with granal stacks
g granal stacks
s starch grains
▲ outer chloroplast membrane
r rough endopasmic reticulum
m mitochondria
w cell wall

bar line 1 μm


m
C
g
s
r
w
A

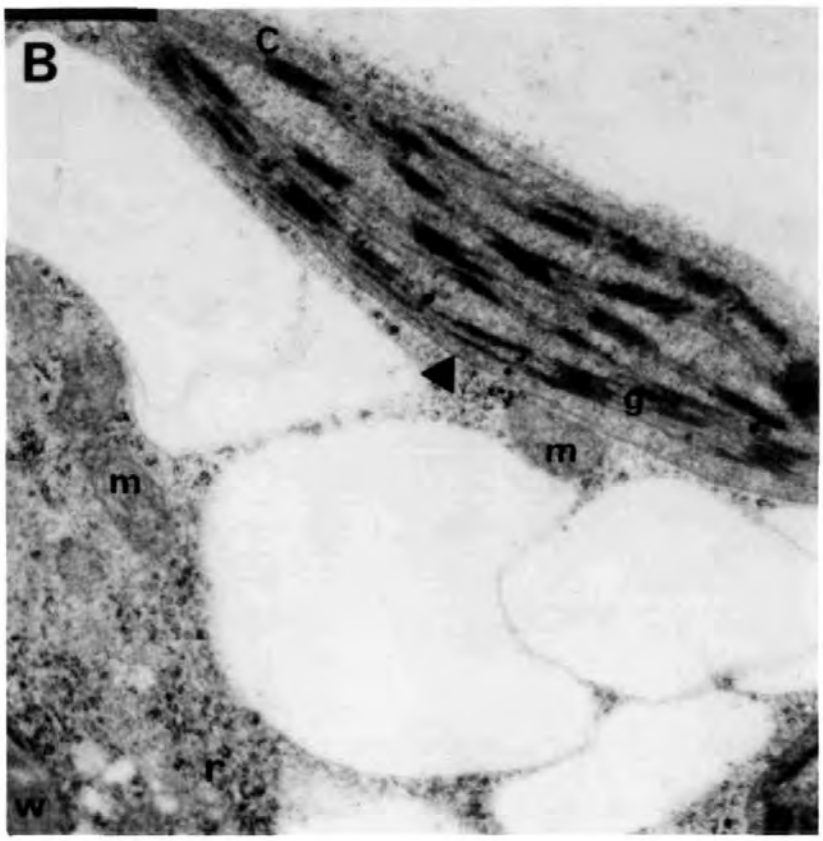

B
C
g
m
m
r
w

TABLE 1.3

Comparison of the partitioning of thylakoid membranes in chloroplasts of osteo-sclereids of Pisum sativum L., near-isogenic except at the r-locus, at two stages of development

Stage	Mean* no. single lamellae	Mean* no. granal stacks per chloroplast	Mean* no. lamellae per granum
II	11.7±4.0	17.7±2.8	11.1±1.8
III	11.2±1.0	20.0±2.5	16.5±4.0

* Each value is the mean of 10 chloroplasts ± its standard deviation from the mean

PLATE 1.23

Comparison of electron micrographs of plastids from osteo-sclereid cells of testas of the green-podded, round-seeded BC/R genotype (GpGp, RR) of Pisum sativum L. at 3 developmental stages

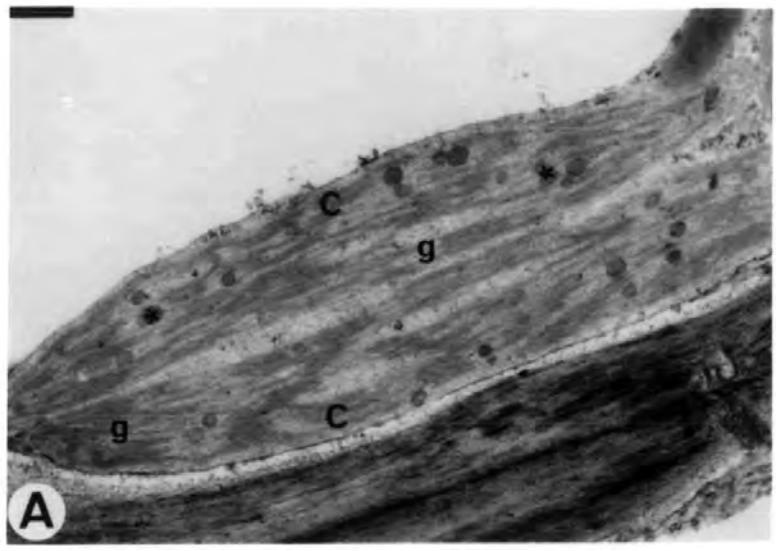
A. Stage II

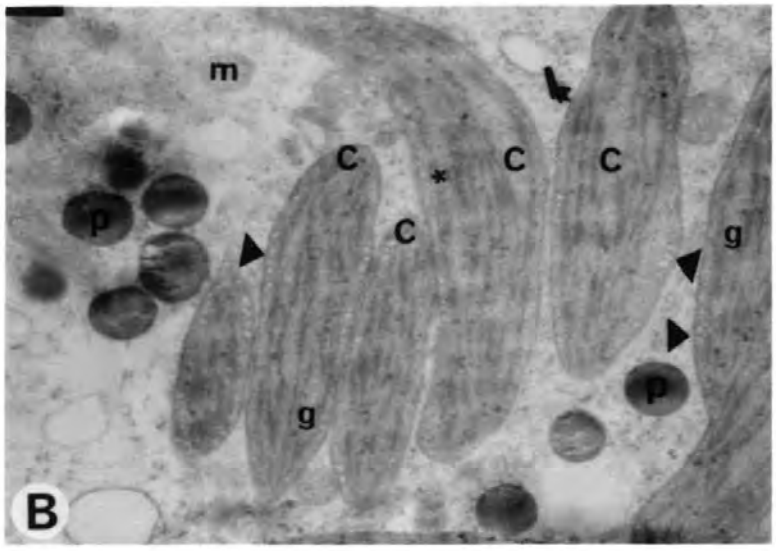
B. Stage III

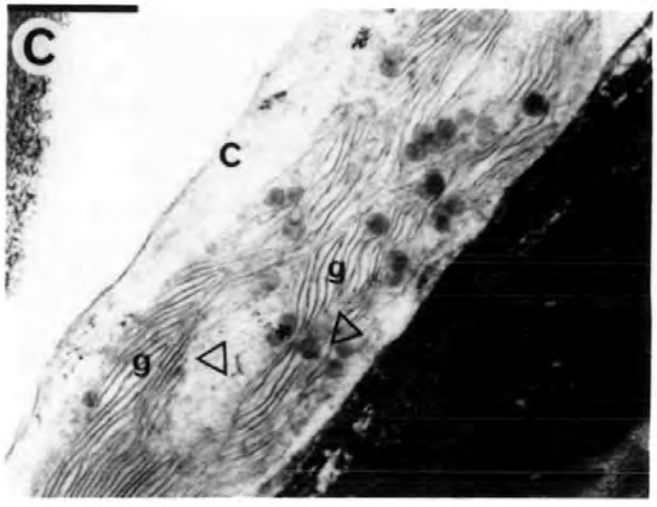
C. Stage IV

c chloroplasts
g granal stacks
| lamellum
m mitochondria
p protein bodies
* lipid (osmiophilic) droplets
▶ peripheral reticulum
▷ diagonally-sloping granal stacks

bar line 0.5 μm


C
g
g
C
A


m
C
C
C
C
p
g
g
p
B


C
C
g
g

Mitochondria and R.E.R. were abundant in these cells throughout development (Plates 1.22) and protein bodies were present at later stages (Plate 1.22 B).

Chloroplasts of the gpgp (yellow-podded) osteo-sclereids had quite extensive thylakoid membranes with large granal stacks (Plates 1.24 A and B). Although the thylakoid membranes were dilated at later stages starch was evident in this tissue. Unlike the corresponding cells of the gpgp testa, plastids in this tissue contained lipid droplets throughout development but contained no protein bodies.

Mitochondria and R.E.R. were not as abundant in this tissue as in the green type, particularly at the later stages.

c. Parenchyma

At the earlier stage (II), these cells in the GpGp (green-podded) testa contained many amyloplasts which had limited thylakoid membranes and large starch grains. Mitochondria and R.E.R. were also present at these stages, with plasmodesmata connecting adjoining cells (Plate 1.25 B and 1.26 A). No protein bodies were observed in this tissue.

As the testa aged and this layer became reduced due to compression caused by expansion of the embryo, the starch disappeared and the cell contents disintegrated (Plate 1.25 A).

In this layer in the gpgp (yellow-podded) testa, amyloplasts were present with limited thylakoids and large starch grains (Plate 1.26 B), similar to those of the GpGp testa. The oldest testa examined contained plastids which were very disorganised (Plate 1.27 A), and contained large numbers of lipid droplets. Such plastids were present throughout this tissue at this stage, but were not found

PLATE 1.24

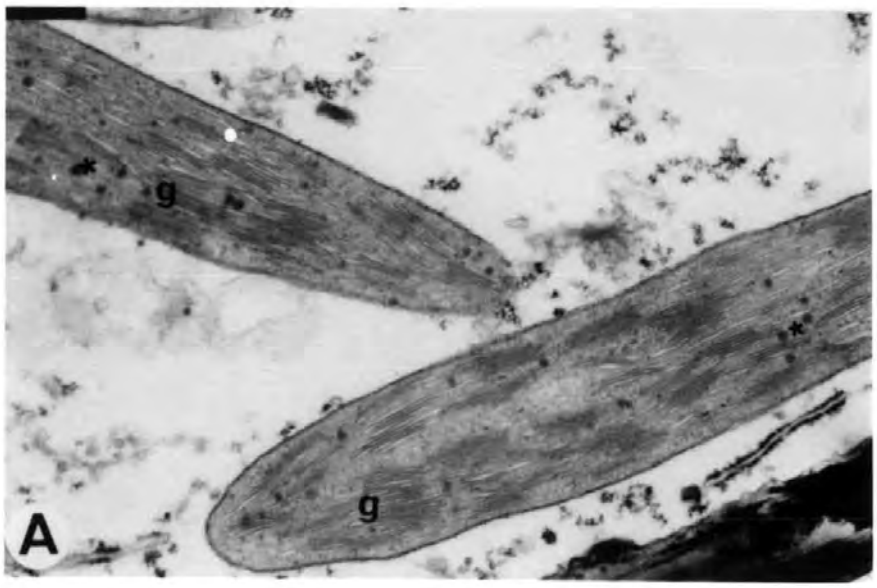
Electron micrographs of sections through chloroplasts from the osteo-sclereid layer of testa of the yellow-podded, round-seeded JI 13 genotype (gpgp, RR) of Pisum sativum L. at 2 developmental stages

A. Stage II

B. Stage III

g granal stack
◀ peripheral reticulum
* lipid (osmiophilic) droplet

bar line 0.5 μm


g
g
A


B
g

PLATE 1.25

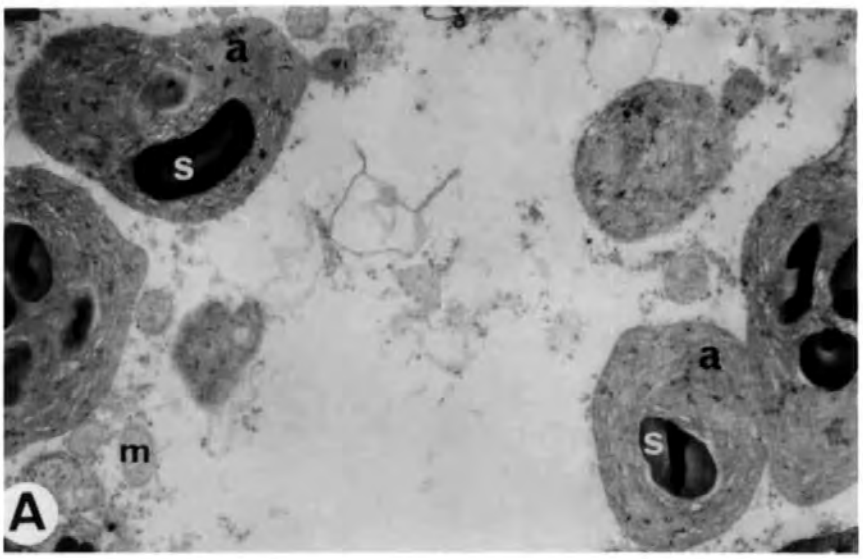
Electron micrographs of characteristic plastids of the parenchyma layer of testas of the green-podded, round-seeded BC/R genotype (GpGp, RR) of Pisum sativum L. at 2 developmental stages

A. Stage II

B. Stage III

a amyloplast
m mitochondria
p◀ plasmodesmata
s starch grains

bar line 1 μm


a
s
a
s
m
A

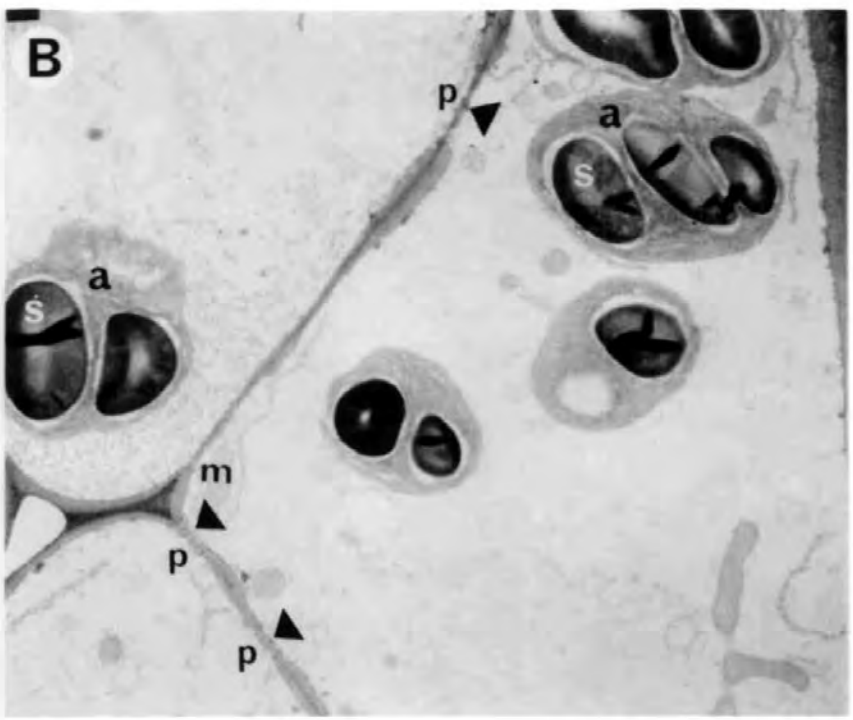

B
p
a
s
a
s
m
p
p

PLATE 1.26

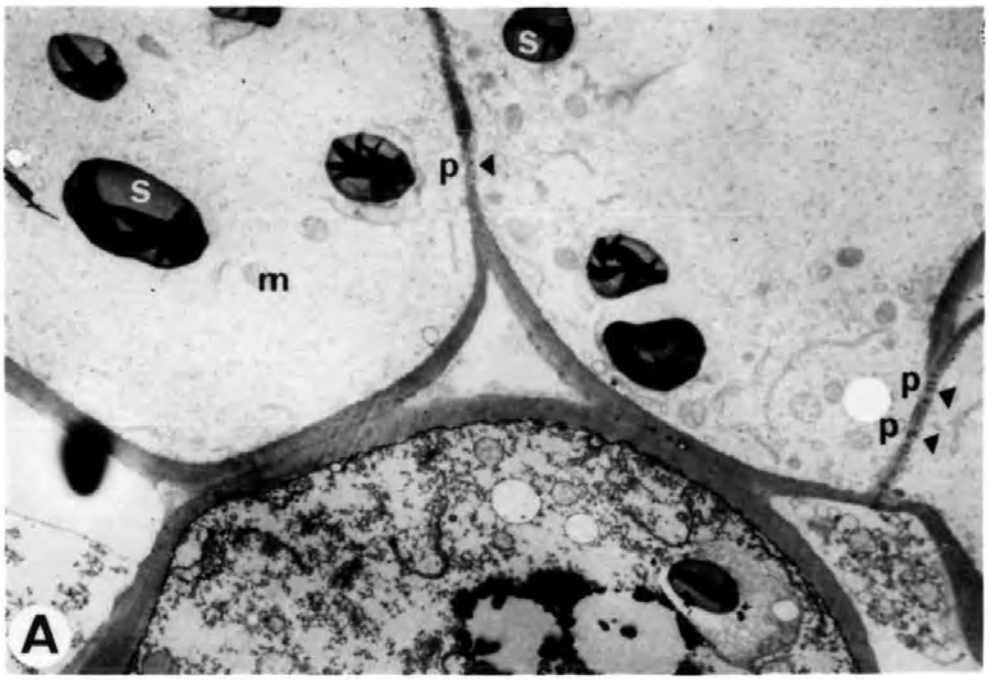
Comparison of electron micrographs of characteristic parenchyma cells of testas from 2 genotypes of *Pisum sativum* L. at stage II in development

A. Green-podded, round-seeded BC/R genotype (*GpGp*, *RR*)

B. Yellow-podded, round-seeded JI 13 genotype (*gpgp*, *RR*)

a amyloplast
m mitochondria
p◀ plasmodesmata
s starch grains
r ribosomes
g Golgi body

bar line 1 μm


A
s
s
p
m
p
p

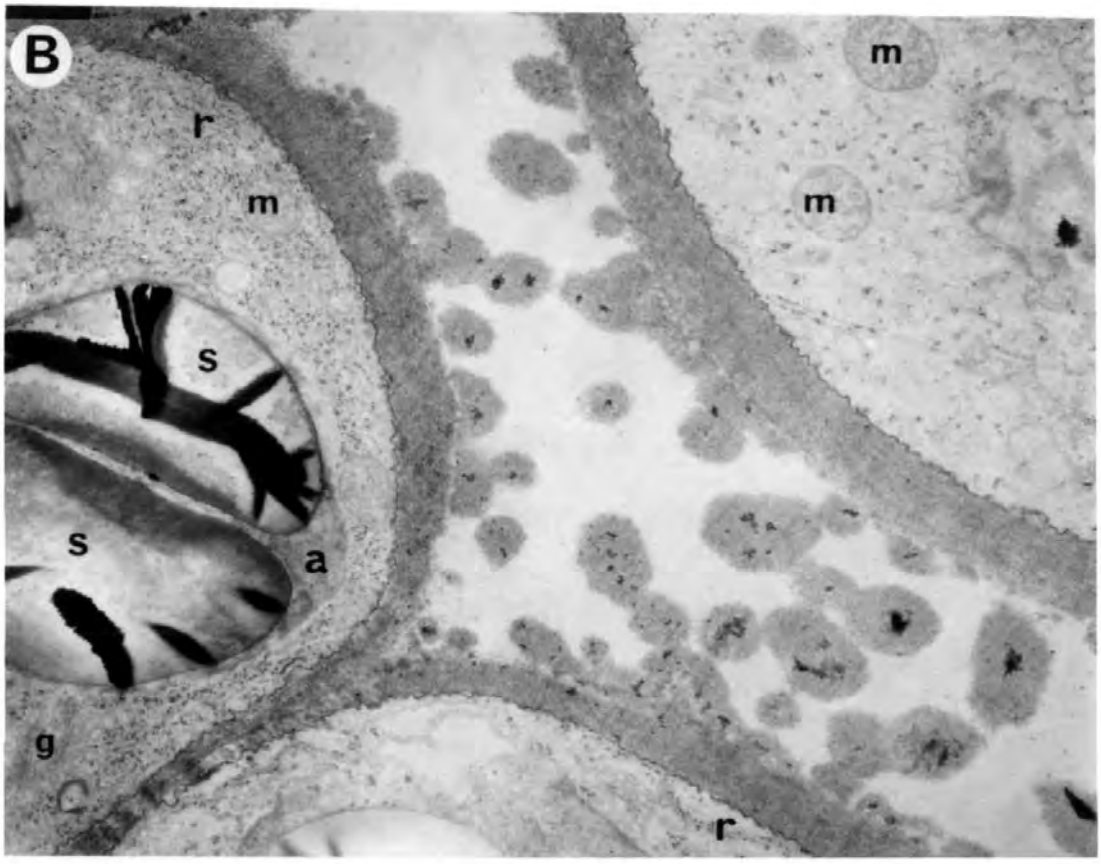

B
r
m
m
m
s
s
a
g
r

PLATE 1.27

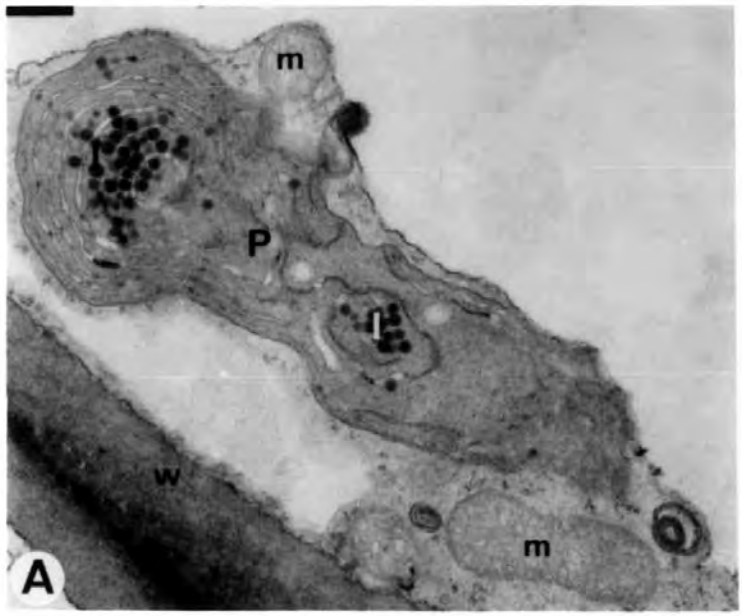
Comparison of electron micrographs of characteristic parenchyma cells of testas of the yellow-podded, round-seeded JI 13 genotype (gpgp, RR) of Pisum sativum L. at 2 developmental stages

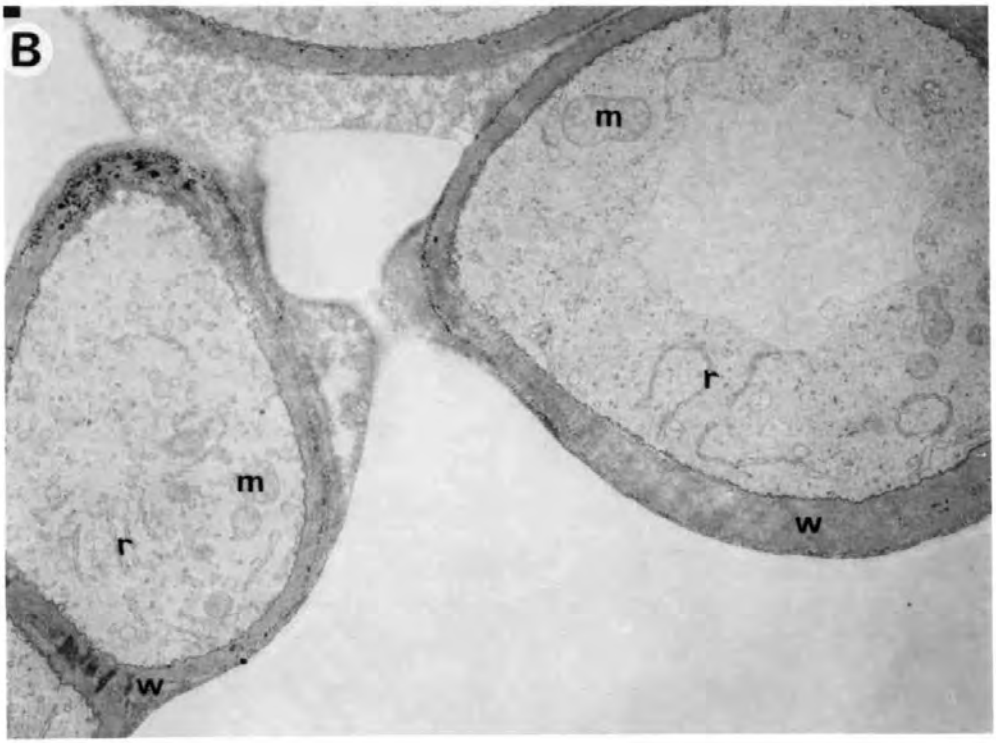
A. Stage IV

B. Stage II/III

| lipid droplets, surrounded by dilated membranes
m mitochondria
p plastid
r rough endopasmic reticulum (R.E.R.)
w cell wall

bar line 1 μm


m
P
w
m
A


B
m
r
w
m
r
w

elsewhere. Mitochondria, R.E.R. and ribosomes were abundant early in development, (stage II/III, Plate 1.27 B) but cell contents degenerated with age.

iii) Cotyledons

Although the cotyledons may appear uniform, there was a gradation of cell development through this tissue; cells near the surface being physiologically less well developed compared with those in the centre. In order to ensure valid comparisons of corresponding cells between lines only the cells of the outer few layers were used.

a. Pre contact-point (stage II)

No differences were observed between plastid stucture in green or yellow, round or wrinkled seed (Plates 1.28 A, B and C), all showing similar thylakoid membrane development and granal stacking. All genotypes contained starch. Cells of these outer layers of the cotyledons were highly organised, with nuclei, mitochondria, R.E.R. and ribosomes.

b. Post contact-point (stage II)

Inter-cellular spaces were still evident at this stage (Plate 1.29) and chloroplasts and mitochondria were abundant (Plate 1.29). The plastids were diverse in the younger types; some had very well-organised internal membranes (Plate 1.30 A, B and C) and resembled chloroplasts of leaf tissue. The internal membranes of some, particularly amyloplasts, were slightly irregular (Plate 1.30 D, 1.31 A and B). Protein bodies were present at this stage (Plate 1.31 A and B).

PLATE 1.28

Comparison of electron micrographs of characteristic plastids from the outer layers of the cotyledons from 3 genotypes of Pisum sativum L. at stage II in development

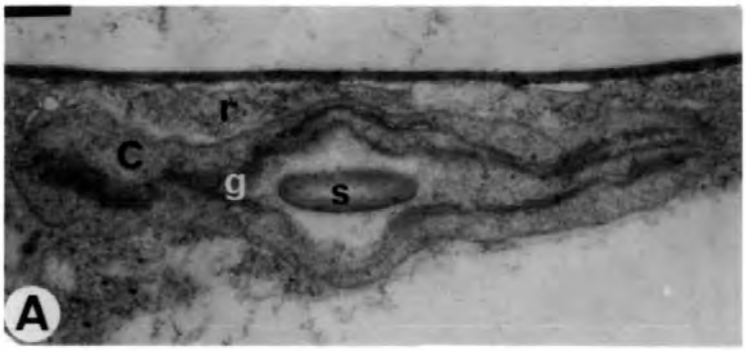
A. Green-podded, round-seeded BC/R genotype (GpGp, RR)

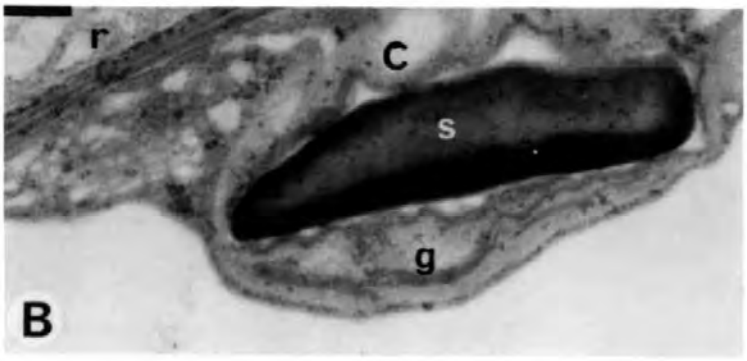
B. Green-podded, wrinkled-seeded BC/r genotype (GpGp, rr)

C. Yellow-podded, round-seeded JI 13 genotype (gpgp, RR)

c chloroplast
g granal stack
n nucleus
r ribosomes
s starch grain

bar line 1 μm


r
C
g
s
A


r
C
s
g
B

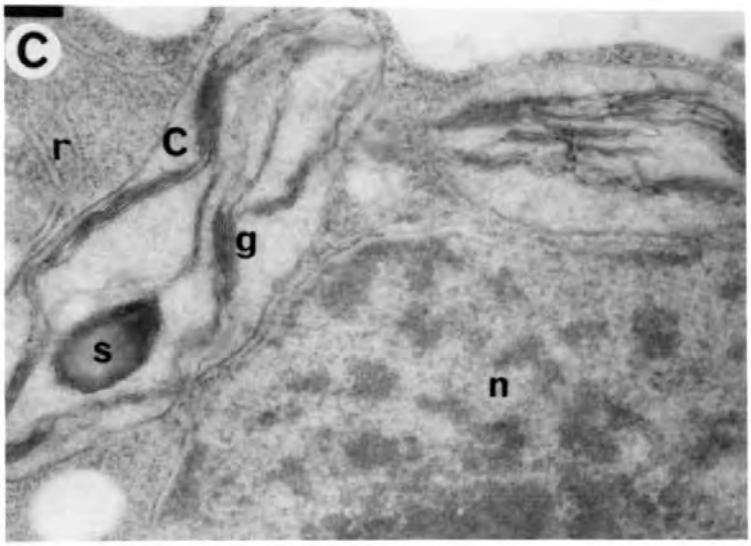

C
r
C
g
s
n

PLATE 1.29

Electron micrographs of the outer layer of the cotyledon from the green-podded, wrinkled-seeded BC/r genotype (GpGp, rr) of Pisum sativum L. at stage I in development

c chloroplasts
i inter-cellular spaces
m mitochondria
n nuclei
r ribosomes

bar line 7 μm

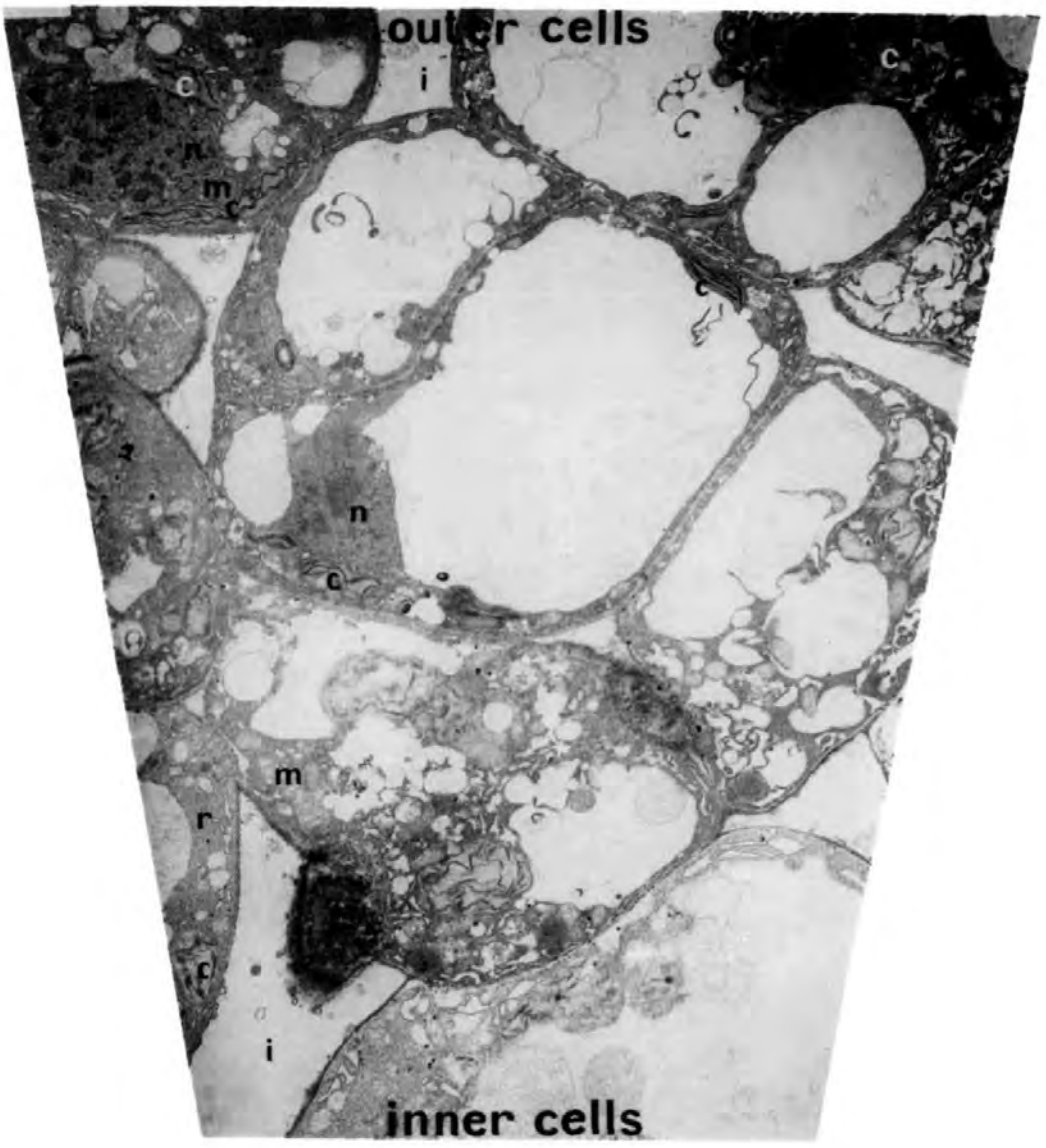

outer cells
c
i
m
n
r
inner cells

PLATE 1.30

Comparison of electron micrographs of the outer cotyledon cells from different genotypes of Pisum sativum L. at stage III in development

A. wrinkled-seeded, green-podded BC/r genotype (rr, GpGp)

B. round-seeded, green-podded BC/R genotype (RR GpGp)

C. round-seeded, yellow-podded JI 13 genotype (RR gpgp)

D. wrinkled-seeded, green-podded BC/r genotype (rr GpGp)

c chloroplast
g granal stack
m mitochondria
r ribosomes
s starch grains
◀ outer chloroplast membrane

bar line 1 μm

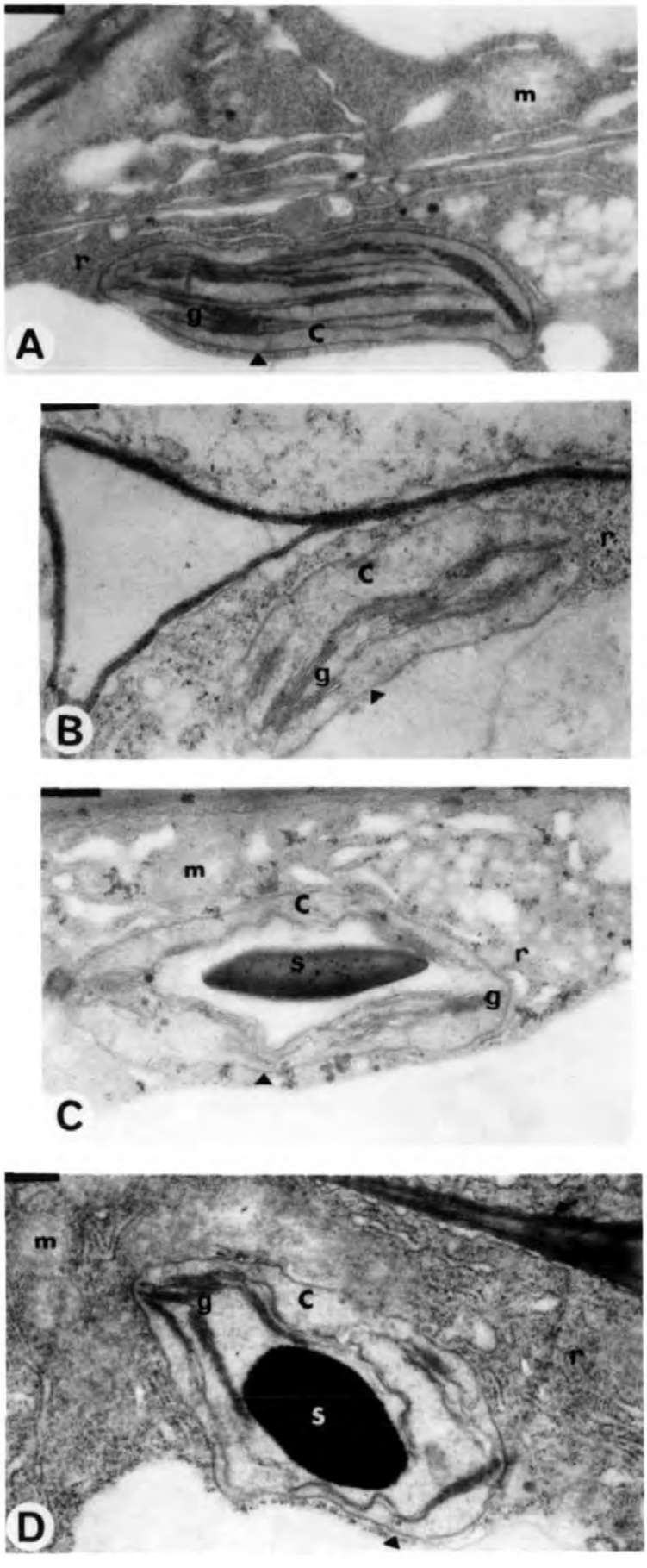

m
r
g
c
A
r
c
g
B
m
c
s
r
g
C
m
g
c
r
s
D

PLATE 1.31

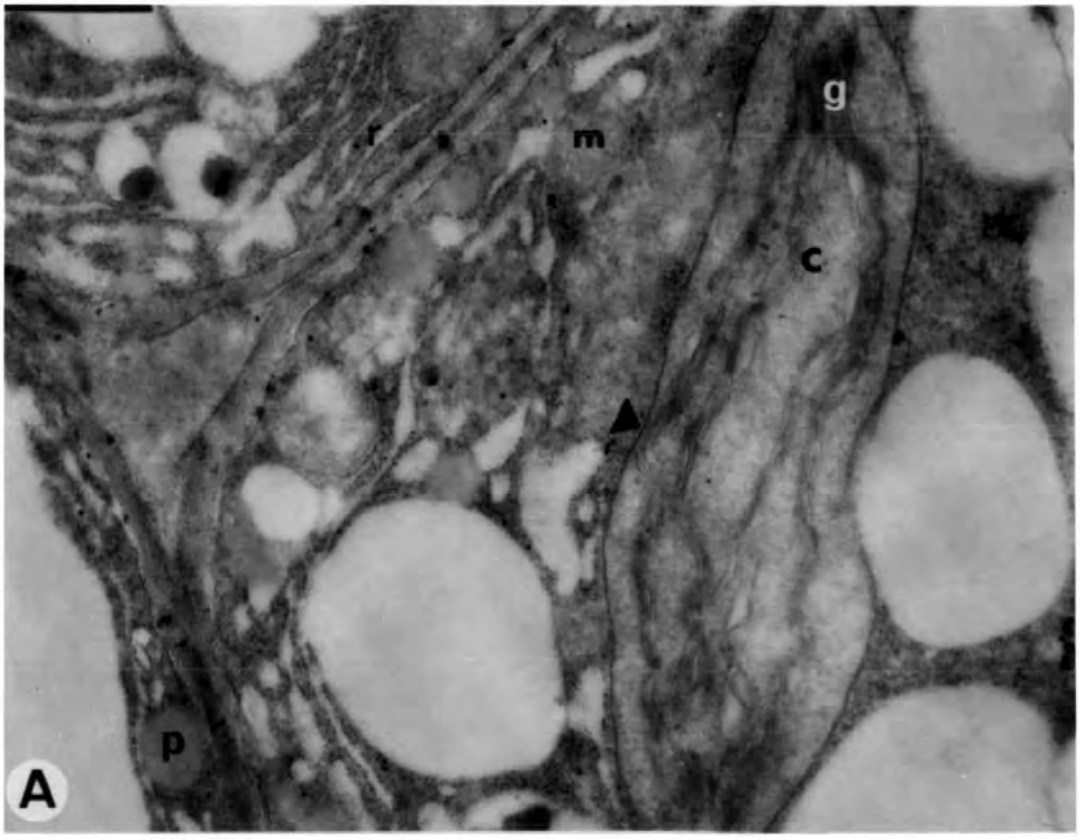
Comparison of electron micrographs of the outer cells of the cotyledon from the 2 green-podded BC/R and BC/r genotypes (GpGp) of Pisum sativum L., at stage III in development

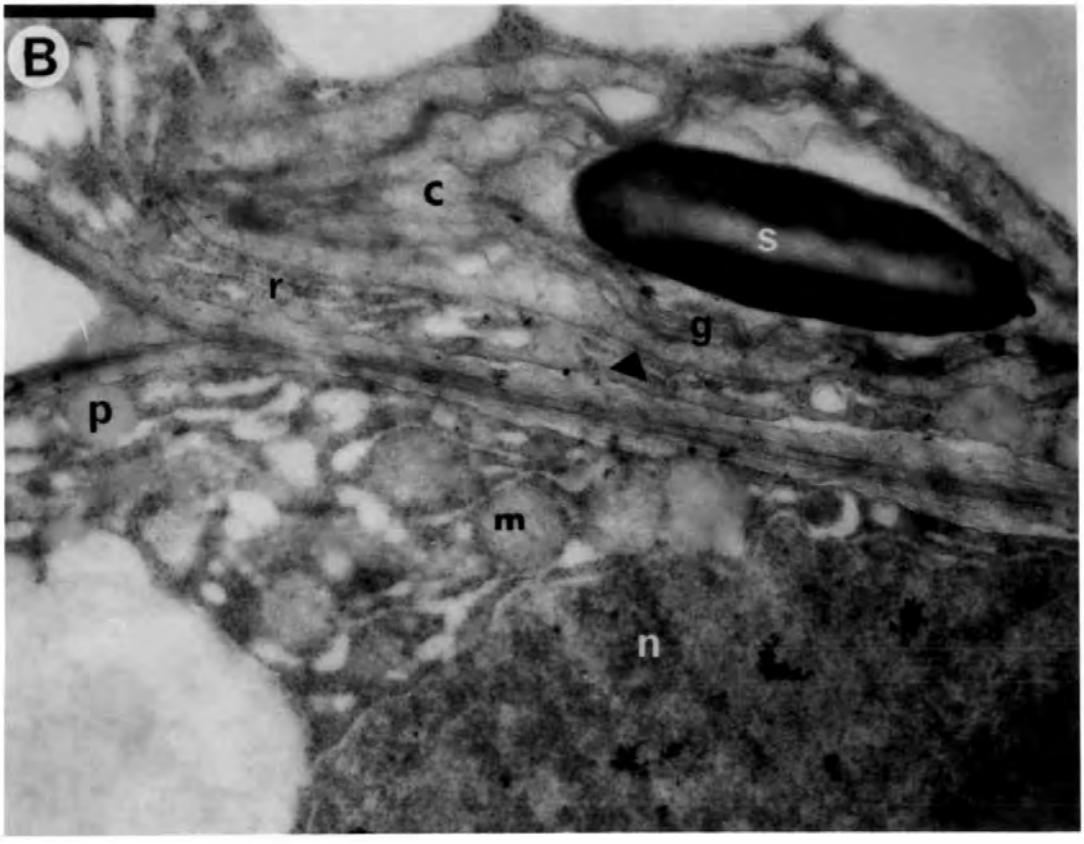
A. wrinkled-seeded BC/r genotype (rr)

B. round-seeded BC/R genotype (RR)

c chloroplast
g granal stack
m mitochondria
n nucleus
p protein body
r R.E.R.
s starch grains
◀ outer chloroplast membrane

bar line 1 μm


A
r
m
g
c
p


B
c
s
r
g
p
m
n

However, the starch grains did appear to be different in the round seeds (RR GpGp and RR gpgp) compared with those in the wrinkled seed (rr GpGp). In the round genotypes they had a distinct outline (Plates 1.32 A), while the ouline of in those of the wrinkled genotype was indistinct (Plate 1.32 B).

Mitochondria, R.E.R. and ribosomes were present in the inner cells. Unlike the younger cells, protein bodies had formed in both rr and RR genotypes at this stage.

D. TRANSMISSION STUDIES

i) Green pod

The general pattern of transmission for the round-seeded and wrinkled-seeded genotypes at stages II and III were very similar (Fig. 1.1). It was low (< 2 %) in the near-ultra-violet (<400nm) region and higher (10 %) in the blue and red regions. Maximum transmission (25-30 %) occurred in the far-red (760nm) with only slightly less (20 %) in the green (540nm) region.

Transmission increased throughout the spectrum during stages III-V. As the pod senesced, transmission at all wavelengths in the visible spectrum (>400nm) increased, particularly in the blue and red regions (Fig. 1.2), corresponding to chlorophyll breakdown (Table 1.5, page 63).

Throughout development the only wavelength which was not transmitted was in the near- U.V. Radiation of this wavelength remained relatively constant at <1 % in both genotypes. Total photon flux density reaching the developing seeds varied between 374 $\mu molm^{-2}s^{-1}$ and 688 $\mu molm^{-2}s^{-1}$ (Table 1.4, from data of H. Smith, 1981).

PLATE 1.32

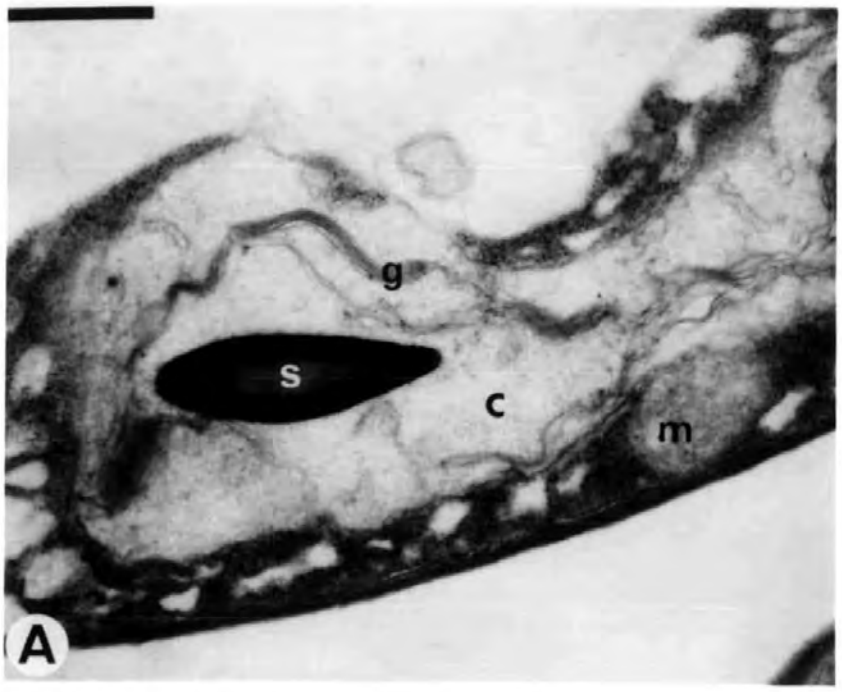
Comparison of electron micrographs of the characteristic outer cells of cotyledons from 2 green-podded genotypes (GpGp) of Pisum sativum L. at stage III in development

A. round-seeded BC/R genotype (RR)

B. wrinkled-seeded BC/r genotype (rr)

c chloroplast
g granal stack
m mitochondria
r ribosomes
s starch grains

bar line 1 μm


g
s
c
m
A

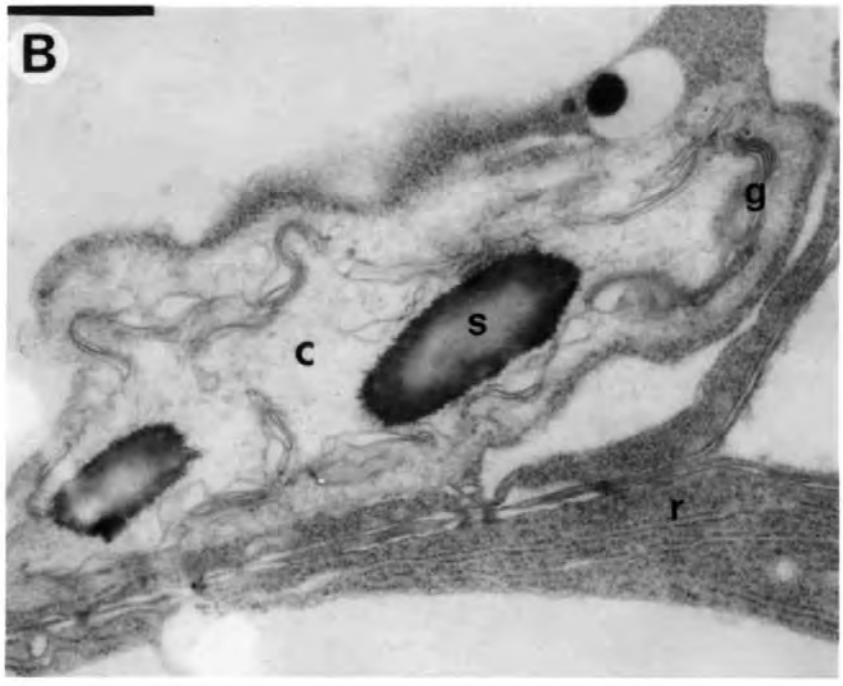

B
g
s
c
r

FIGURE 1.1

Comparison of the percent radiation over the visible spectrum transmitted through the pod wall of two green-podded genotypes (GpGp) of Pisum sativum L. at two developmental stages (A. stage II and B. stage III), before and after the endosperm has been absorbed in the seed

x-x wrinkled-seeded BC/r genotype (rr)

o-o round-seeded BC/R genotype (RR)

Integration over the whole spectrum gives the value of total radiation transmitted as 14.8 % (RR) and 13.8 % (rr)

L.S.D. at 5 % level = 1.26 % (RR) and 4.96 % (rr)

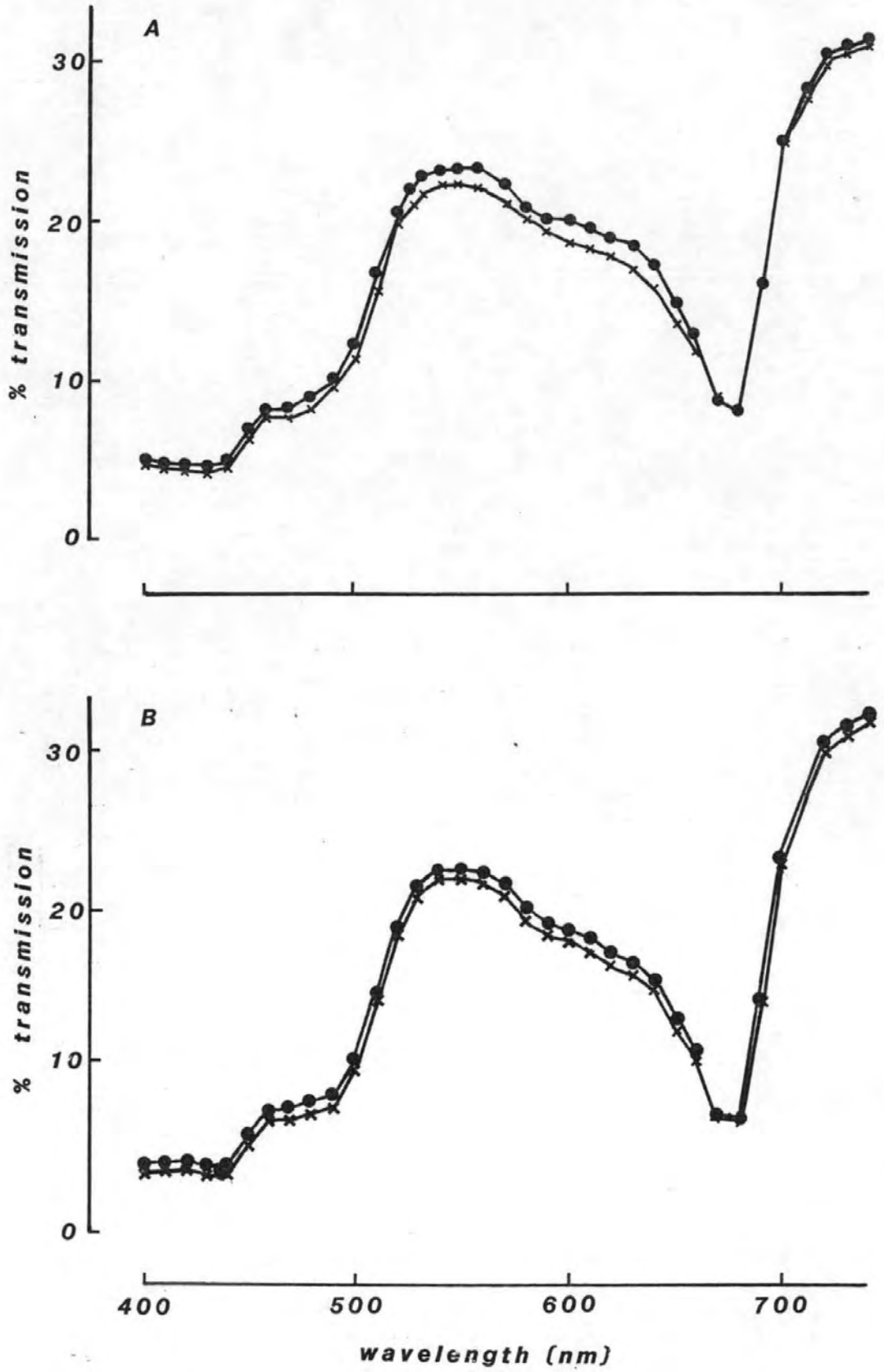

A
B
30
20
10
0
% transmission
% transmission
400
500
600
700
wavelength (nm)

FIGURE 1.2

Percent radiation transmitted over the visible spectrum through the pod wall of the senescing (stage V) green-podded, round-seeded BC/R genotype (GpGp, RR) of Pisum sativum L.

Integration over whole spectrum = 18.4 % total

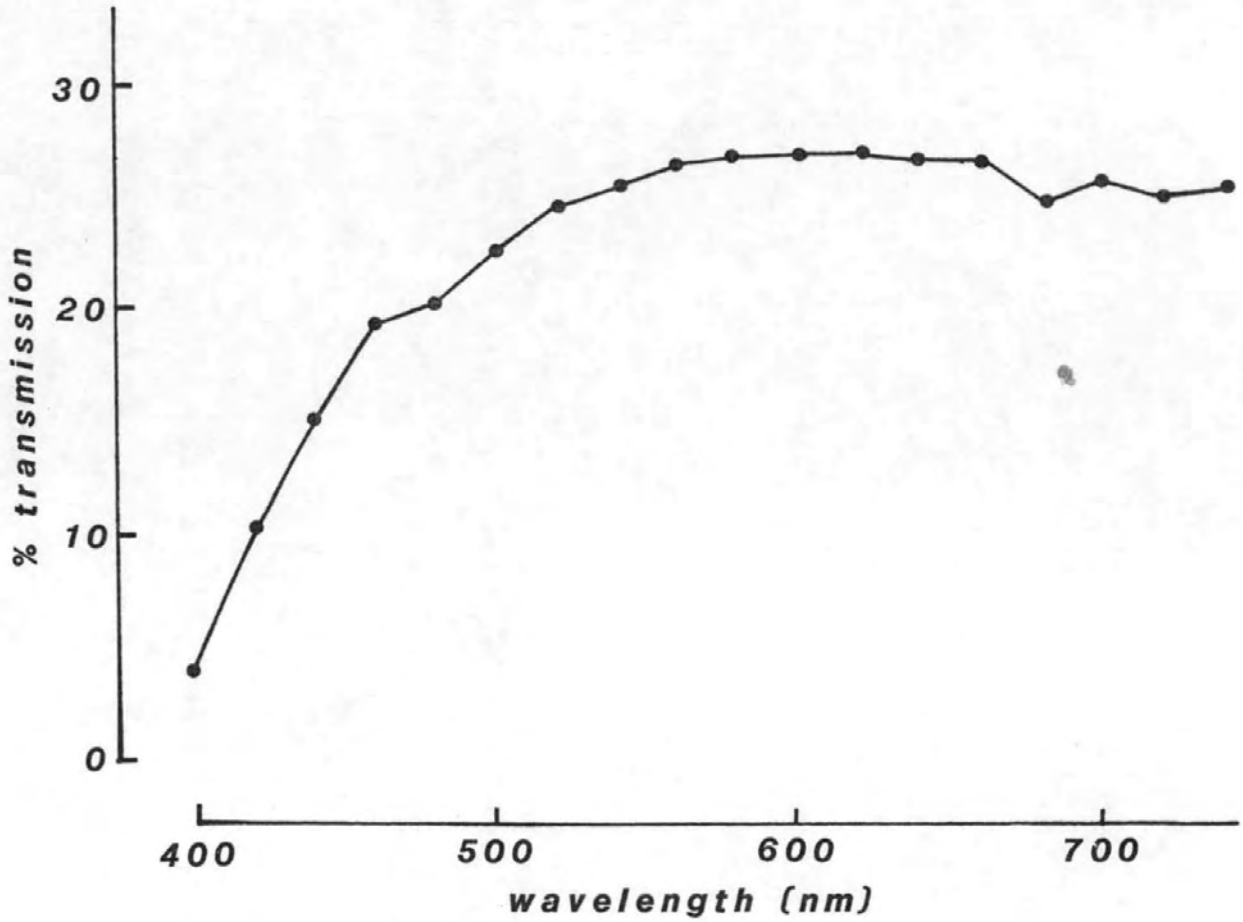

30
20
10
0
% transmission
400
500
600
700
wavelength (nm)

TABLE 1.4

Photon flux density (over the visible spectrum) of solar radiation reaching the earth's surface and of radiation filtered through leaves, pods and testas of *Pisum sativum* L. plants

	Stage	Photon flux density $\mu molm^{-2}s^{-1}$
Radiation reaching earth's surface *	-	2345
pea leaf	fully-expanded	140
green (GpGp) pod	II / III	347
	V	431
yellow (gpgp) pod	II / III	688
	V	942
green (GpGp) pod plus testa	V	83
yellow (gpgp) pod plus testa	V	80

* Data from Smith, 1981

Total values of each tissue type were obtained by integrating the area under the curves of Fig. 1.1, 1.2, 1.3, 1.4 and 1.5 and using the value of 2345 $\mu molm^{-2}s^{-2}$ as a characteristic value reaching the leaf / pod surface

ii) Yellow pod

The general transmission spectrum of the yellow pods (Fig. 1.3) showed several differences to that of the green pods (Fig. 1.1). Although they transmitted least radiation in the blue and most in the far-red, overall transmission was significantly higher than the green-podded type.

The yellow pod at stage III transmitted 2.5-times more radiation in the blue region (420-440mn) and in the red (660-680nm) (Fig. 1.3) than did the green pod (Fig. 1.1). Levels of radiation in the green region were fairly constant throughout at 26-30 %, which was similar to the senescent stage (V) of the green pods. Later in development (stage V) (Fig.1.3 B), the spectral composition of transmitted radiation different, with more radiation transmitted in the blue and red regions, 1.2 and 1.9 $\mu mols^{-1}m^{-2}nm^{-1}$ respectively, corresponding with a breakdown of the (relatively small amount of) chlorophyll (Table 1.5). Thus overall, much more radiation is transmitted by the yellow pod and compares with that for the senescent green pod (Fig. 1.1).

Transmission in the near-U.V. varied little and was similar to that of green pods at 1%. Total photon flux density reaching the developing seeds was 688 and 942 $\mu molm^{-1}s^{-1}$ at stage III and V respectively.

iii) Testa

Total radiation transmitted through the pod and testa of an older (stage V), green-podded carpel wall and testa (Fig. 1.4) was of similar quality to that of the green pod at stage II (Fig. 1.1).

FIGURE 1.3

Comparison of the percent radiation transmitted through yellow pods of the JI 13 genotype (gpgp) of Pisum sativum L. at 2 developmental stages

A. Stage III

B. Stage V

Total integrated value = 24.9 % (stage III)

= 34.8 % (stage V)

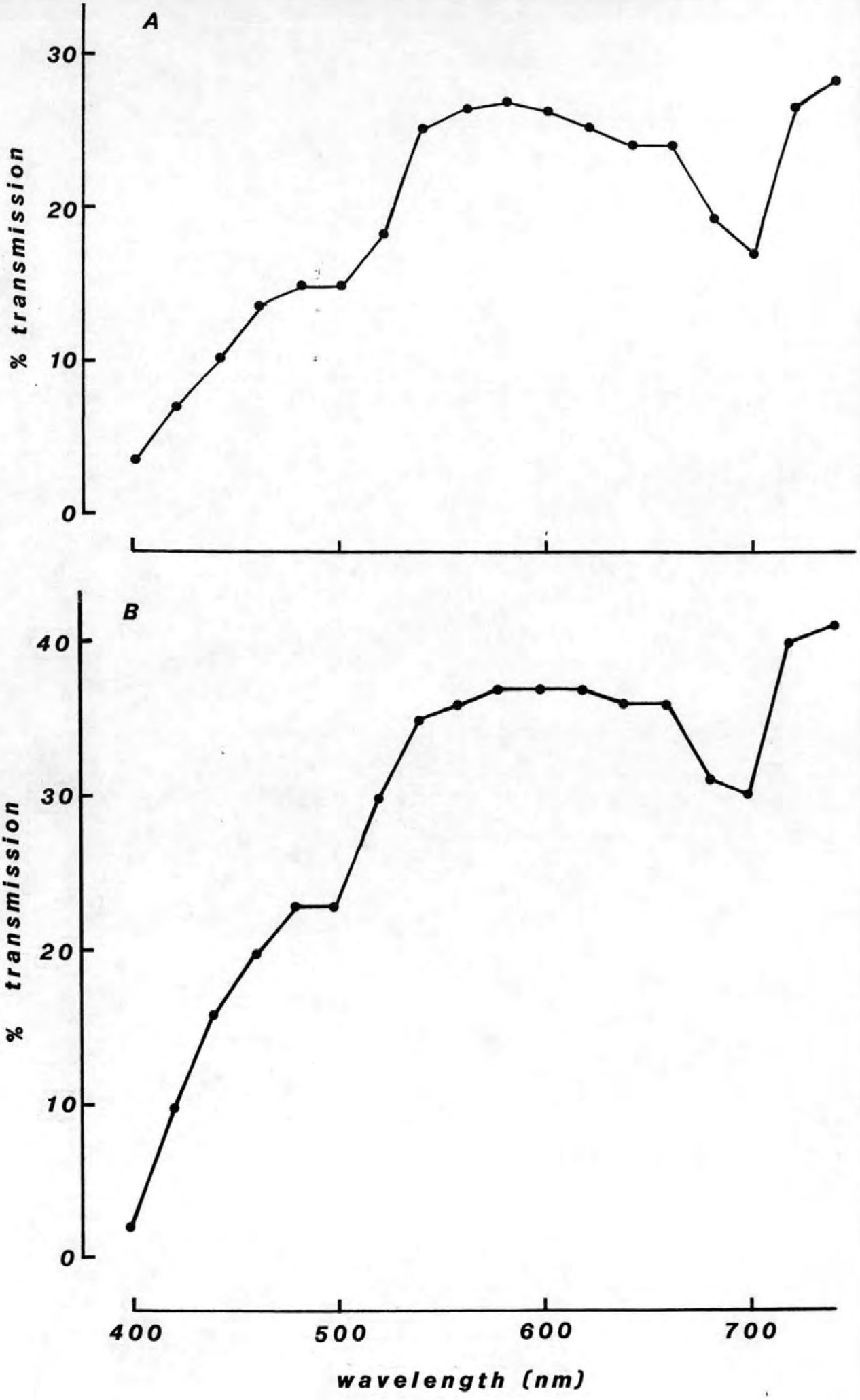

A
30
20
10
0
% transmission
B
40
30
20
10
0
% transmission
400
500
600
700
wavelength (nm)

TABLE 1.5

Comparison of chlorophyll contents of 3 genotypes of *Pisum sativum* L. at 4 developmental stages

Stage	mg chlorophyll/ pod			mg chlorophyll/ g fresh wt. pod		
	BC/R	BC/r	JI 13	BC/R	BC/r	JI 13
II	0.041	0.052	0.016	0.0136	0.0173	0.0054
III	0.084	0.104	0.016	0.014	0.0200	0.0076
IV	0.057	0.070	0.007	0.0095	0.0147	0.0020
V	0.026	0.026	0.008	0.0064	0.0063	0.0028

BC/R green-podded round-seeded genotype (*GpGp*, *RR*)

BC/r green-podded, wrinkled-seeded genotype (*GpGp*, *RR*)

JI 13 yellow-podded, round-seede genotype, (*gpgp*, *RR*)

FIGURE 1.4

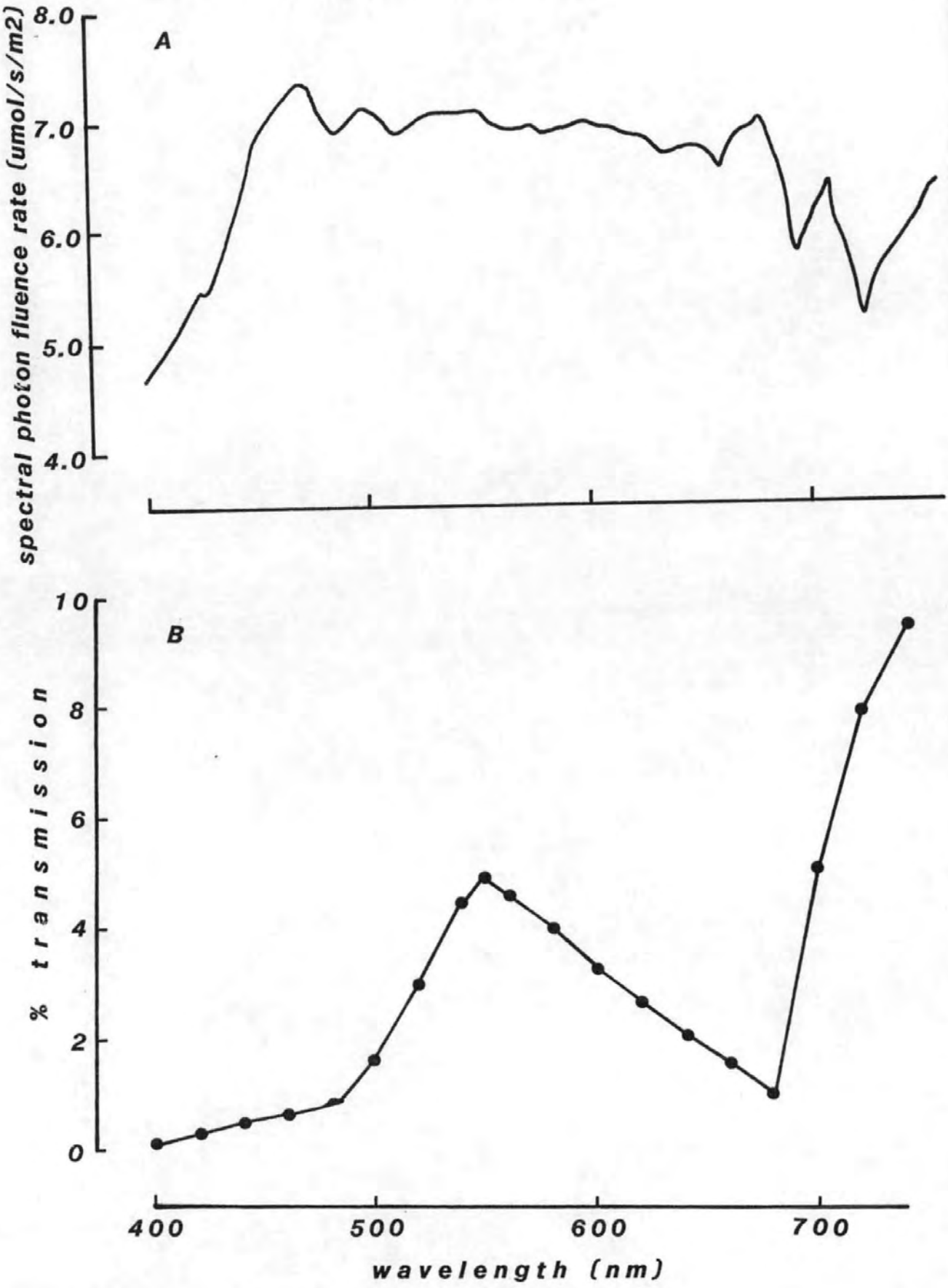
A. Spectrum of radiation from the sun on a sunny but not bright summers' day in Leicester, England (Data from H. Smith, 1981)

Integration of the area under the curve indicates that the total radiation reaching the earths' surface is 2345 $\mu molm^{-2}s^{-1}$

B. Percent radiation transmitted through the pod wall and testa of a maturing (Stage V) fruit of the green-podded, wrinkled-seeded BC/r genotype (*GpGp*, *rr*) of *Pisum sativum* L.

Integration of the area under the curve indicates that the total radiation transmitted is 3 % of that reaching the pod surface

i.e. 3 % of 2345 $\mu molm^{-2}s^{-1}$ ($80\mu molm^{-2}s^{-1}$) reaches the embryo


A
8.0
7.0
6.0
5.0
4.0
spectral photon fluence rate (umol/s/m2)
B
10
8
6
4
2
0
% transmission
400
500
600
700
wavelength (nm)

It was very low (3-5 %) in the blue and red wavelengths and relatively high in the green and far-red (16 % and 36 % respectively). The younger pod thus effectively screened out most of the radiation of the blue and red wavelengths and very little reached the embryo until the pod senesced. Total photon flux density reaching the developing embryo at stage V was 80 $\mu molm^{-2}s^{-1}$ (Fig. 1.4).

iv) Leaf

In contrast to pods and testas, negligible amounts of radiation were transmitted in the blue and red wavelengths (Fig. 1.5). Transmission in the near-U.V. region was higher than that through pods, being 8 %. Transmission in the green region of the leaf was nearly 3-times that through pods. Transmission was also greater through the leaf than through the pods in the far-red, where 50 % radiation was transmitted. Total photon flux density transmitted through the leaf was 149 $\mu molm^{-2}s^{-1}$ (Table 1.5), i.e. 2105 $\mu molm^{-2}s^{-1}$ of radiation were used by the leaf.

E. CHLOROPHYLL CONTENT

i) Pods

When expressed on a per pod basis, there were no differences in chlorophyll content between the RR (round-seeded) and rr (wrinkled-seeded) green-podded GpGp genotypes at any stage of development (Table 1.5). Total chlorophyll content per pod increased to a peak of 90-100 mg at stage III which corresponded to maximum pod fresh weight and then declined during senescence (stages IV and V).

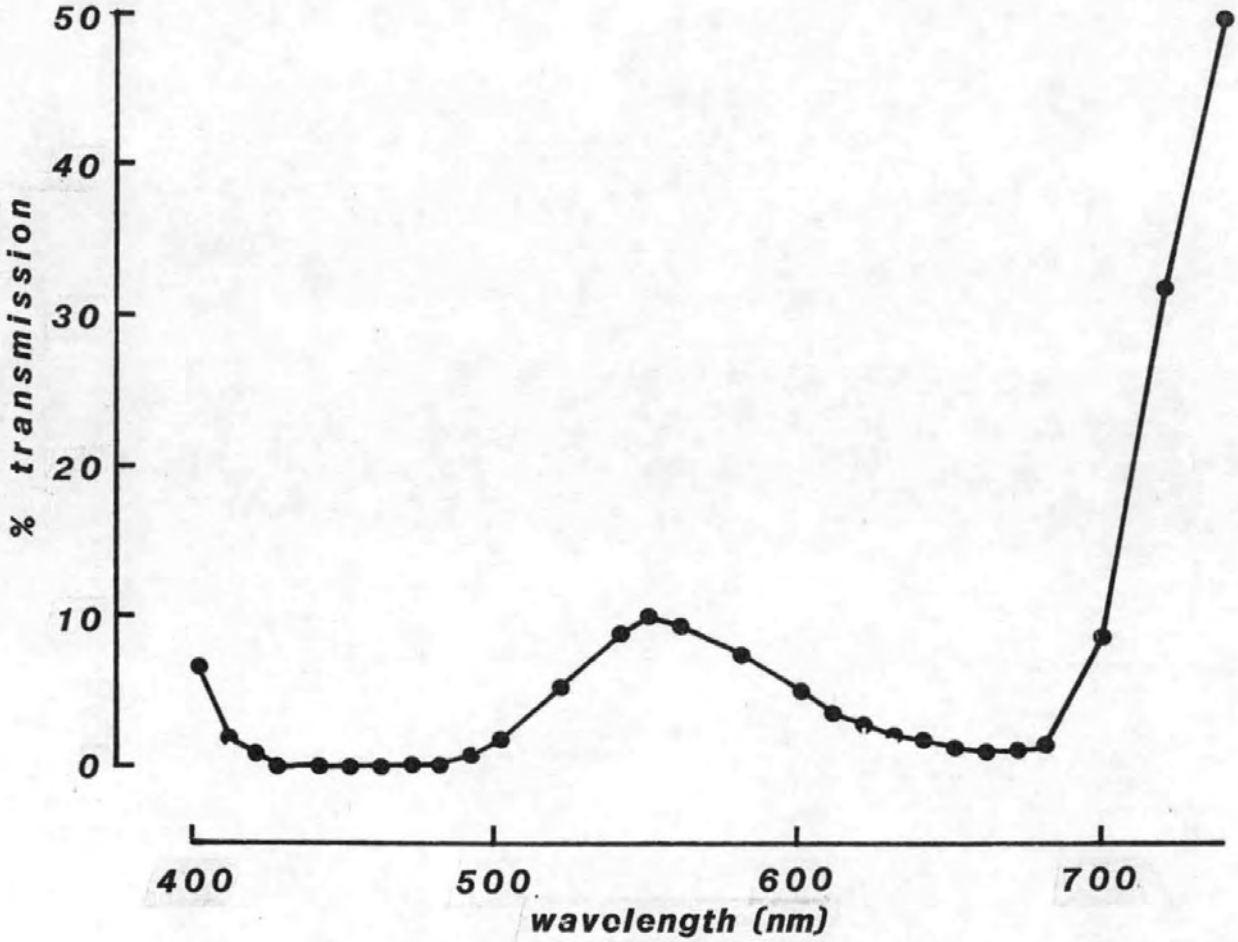
FIGURE 1.5

Percent radiation transmitted over the visible spectrum through a fully-expanded green leaf of the green-podded genotype (GpGp) of Pisum sativum L.

Integration of the area under the curve = 6.1 %

Thus the total photon flux density = 6.1 % of 2345 $\mu molm^{-2}s^{-1}$

= 141 $\mu molm^{-2}s^{-1}$


50
40
30
20
10
0
% transmission
400
500
600
700
wavelength (nm)

By comparison, the yellow gpgp pod had attained its maximum amount of chlorophyll per pod by stage II. It remained constant during stage III and declined during senescence (stages IV and V).

Expressed as mg chlorophyll per g fresh weight of pod, it appears that there is some difference between the GpGp lines. At stages II and III the wrinkled type contained slightly higher amounts of chlorophyll than the round type. The amount of chlorophyll per g fresh weight remained virtually constant during these two stages and then declined during senescence (stages IV and V).

The yellow pod showed a similar pattern of chlorophyll decline. Examination of the yellow pods showed the endocarp to be darker green than the mesocarp, suggesting that most of the chlorophyll was present in this layer.

ii) Testas

In all 3 genotypes, total chlorophyll content per testa increased to a maximum at stage IV and then declined (Table 1.6). Initially, the gpgp testa contained much less chlorophyll than the GpGp testa of similar age, but its maximum was comparable to that of the GpGp lines.

On a mg chlorophyll per g fresh weight basis, the results are rather variable but there was little difference between genotypes all of which exhibited a decline in chlorophyll content through development except at the first stage examined.

iii) Embryos

In all 3 genotypes, total chlorophyll content per embryo increased during development to a maximum at stage IV (Table 1.7)

TABLE 1.6

Comparison of chlorophyll contents of testas of 3 genotypes of Pisum sativum L. at 4 stages of development

Stage	mg chlorophyll x 10^{-3} / testa			mg chlorophyll / g fresh wt. testa		
	BC/R	BC/r	JI 13	BC/R	BC/r	JI 13
II	1.45	1.81	0.495	0.0135	0.0201	0.00491
III	1.73	1.484	0.970	0.0122	0.00795	0.00962
IV	2.133	2.615	2.617	0.0138	0.0172	0.0123
V	1.868	1.087	2.13	0.0133	0.0103	0.0147

BC/R green-podded, round-seeded genotype (GpGp, RR)

BC/r green-podded, wrinkled-seeded genotype (GpGp, rr)

JI 13 yellow-podded, round-seeded genotype (gpgp, RR)

TABLE 1.7

Comparison of chlorophyll contents of embryos of 3 genotypes of Pisum sativum L. at 4 stages of development

Stage	mg chlorophyll / embryo			mg chlorophyll / g fresh wt. embryo		
	BC/R	BC/r	JI 13	BC/R	BC/r	JI 13
II	0.0036	0.0033	0.00174	0.060	0.0726	0.0575
III	0.0135	0.0164	0.0100	0.065	0.0725	0.0672
IV	0.0229	0.0234	0.0185	0.058	0.0419	0.0415
V	0.0162	0.0144	0.0105	0.036	0.0280	0.0246

BC/R green-podded, round-seeded genotype (GpGp, RR)

BC/r green-podded, wrinkled-seeded genotype (GpGp, rr)

JI 13 yellow-podded, round-seeded genotype (gpgp, RR)

During stages II and III there was variation in chlorophyll content, but at later stages (IV and V) there was no significant difference between chlorophyll content of the two GpGp lines. They both contained significantly more chlorophyll than the gpgp line. However, when expressed in terms of chlorophyll per g fresh weight of embryo, no significant difference was found between the 3 genotypes at these later stages (IV and V). At the earlier stages (II and III), the gpgp embryo contained slightly less chlorophyll per g fresh weight than either of the GpGp embryos.

DISCUSSION

The most marked difference between the green- and yellow-podded genotypes was in the structure of the chloroplasts of the pod and to a lesser degree the testa. The major visible effect of the gpgp genotype in the mesocarp chloroplasts was a reduction in the number of thylakoids and in particular the reduction of granal stack formation. It appears that the gp gene affects membrane cohesion rather than membrane formation, since stromal thylakoids were present.

Chlorophyll is produced by granal thylakoids (Kirk and Tilney-Bassett, 1968) and their depletion in the yellow pod mesophyll chloroplasts probably accounts for the lower chlorophyll content observed here. Lower chlorophyll contents were also found in other yellow-podded genotypes (Price and Hedley, 1980). Since no abnormal chloroplasts were found in the inner epidermis of the pods, it seems likely that the inner epidermis chloroplasts produce chlorophyll at normal levels. However, the total chlorophyll content in the pod wall does not allow for differences between the different tissues. Price and Hedley (1980) found that relatively high levels of chlorophyll were present in the inner epidermis of yellow pods, but not in the mesocarp.

From personal observations, the inner epidermis of the yellow (gpgp) pods remained green even after the mesocarp was losing its green colour (chlorophyll). The percentage transmission through the yellow pod wall at stage V reflects this presence of chlorophyll. By comparison, the green (GpGp) pod at stage V has a much flatter curve, suggesting a lower chlorophyll content.

Thus differential expression of the gp gene has occurred, but

its mechanism is complex. It could be a failure to produce the required factor and / or the production of inhibitor(s) or an inhibitory condition. It may be selective within tissues because the chloroplasts of the mesocarp are differentially more sensitive than other tissues. Alternatively, inhibitors of chlorophyll production or membrane formation may occur in this layer. The gp gene could invoke a general shift in cell environment, affecting pH or ion availability and hence alter the membrane formation; a differential production of hormone, response to water-stress or temperature and nutrient status could have a similar effect. For example, phytohormones not only control the biogenesis of thylakoids but also their rate of turnover (Lichtenthaler and Grumbach, 1974). Abscisic acid (ABA) is present in chloroplasts of greening barley seedlings, but a further addition of ABA decreases chlorophyll production (Lichtenthaler and Becker, 1970). It was suggested from this work that ABA depresses the light-induced gene activation, leading to decreased chlorophyll and thylakoid formation. El-Antably, Wareing and Hillman (1967) also observed that ABA accelerates senescence of chloroplasts in leaf discs of a variety of species, including Prunus spp., Populus alba, Taraxacum officinale and Pteridium aquilinum. It may be that the gp gene increases ABA production which then either breaks down chlorophyll or prevents thylakoid formation.

It has also been found (Duysen and Freeman, 1974) that water-stressed growth conditions can result in a reduction in the number of thylakoids per granum and also in a reduction in the number of grana per plastid when compared with chloroplasts of non water-stressed plants. In addition, chlorophyll production was lower and the plastids of the stressed leaves showed decreased length and dilation

of the membranes in the granal and stromal thylakoids. These are similar effects to those shown in the gpgp pod and thus the gp gene may cause an increase in susceptibility to water-stress, to produce such a response.

Alternatively, micro-nutrient deficiency can cause membrane disruption. Manganese has been suggested as having a structural role in the stacking of lamellae in spinach chloroplasts (Possingham, Vesk and Mercer, 1964) and zinc deficiency leads to a reduction of granal and inter-granal connections. Copper deficiency results in swelling of grana in spinach chloroplasts (Bottril, Possingham and Kriedman, 1970), iron deficiency causes granal lamellae of Phaseolus leaves to swell (Whatley, 1971) and in severe cases in Tradescantia, a decrease in the number and size of grana, together with a dissolution of granal and inter-granal thylakoids. Iron deficiency also inhibits chlorophyll synthesis in green plants and photosynthetic bacteria (Price, 1968). The gp gene in yellow pods could thus cause a block in the utilisation pathway of any of these nutrients or it could produce an inhibitor by disruption of some other metabolic pathway or disrupt the nutrient supply to the mesocarp.

Armond and Arntzen (1977) found that the grana partition regions in pea leaf chloroplasts contain 75-80 % of all photosystem II (PS II) centres (the other 20-25 % being on the stroma lamellae) and that the stroma lamellae required higher light intensities to attain maximal rates of PS II activity than did the granal membranes. This was also observed by Koenig, Menke, Craubner and Schmid (1972), who found that the photosystem I (PS I) activity was approximately equal between the stromal and granal lamellae. The structure and high number of the chloroplasts observed in the inner epidermis of both gpgp and GpGp

pods thus suggests that they are capable of photosynthesis, particularly as the amount of radiation reaching this layer (in excess of 688 and 347 $\mu mols^{-1}m^{-2}$ respectively) is sufficient to support photosynthesis. This would be true of both green and yellow pods since no abnormal chloroplasts were found in the gpgp inner epidermis.

The thin cuticle of the inner epidermis and the position of the chloroplasts around the periphery of the cells make them well-situated to obtain CO_2 from the pod space. Harvey, Hedley and Keely (1976) found that the CO_2 concentration in the pod space was much higher than ambient, varying between 0.1% and 4.3 % according to age. Similarly higher levels were observed in the pod space of developing lupins (Atkins and Flinn 1978). This is probably a result of seed respiration since the pod was capable of reducing this level when illuminated (Harvey, et al., 1976; Flinn, Atkins and Pate, 1977). It would appear that the pod and in particular the inner epidermis, is able to photosynthesise. This inner layer would be most important in the gpgp pod, as it receives relatively high levels of radiation and contains high levels of chlorophyll.

The chloroplasts of the inner epidermis of green and yellow pods contained little if any starch and it seems likely that the sugar they produce photosynthetically is transported for utilisation elsewhere, possibly in the seed. The first barrier to be met would be the parchment. Although eventually it is thick, water-repellant and apparently dead tissue, earlier in development it contained some plastids and mitochondria suggesting that it may play an active role in transport of metabolites between the inner epidermis and the mesocarp. Transport through the mesocarp is implied by the abundance of plasmodesmata in both GpGp and gpgp pods and also by the position

of plastids around the vascular bundles.

While chloroplasts are responsible for assimilating CO_2 into carbohydrate by photosynthesis, amyloplasts are present in storage tissue to synthesise starch from imported sugar (when carbohydrates are available to be laid down as a reserve) and then to mobilise the starch when the plant is again in need of carbohydrate (Kirk and Tilney-Bassett, 1967). The plastids of the mesocarp of both genotypes contained starch, further illustrating its role as a temporary store. This is supported by Flinn, Atkins and Pate (1977) who established that there was a large accumulation of carbon as pod dry matter followed by transport into the seeds, the seeds incorporating 86 % of the carbon acquired by the fruit. This was carried out on green-podded fruit. In yellow fruits, it is likely that the source of starch is from imported sugar rather than that produced *in situ*. However, they also found that the net daytime gain of carbon from the surrounding atmosphere was insignificant, suggesting that the mesocarp supported little photosynthesis. Data of Hayward, Price and Smith (1982) however shows that during the period of pod growth the illuminated pods of *GpGp* genotypes are capable of a net uptake of CO_2, while yellow pods are only capable of net CO_2 output similar to that observed in the dark.

The chloroplasts of the *GpGp* mesocarp had internal membranes arranged similarly, if slightly reduced, to those of pea leaves, suggesting only slightly lower photosynthetic potential. The chloroplasts of the *gpgp* mesocarp however were very inferior compared with those of a leaf, suggesting that they are inefficient at photo-synthesising. It thus seems likely that the mesocarp plastids of green pods photosynthesise and produce starch *de novo*. In view of the

paucity of thylakoid membranes of plastids in the mesocarp of yellow pods and hence the lack of necessary enzymes for starch synthesis, it seems unlikely that these plastids are capable of photosynthesis, although they may act as stores for starch. However, the inner epidermis seems likely to photosynthesise in both green and yellow pod types.

Another effect of the gp gene in the yellow pod mesocarp was an increased lipid content in the plastids which had few grana. Approximately 26 % of the total lipids present represent the photosynthetic pigments, which must be held in the correct orientation to allow energy transfer to occur in the light-harvesting complexes. The chloroplast membranes are essentially a lipid bilayer into which various proteins are embedded (Singer and Nicolson, 1972). The increased amount of lipid droplets observed in the gpgp pod may have been simply the result of lack of membrane formation, i.e. those lipids unable to contribute to thylakoid development. Alternatively the gene may regulate the production of lipid by affecting an enzyme or substrate involved in lipid synthesis. It may be an effect of ABA, which has been shown to increase lipid production in chloroplasts of wheat leaves (Mittelheuser and Van Steveninck, 1971).

Lipid is used by the seed during germination, small amounts having been laid down during development. Lipid droplets were observed in the green and yellow podded cotyledons, suggesting that the gp gene does not affect lipid in the seeds early in development. Although lipid content of dried pea seed has been recorded as 1-3 %, depending on the genotype (Miyazawa, Ito, and Fujino, 1974; Ito, Miyazawa, Minamide and Fujino, 1975; Coxon and Wright, 1985; Davies, Coxon, Gavrel and Wright, 1985) and crude lipid may be as high as 6 %,

no reference has been made to lipid content in relation to pod type. However, data from Coxon and Wright (1985) shows seed of the yellow-podded type to have a lipid content of 1.92 %, and seed of the parents of the green-podded lines isogenic except for the r-locus (JI 430 and JI 145) to have a lipid content of 3.37 %. This suggests that the gp gene does not affect the cotyledons. It may be that the gp gene reduces lipid production in the seed, or it may be that any increase in lipid in the pod is reflected by a decrease in the seed. Any pod lipid mobilised would be transported as sugar; it may then be re-assimilated as carbohydrate, not necessarily as lipid, there being no prima facie reason for gp to affect lipid of the cotyledons.

From results presented in this thesis, it appears that there was no difference in chlorophyll content per g fresh weight of the embryo between the GpGp and gpgp genotypes, suggesting that the gp gene has no effect on chlorophyll content of the embryo. The difference observed between pod genotypes indicated that the gp gene affects a pod character. However, any measurement is dependent upon the precise stage at which the seed or pod was harvested and during exponential growth even a matter of hours can produce very different results. Ideally it would be desirable to make measurements of chlorophyll content on several seeds from many ages and express chlorophyll content in terms of fresh weight of seed or embryo over a longer period than was described here. This would necessitate the use of very many seeds and unfortunately could not be included in the scope of this thesis.

A further complication is the r gene. The maternal parent of the lines isogenic except for the r-locus to which progeny were back-crossed was wrinkled (rr), whereas the yellow podded seed were

round (RR). It has been shown (Coxon and Wright, 1985) that wrinkled seeds have a higher lipid content than round seeds. The apparent increased lipid resulting from the double recessive gpgp gene may therefore be influenced by the presence of the gouble recessive rr gene. It would be interesting to look at the lipid content of yellow-podded wrinkled-seeded types, which from these data might be expected to contain higher levels of lipid. This is particularly pertinent at the current time, in view of the interest in increasing the storage product content of pea seeds in order to improve their nutritional value.

All pea fruits act as a portal for nutrient flow from the vegetative parts of the plant as well as synthesising their own metabolites. Although it has been established that pod metabolism is significant in terms of importing nutrients into the developing seeds, the extent of dependance upon the pod by the seed and the seeds ability to produce its own nutritional requirements is unclear.

The extent of pod transport to the seeds increases in proportion to the mass of seeds present (Lovell and Lovell, 1970) and is transferred via the vasculature of the pod and the funiculus.

Whether the testa is capable of fixing CO_2 by photosynthesis is unclear. Agarawal and Maitra (1975) suggest that if insufficient levels of radiation are available, chloroplast formation does not occur. However, radiation levels of 347-688 $\mu mol.m^{-2}s^{-1}$ were reaching the outer cells of the testa which would be sufficient to induce chloroplast production. The chloroplasts present in the outer layer of the testa of both the green- and yellow-podded types had several well-developed thylakoid membranes and small granal stacks, which although not as large as those of pods and leaves does further suggest

that they may be able to function photosynthetically.

The CO_2 concentration in the pod space is high since the seeds are respiring. There must therefore be gas exchange from the cotyledons to the testa and again from the testa to the pod space i.e. a CO_2 gradient through the fruit. The thin area of cell wall at the top of each of the palisade cells suggests that gas exchange is feasible, although no stomata were present. The CO_2 could then be utilised by the inner layer of the pod.

However, the osteo-sclereid cells contained chloroplasts which had quite large granal stacks, suggesting that they may be capable of photosynthesis. Flinn, Atkins and Pate (1977) reported that pea seeds were not capable of significant photosynthesis, but they only examined a single GpGp (green-podded) genotype. Bearing in mind the variation in pod and testa structure, it may be that other genotypes, particularly yellow-podded types which allow more radiation through, have testas if not embryos which are capable of photosynthesis. Seeds of lupin have been found to photosynthesise (Atkins and Flinn, 1978). This may be due to differences in pod morphology, for example thinner pod walls or lower chlorophyll content, which would allow greater amounts of useful radiation to reach the testa. Alternatively they may contain different enzymes necessary to support photosynthesis. Although the key enzyme responsible for CO_2 fixation, ribulose-1,5-bisphosphate carboxylase (RUBPcase) was found only in low levels in pea cotyledons early in development (Hedley, Harvey and Keely, 1975), more significant levels were found in lupins. Similarly, phosphoenolpyruvate carboxylase (PEPcase), the enzyme responsible for utilising CO_2 by non-photosynthetic pathways may be more abundant in lupins than in peas. It has however been recorded in the round and

wrinkled lines used here (Price and Taffs, 1984) and in other genotypes later in development (Hedley, Harvey and Keely, 1975). However, they suggest that it plays a role in seed germination rather than seed development.

The level of irradiation reaching the outer cotyledon cells was in the order of 80 $\mu molm^{-2}s^{-1}$. Hence it seems quite likely that these chloroplasts are capable of carbon-fixation. This seems particularly likely later in development when the chlorophyll in the pod is breaking down and there is more radiation of the blue and red wavelengths reaching these cells.

The plastids of all cells in the testa contained starch early in development. The role of the plastids of the outer palisade cells was probably one of temporary starch storage, as they did not appear to be structurally equipped for photosynthesis.

It was in the palisade cells that a structural difference was observed between the round and wrinkled genotypes. There were ingrowths of sclerified tissue in the RR but not in the rr testas. Similar ingrowths have been observed (Reeve, 1948) in other genotypes, although it was not stated whether the genotypes were round or wrinkled. Reeve (1948) suggests that the function is merely mechanical to prevent pathogens from penetrating the mature seed and also to prevent desiccation of the embryo. However, the many mitochondria, R.E.R. and plastids in this layer prior to contact point suggests a more active role, at least initially. Later in development, the round- and wrinkled-seeded genotypes seemed equally sclerified, so it may have been that only the rate of sclerification differed between the two genotypes. If this were true, then it may partially explain the different water content of the two genotypes

during development (Section 2). Further work needs to be done to establish whether or not this is the case by sectioning testa tissue over this developmental period daily so as to detect precisely when sclerification occurs in each genotype.

In the older osteo-sclereid cells some chloroplasts were found with a diagonal stacking arrangement of grana. These were similar to those found by Wellburn and Wellburn (1976) in the resurrection plant, Myrothamnus flabellifolia. It has been suggested that in this plant the displaced thylakoids allow a more effective presentation of photosynthetically active membrane to incoming radiation and hence increased efficiency. This plant has to develop under extreme conditions of water stress, which is not true for developing pea seeds although later in development they dehydrate. It may be that the unusual arrangement of the chloroplast membranes allows most efficient usage of the enzymes present. Alternatively, the arrangement may be a direct consequence of desiccation and in fact merely be a preliminary to the disintegration of the membrane. There is a paucity of information regarding such plastids, but as no such chloroplasts have been found in ordinary senescent plants it seems more likely that they are a special adaptation to adverse conditions. To determine their role in pea tissue would require a study of the gas exchange specifically of these cells. This would be very difficult to assess due to the position of the cells within the testa and of the testa within the fruit. However, it is interesting to postulate that this unusual granal arrangement would allow greater photosynthesis by the testa, particularly during pod senescence.

Another interesting feature of these osteo-sclereid plastids later in development was the presence of peripheral reticulum. This

was originally observed in C_4 plants (Laetsch, 1968; Laetsch and Price, 1969; Osmond, Troughton and Goodchild, 1969), but has since been found in various C_3 plants (Laetsch 1970; Taylor and Craig, 1971). Its precise function is unknown but there is evidence (Hilliard and West, 1971) that it decreases the rate of photo-respiration. Also, it may increase the CO_2-affinity of chloroplasts, by enhancing the transport of CO_2 from the chloroplast surface to the stroma (Gracen, Hilliard, Brown and West, 1972). This would allow partial recapture of photorespiratory CO_2 from the cytoplasm and thereby reduce apparent photorespiration rates. Normal green C_3 and C_4 plants can photosynthesise in the light even under low levels of CO_2 as part of a compensatory mechanism (Laetsch, 1974). However, the high levels of CO_2 present through the pod and testa make it extremely unlikely that photorespiration would occur as CO_2-loss would be restricted. Peripheral reticulum may be a feature of chloroplasts of cells involved in CO_2 transport without actually fulfilling an active role. Much more work needs to be carried out before such a suggestion could be confirmed, especially as peripheral reticulum is partially controlled by environmental conditions (Gracen et al., 1972).

The large quantities of starch present in the parenchyma cells of the testa suggest an immediately exploitable reserve when the sugar supply is restricted. It is also possible that it is a transport route from the pod to the developing embryo. Thorne (1980) has shown that in soybeans there is a lag phase in sucrose appearence in the seeds, suggesting that the integument - embryo interface may be a control point for sucrose accumulation by the cotyledons. Similar observations have have been made by Lichtner and Spanswick (1981). They found that starch accumulation in the plastids of the testa and

coytledons of soybean was transient and that it disappeared as protein and lipid bodies were formed. Sugars were found to be used as a means of osmotic adjustment, providing a driving force for water uptake, turgor maintenance and cellular enlargement (Lichtner and Spanswick (1981) and it is possible that sugars are used similarly in the developing peas. It is possible also that a passive transport of photosynthate occurs by diffusion down a concentration gradient through the cells, established at one end in the outer layers by phloem unloading and maintained at the other end by utilisation by the embryo. Thorne (1982) showed that the rates of unloading of photosynthate into soybean embryos were dependent upon the photosynthetic rate of the plant. Thus, in addition to being controlled by the testa and its apoplast and symplast, the embryo is also affected by the photosynthetic regime of the whole plant. The similarity between pea and soybean anatomy suggests that this may also occur in peas. Since the cotyledons of peas only contain a provascular system (Craig, Goodchild and Hardham, 1979), metabolite transport through the seed must be by a different route and the possibilities of apoplastic or symplastic transport are further discussed in Section 2.

The abundance of mitochondria and R.E.R. in the parenchyma suggest that this tissue is responsible for producing nutrients which may then be secreted via the endosperm for utilisation by the embryo. The parenchyma cells of the testa early in development have large inter-cellular spaces, not dissimilar to plants of wet, oxygen-deficient habitats (Thorne, 1981) and similar to those of the cotyledons. The advantage of these spaces may be to facilitate oxygen movement through the seed-coat to the embryo, there being a ten-

thousand-fold increase in diffusion through inter-cellular air compared with air dissolved in water within the cell-walls (Thorne, 1981). This oxygen would then be utilised by the abundant mitochondria which produce ATP and NADP in these cells. These air spaces also result in the testa being spongy and it may therefore act as a cushion to protect the expanding embryo.

An interesting feature was that neither the gpgp nor the rr genotype appeared to have any effect on the plastid structure of the cotyledons. The diversity of structure of the cotyledon plastids could suggest various roles. Many, particularly in the outer layers, were well-developed and resembled chloroplasts found in leaves and pods. The amount of radiation reaching these cells early in development (approximately 30 $\mu molm^{-2}s^{-1}$), may not be sufficient to support photosynthesis but would possibly play a role in chloroplast development. However, once the pod is senescing the levels of radiation reaching the outer cells of the cotyledon would be in the region of 80 $\mu molm^{-2}s^{-1}$, which could support photosynthesis. This would then reduce the seeds nutritional dependance during development (Flinn, Atkins and Pate, 1977).

Chlorophyll present in the cotyledons, testa and pods may serve to control the development of the inner layers of the developing embryo by modifying the photoenvironment. In particular, it may modulate the amount of red and / or far-red radiation reaching the embryonic axis, or it may screen out harmful radiation. A window cut in the pod wall to the sclerenchyma results in the cotyledons appearing darker green in subsequent development (D.N. Price, personal communication). This may just be a response to increased radiation, increasing chlorophyll production, but it illustrates the point that

the light regime of the embryonic axis can be modified and regulated by any part of the fruit.

Amyloplasts were not very abundant in these outer cotyledon layers, but irregularly shaped plastids were present with dilated membranes in the inner cells. Although only the outer few layers of cells were examined, it was still apparent that there was a gradation of storage material, as described by Smith (1973). Mitochondria and ribosomes were abundant in all cells of the cotyledons examined, reflecting their highly active role.

The cotyledons become filled with protein bodies and starch grains (Bain and Mercer, 1966; Horowitz, 1983). Since the r locus is known to affect starch synthesis (Kooistra, 1962; Biliaderis, 1981; Boyer, 1981), it was expected that this difference might have been apparent under the electron microscope. However, no difference was observed except in the outline of the starch grains. The starch grains of the wrinkled (rr) cotyledons had an indistinct outline, similar to iron filings round a magnet. Since all tissues underwent identical fixing, embedding and staining procedures and the surrounding membranes do not appear abnormal, it seems unlikely that this effect is a result of the technique employed. This phenomenon was possibly a result of the different amylose / amylopectin ratio affecting the stain binding. The overall shape of the starch grains did not appear to be different between these two genotypes, unlike the view obtained under the light microscope (Section 2), where their outlines are significantly different. The sectioning procedure may in fact break up the starch and thus mask the shape, but cannot alter the composition which may affect the stain. Electron micrographs of mature pea seed by other workers (Bain and Mercer, 1966; Horowitz,

1981) has not shown this effect. This may be due to the genotypes used or possibly be an effect of the procedures employed.

Most of the storage material, protein bodies and starch grains, was present after the endosperm had been absorbed by the cotyledons. It may be that the endosperm metabolites are utilised directly in the production of these storage products, or it may be that uptake is a signal for storage product synthesis. Correlating with this, the cells of the outer epidermis develop wall ingrowths classified as "transfer cells" (Gunning and Pate, 1974). These are believed to increase the efficiency with which the cotyledons absorb solutes secreted by the endosperm. Although not observed in this tissue, it may be that they only develop at a specific stage, and / or that they are genotype-dependant. Further investigations over the period of growth when endosperm is present would be needed to determine more precisely its role.

SECTION 2

GROWTH ANALYSIS & OSMOTIC STUDIES *IN VIVO* AND *IN VITRO*

"One side will make you grow taller," remarked the caterpillar,
"the other side will make you grow shorter."

"One side of *what*? The other side of *what*?" thought Alice.

Alice in Wonderland

INTRODUCTION

Having established that the r locus has no apparent effect on chloroplast structure of pod, testa nor cotyledon (Section 1) despite affecting starch formation (Kooistra, 1962; Boyer, 1981; Colonna and Mercier, 1984), further investigations were carried out in an attempt to determine how the r locus affects fruit and especially seed development.

The general growth pattern of pea fruits has been well documented (Bisson and Jones, 1932; McKee, Robertson and Lee, 1955; Bain and Mercer 1966; Flinn and Pate, 1968; Smith 1973; Hedley and Ambrose, 1980). Initially the pod wall grows faster than the seeds (Eeuwens and Schwabe, 1975), the pod having reached more than half its final fresh weight before the seeds enter their exponential phase of growth (Flinn and Pate, 1968). Growth of the pod wall follows a sigmoid curve (Eeuwens and Schwabe, 1975), an exponential period of rapid growth followed by a decline in rate of growth and finally dehydration. During this latter phase total nitrogen, minerals and dry matter decrease, indicating that more than a loss of water is involved (Flinn, 1969).

The initial rapid growth phase of all seeds of a variety of genotypes was found to be exponential, resulting in the relative seed growth rates being linear. Comparisons between genotypes demonstrated differences in relative growth rate during this phase (Hedley and Smith, 1985). The seed-coat initially grows at a faster rate than the embryo and almost attains its maximum fresh weight prior to the embryos' exponential phase of growth (Hedley and Ambrose, 1980). The difference in initial growth rate of the testa

and embryo results in a space around the embryo within the embryo sac, which becomes filled with liquid endosperm secreted by the cellular endosperm and the testa. During subsequent cell division and expansion the liquid endosperm is absorbed by the developing embryo which ultimately fills this space.

Initially the embryo grows mainly by cell division followed by increasing amounts of cell expansion (Bisson and Jones, 1932; Bain and Mercer, 1966; Hedley and Ambrose, 1980) during which most of the storage products are laid down (Smith, 1973). The allometric relationship between embryo cell number and embryo size therefore changes significantly when the cell population changes from mainly dividing to mainly expanding cells (Hedley, Ambrose, Smith, Cook and Wang, In Press). The growth pattern changes according to the genotype, a diauxic pattern occurring once the embryo has filled the embryo sac, although in some cases this lag occurs before embryo sac filling. Eeuwens and Schwabe (1975) showed the growth pattern of the embryo to be a double sigmoid curve separated by a lag phase. The lag corresponded to a decline in the growth rate of the testa and to the expansion phase of the embryo, which occurred when the seed was less than half its maximum fresh weight. Whether this lag was due to a decline in the number of cell divisions or amount of cell expansion is unclear as there has been little work on cell numbers of developing pea seeds.

Growth of the seeds also exhibits a double sigmoid curve (Eeuwens and Schwabe, 1975). The onset of the lag phase is closely associated in some genotypes with a sharp decline in the growth rate of the testa and the disappearance of the endosperm. Although both the embryo and the testa increase in fresh weight at the end of the

lag phase, only the embryo increases in dry weight. This accumulation of dry matter by the embryo, partly at the expense of the pod wall and testa (Bisson and Jones, 1932; McKee, Robertson and Lee, 1955), results in a peak in relative growth of the seed during the post-lag period (Eeuwens and Schwabe, 1975).

The absolute rate of development of the pea fruit varies according to genotype and the environment in which the plants are grown (McKee, Robertson and Lee, 1955; Carr and Skene, 1961; Flinn and Pate, 1968; Burrows and Carr, 1970; Hedley and Ambrose, 1980). In order to determine solely genetic differences it is necessary to adopt stringent environmental controls. Eeuwens and Schwabe (1975) reduced within-genotype variation by using a single seed from each of 50 pods of known age. Hedley and Ambrose (1980) used 3 seeds from similar positions in pods from a given node and found that the variation between seeds from the same position from several pods was similar to the variation between the three seeds from the same pod. A similar sampling method was used in the course of this thesis to minimise within-plant variation.

Relatively little has been reported on the physiological effects of the r locus. It was first reported by Mendel (1865) to affect the appearance of the mature dried seed, which is wrinkled only in the homozygous recessive (rr) genotype. Starch grains of the round (RR or Rr) type are simple, oval-shaped and tend to be larger than the complex, fissured grains of the wrinkled genotype (Gregory, 1903; Darbishire, 1908). The mature wrinkled seeds contain approximately twice as much sugar as the round type and take up more water upon germination (Gregory, 1903; Darbishire, 1908).

Since round-seeded lines can show indentations on their

surface which often makes them appear "wrinkled" (a consequence of the di gene, see Plate 1.10), a better criterion for distinguishing between round- and wrinkled-seeded peas is the structure of the starch grains in mature dried seeds. However, Kooistra (1962) reported a third type of pea seed indistinguishable from seeds with the rr genotype in having a very wrinkled seed surface but having simple/ oval starch grains. Test crosses showed this to be due to a gene at a different locus and on a different chromosome to the r locus, and this new locus was given the label rb. There are therefore four types of pea seed, differing in starch and external morphology depending on the combination of genes at the r and rb loci:

RR RbRb : round, simple starch grains

rr RbRb : wrinkled, compound starch grains

RR rbrb : wrinkled, simple starch grains

rr rbrb : wrinkled, compound starch grains

The dominant factor R is responsible for simple starch grains. Round seed is only obtained if both R and Rb are dominant, thus since the round near-isogenic line used in this thesis is known to have simple starch grains, these lines must have the dominant Rb gene.

Most plant starch consists of two major components: amylose and amylopectin (Kellenbarger, Silveira, McCready, Owens and Chapman, 1951). Both are α-glucosides but amylopectin has a branched chain structure while amylose has an essentially straight chain structure.

In most plant species, starch is composed of approximately 30% amylose and 70% amylopectin. However, in several species mutants have been identified having more than 60% amylose. Starch from roots, cereals and smooth round-seeded (RR) peas usually contain 20-25% amylose (Nielsen and Gleason, 1945; McCready, Guggolz, Silveira and Owens, 1950), although Kellenbarger et al. (1951), Schneider (1951), Yarnell (1962) and Colonna and Mercier (1984) recorded values of 38% amylose in various round-seeded pea genotypes. Much higher values of 70% (Nielson and Gleason, 1945; Hilbert and MacMasters 1946; McCready et al., 1950) and of 90% (Peat, Bourne and Nicholls, 1948), have been found in the wrinkled-seeded lines. However, the method used by Peat et al. (1948) is likely to indicate higher levels than those actually present, and so may be disregarded.

The composition of the amylose in both round and wrinkled seeds is similar (Potter, Silveira, McCready and Owens, 1953). However, the amylopectin component from wrinkled seed was found to have an abnormal chain length of 36 glucose units instead of the normal 26 units and to also have a smaller molecular size than that of the round seed (Potter et al., 1953). The amylopectins from wrinkled seeds could however be separated by differential ultra-centrifugation into normal amylopectins with chain length of 26-27 glucose units and those containing short chain amylose (Greenwood and Thomson, 1962).

The increased linearity of starch from wrinkled-seeded cotyledons is due to increased amylose content as well as decreased branching in the amylopectin molecules (Hilbert and MacMasters, 1946; Boyer, 1981). Alterations in the starch synthetic pathway due

to genetic variation have been used in other species to study starch synthesis and investigate the physiological basis for gene action. Work carried out on a high-amylose maize mutant amylose-extender (ae), focused on the soluble starch synthetic enzymes starch synthase (E.C.2.4.1.21) and starch branching enzyme (E.C.2.4.1.18) (Boyer and Preiss, 1978b). The high amylose content of ae starch was found to be partially attributable to an enzymic lesion in one of the three forms of starch branching enzyme, specifically an absence of branching enzyme IIb. Each of the three forms of enzyme in maize appears to be under independant control (Boyer and Preiss, 1978b). Boyer, Damewood and Matters, (1980) found similarities between the ae in corn and wrinkled-seeded cultivars of pea with regards to starch characteristics. The possibility of a comparable enzymic deficiency in pea was investigated by Matters and Boyer (1982) who found that branching enzyme activity in cotyledons of the wrinkled rr genotype was 10% the level of the round RR cotyledons. In addition, a single soluble starch synthase was found in the rr extracts whereas two were found in the RR extracts.

A similar reduction in the amount of branching enzyme present in the wrinkled-seeded cotyledon was reported by Edwards (1984). He found that fructose-2,6-bisphosphate was more abundant in RR than rr embryos during development and that this correlated with higher amounts of starch found in the RR embryo. Whether the r locus acts to regulate the action or structure of the branching enzyme is unclear. Sucrose, glucose, fructose, hexose phosphates, triose phosphates and fructose-1,6-bisphosphate were all present in greater amounts in rr than RR embryos (Edwards, 1984) although the lines used in this were work were not isogenic except for the r locus.

The difference in appearance of the starch grains has been suggested by Schneider (1951) to be a result of the different amylose / amylopectin ratios in the two types of seed. He also suggested that this ratio accounts for the difference in water content of immature round and wrinkled seeds, although there is no evidence to support this. From observations, Boyer (1981) suggests that the wrinkled appearance results from the splitting of the rr starch grain as the seed matures, cracking and exposing the amorphous interior presumably high in amylose.

The amount of starch present also differs between the two genotypes; 33-36% in wrinkled compared with 45-49% in round seed (Kappert, 1915; Kellenbarger, et al., 1951).

Protein content of the mature dry seed is also affected, rr types containing approximately 3% more protein in the meal, although the absolute amount per plant does not appear to be affected (Shia and Slinkard, 1977; Jermyn and Slinkard, 1977). As the round-seeded types have larger seeds than the wrinkled (Shia and Slinkard, 1977), the total yield of protein per seed is likely to be similar in both. However, using the same lines (BC/R and BC/r) near-isogenic except at the r-locus as are used in this thesis, Domoney and Casey (1985) found more legumin per unit protein present in mature dried seeds of the round (RR) than in the wrinkled (rr) genotype. Davies (1980) using a series of other lines near-isogenic except for the r locus obtained similar results. The size of this difference ranged from a 9% decrease to a 79% decrease in legumin depending on the genetic background of the isoline (Davies, 1980). This reduction of legumin may be a secondary effect related to the overall metabolism of the cotyledons (Davies, 1980; Matters and Boyer, 1982). Davies (1980)

further suggests linkage between r and the locus for the sub-units of legumin, but the true interaction of starch and protein accumulation has yet to be elucidated.

In general, the proportion of legumin to vicilin is higher in the RR than in the rr isolines (Davies, 1980; Davies and Domoney, 1983). The percentage of legumin in the globulin of these round and wrinkled seeds was very variable, but the mean values for the wrinkled-seeded genotypes (18.6%) was significantly lower than that for the round (37.7%). There is also an inverse relationship between albumin and legumin; a high albumin content being found in association with low levels of legumin such as in rr genotypes (Schroeder, 1982).

The r locus has been shown to affect lipid content, the rr genotype having approximately 5% crude lipid compared with about 3% in the RR genotypes (Coxon and Davies, 1982). The rb gene has also been shown to affect lipid content. Although only examining one example of each genotype, Coxon and Davies (1982) found that the rb rb genotype had a significantly higher lipid content than the Rb Rb genotype.

The water content during development is greater in wrinkled genotypes than in round (Kappert, 1915; Ottosson, 1958; Hedley and Ambrose, 1980) and water loss during ripening at the end of development is higher in wrinkled genotypes. Cell enlargement occurs when a demand for water is created by extension of the cell walls under action of turgor and water is supplied by gradients in water potential (Boyer, 1968; Lockhart, 1968; Cleland, 1981; Cosgrove, 1981). As extension occurs, turgor decreases and creates a lowered water potential within the cells. This low water

potential causes water to enter the cells, enlarging them. During this process solute must continually accumulate in order to prevent dilution and maintain the osmotic forces necessary to induce enlargement and supply metabolites for wall synthesis (Boyer, 1985).

The sugar content of pea seeds has been found to increase to a maximum and then remain fairly constant before declining during maturation (Bisson and Jones, 1932). Sucrose is the major sugar in developing pea seeds (Stickland and Wilson, 1983). It thus seems likely that the sucrose and water contents of the developing pea fruits are important in determining final seed size and more pertinently any differences between round- and wrinkled-seeded genotypes. In particular, since starch has been found to differ between the two types, the results of a study of sucrose and osmotica may help to elucidate its effects.

Such studies are more easy using in vitro systems in which it is possible to vary the osmotica in which embryos are grown and hence deduce the effect of osmotica on in vivo growth.

Several attempts have been made to culture whole fruits, seeds and cotyledons of legumes. Baldev, Lang and Agatep (1965) successfully cultured intact pea pods in simple solid medium containing mineral nutrients plus 5% sucrose and demonstrated that seeds developing within these pods could synthesise gibberellins. Millerd, Spencer, Dudman and Stiller (1975) developed a culture system whereby immature pea cotyledons could grow and synthesise DNA, RNA, starch and storage proteins, albeit at a slower rate than in vivo. A similar decrease in in vitro growth of pea pods was also found by Srivastava, Varga and Bruinsma (1980). Soya bean cotyledons have been shown to accumulate dry matter and storage

protein at a faster rate than *in vivo* (Thompson, Madison and Muenster, 1977), although this was probably a result of increased temperature.

No benefit to growth was derived by adding kinetin (a commercial cytokinin known to increase cell division) (Stafford, 1978) or gibberellic acid (Thompson *et al.*, 1977) to the culture medium of pea seeds.

Both pea and soya bean cotyledons have been shown by culturing experiments to be intrinsically capable of synthesising their own structural and metabolic components at the stages examined (Millerd, Thomson and Schroeder, 1977). Stafford (1978) found that higher osmotic potential of the culture medium facilitated the *in vitro* growth of younger embryos. In addition to inhibiting germination of the embryonic axis, this medium allowed an increase in fresh weight, dry weight, protein, DNA and starch. She found that in one genotype (JI 181) the rate of growth *in vitro* matched that *in vivo*. Media of different osmotic potentials were required to culture flax embryos at different developmental stages (Pretova, 1974).

The osmotic state of the whole fruit thus seems to play an important part in embryo development and in particular in the transport of solutes. A high sucrose concentration in the seed coat apoplast did not inhibit sucrose and amino acid transport into an empty ovule when compared with a control solution of the same molarity (Wolswinkel and Ammerlaan, 1984) and phloem transport into the seed coat of developing pea seeds is sensitive to the osmotic environment. Wolswinkel and Ammerlaan (1984) also showed that increasing concentrations of mannitol greater than 200 mM increased

transport into empty ovules, but that above 700 mM unloading from the seed-coat decreased, i.e. there is an optimum osmoticum.

Ideally, any culture medium should resemble the environment within the seed. Solutes in the endosperm are likely to be in a constant state of flux during rapid embryo growth; low concentrations of metabolites could result from extensive absorption and assimilation, whereas a high concentration could result from synthesis of products exported from the embryo. Such variation in endosperm composition has been found in Phaseolus vulgaris L. seeds during development (Smith, 1973) and the endosperm of Lupinus spp. and Pisum sativum L. has been shown to modify translocates entering the seed (Hocking and Pate, 1977). The liquid endosperm in which embryos are constantly bathed has a lower (more negative) osmotic potential (Ryczkowski, 1969) and low osmotic values are beneficial to the growth of young embryos (Pretova, 1974; Raghaven, 1977). Once the endosperm has been absorbed the embryo is in close contact with the cellular endosperm and testa, thus synthesis of storage materials in the cotyledons would be expected to make new demands on the surrounding tissue. A culture medium based on the biochemical composition of the embryo would not take into account differences occurring in the rates of uptake from surrounding tissue. However, comparison of the embryo and immediate extra-embryonic environment may give some indication of suitable media for embryo culture.

Factors other than the composition of the medium affect soyabean cotyledon growth in culture (Thompson et al., 1977). These include pH and volume of media, amount of light, number of cotyledons per vessel and whether or not they were shaken. Stafford (1978) found that the gas volume to liquid volume ratio was

important when culturing pea embryos.

The preceding examples of pea fruit development have included a variety of different genotypes and pea cultivars of different backgrounds. The genes involved are likely to have a pleiotropic effect and precise gene effects are thus difficult to ascertain. The remainder of this section deals with two lines, near-isogenic except for the r locus, in an attempt to remove other gene effects and determine the effect of the r gene. Growth analysis was carried out on pods, testas and embryos of these two lines to determine the effect of the r gene on development. In addition cell numbers per embryo and cell size were investigated at specific stages to determine whether these varied according to genotype.

Solute potentials of the different tissues in the fruit were therefore studied in greater detail using lines near-isogenic for the r-locus.

Since the aim of the studies in this thesis was to determine differences between two different genotypes rather than the effect of different environments, only a single culture system was used, having a constant gas volume to liquid volume ratio.

A study of the *in vitro* growth of RR and rr embryos was subsequently carried out to determine whether different environments invoked different growth responses in the two genotypes. Some work on reciprocal crosses was also carried out to determine whether there was a gene dosage effect. These data were then related to the *in vivo* data acquired to further establish the mechanism of the r gene action.

RESULTS

A. GROSS MORPHOLOGY

From observations, growth of the two near-isolines appeared similar: height, colour, time and node of first flower (Plate 2.1); colour (Plate 2.2 A) and size of pods (Plate 2.3) and seeds (Plate 2.2 B).

B. GROWTH ANALYSIS IN VIVO

From a two-way analysis of variance, no significant difference was found in fresh weight nor dry weight accumulation between nodes of a single genotype and hence data was pooled to give one set for each tissue (pod, testa, embryo) per genotype.

i) Carpel Growth

The overall trend in fresh weight was a steady increase over the time examined (Fig. 2.1), although after day 22 there was relatively little increase in fresh weight. The main increase occurred between day 16 and 22 and the main difference in fresh weight between round- and wrinkled-seeded genotypes at days 16, 17, 24 and 26. The standard error at these ages was higher than at other stages suggesting greater variation. There was no exponential phase of pod growth, this would have occurred prior to day 10.

The percentage water in the pod decreased from a maximum of 90% at day 10 to a minimum of 85% during days 16-30 (Fig. 2.2). Thus the increase in fresh weight after day 16 was due to increase in dry weight as well as an increase in absolute water content.

The overall trend in pod dry weight was a steady increase prior to day 23, after which there was no increase in dry weight

PLATE 2.1

Characteristic plants of the round-seeded (RR) and wrinkled-seeded (rr) lines of Pisum sativum L. near-isogenic except for the r-locus, grown in a greenhouse under natural illumination in the summer 1983 and transferred to a controlled environment room ($15^{o}C \pm 2^{o}C$, 16h photoperiod) just before the first flower opened

Ruler = 300 mm


RR
rr

PLATE 2.2

Comparison of pods and seeds characteristic of the two lines of *Pisum sativum* L. near-isogenic except for the *r*-locus at stage II in development

A. Pods: RR round-seeded (*RR* genotype)
rr wrinkled-seeded (*rr* genotype)

B. Pods of A. (above) opened to reveal the developing ovules

Note that the seeds are alternate along the mid-rib of the pod

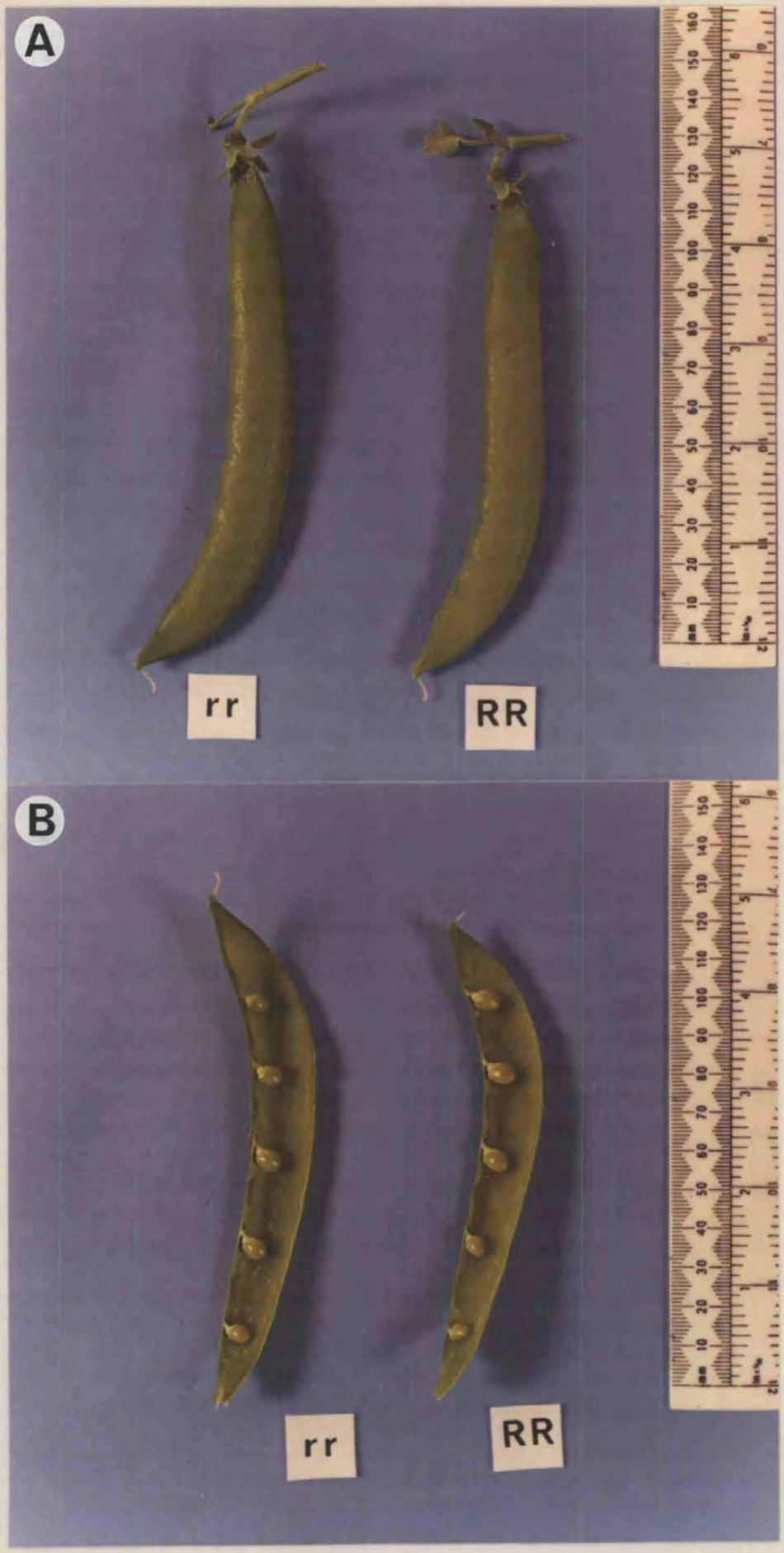

A
rr
RR
B
rr
RR

PLATE 2.3

Comparison of pods characteristic of the two lines of Pisum sativum L. near-isogenic except for the r-locus at 3 developmental stages

A. round-seeded RR genotype

B. wrinkled-seeded rr genotype

a) stage I (pre pod inflation)

b) stage II (post pod inflation)

c) stage IV (prior to senescence)

These pods are described in detail
in Table 1.2, p.26 of Section 1


A
c
b
a
11cm 12 13 14 15 16 17 18 19 20 21 22 23 24


B
c
b
a
10 mm 11cm 12 13 14 15 16 17 18 19 20 21 22 23 24

FIGURE 2.1

Comparison of the change in pod fresh weight (A) and pod dry weight (B) with time of the two lines of Pisum sativum L. near-isogenic except for the r-locus

o——o round-seeded BC/R genotype (RR)

x- - x wrinkled-seeded BC/r genotype (rr)

Each point is the mean of 15 samples

Bar lines represent the standard error of the mean

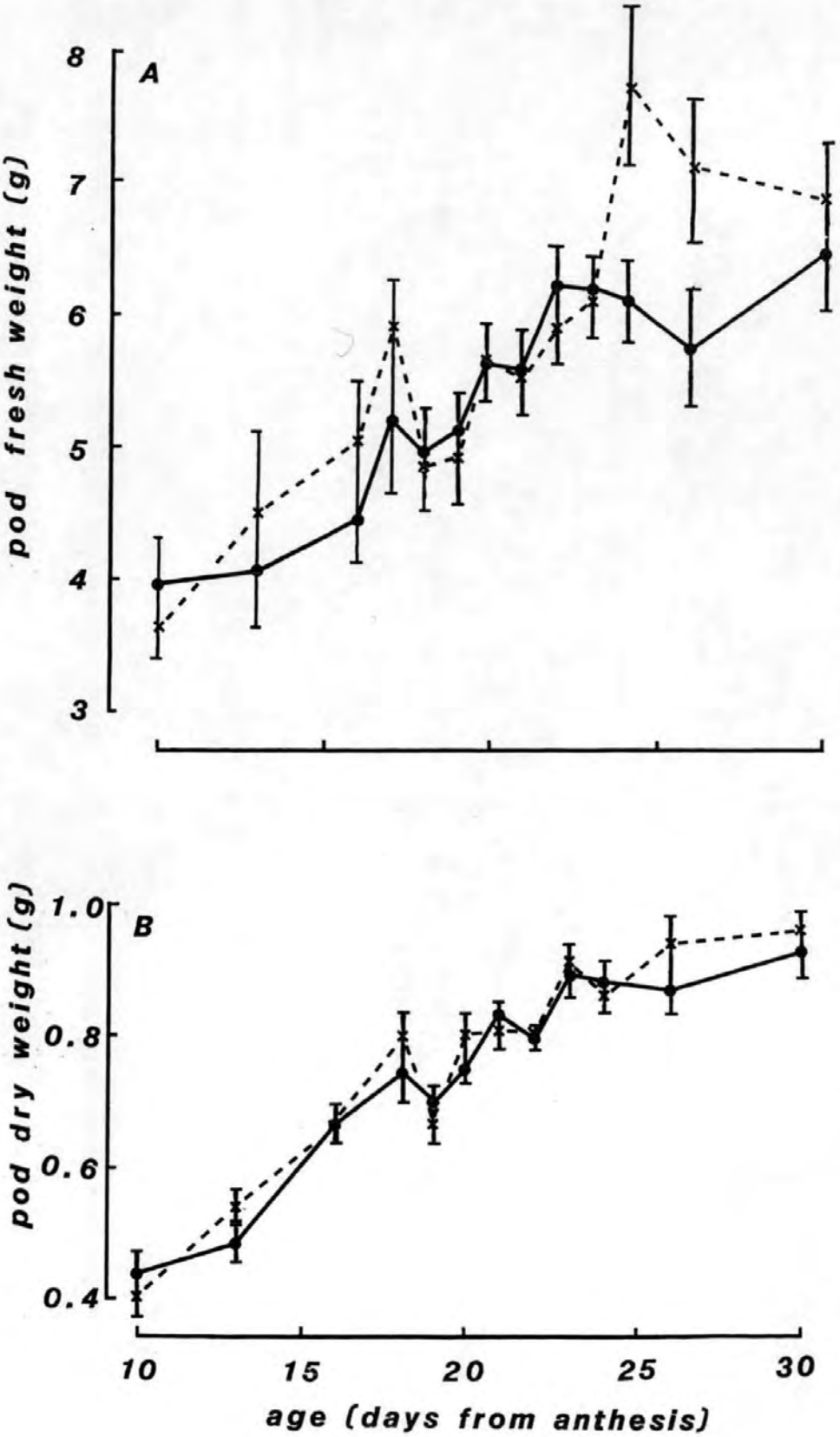

A
pod fresh weight (g)
8
7
6
5
4
3
B
pod dry weight (g)
1.0
0.8
0.6
0.4
10
15
20
25
30
age (days from anthesis)

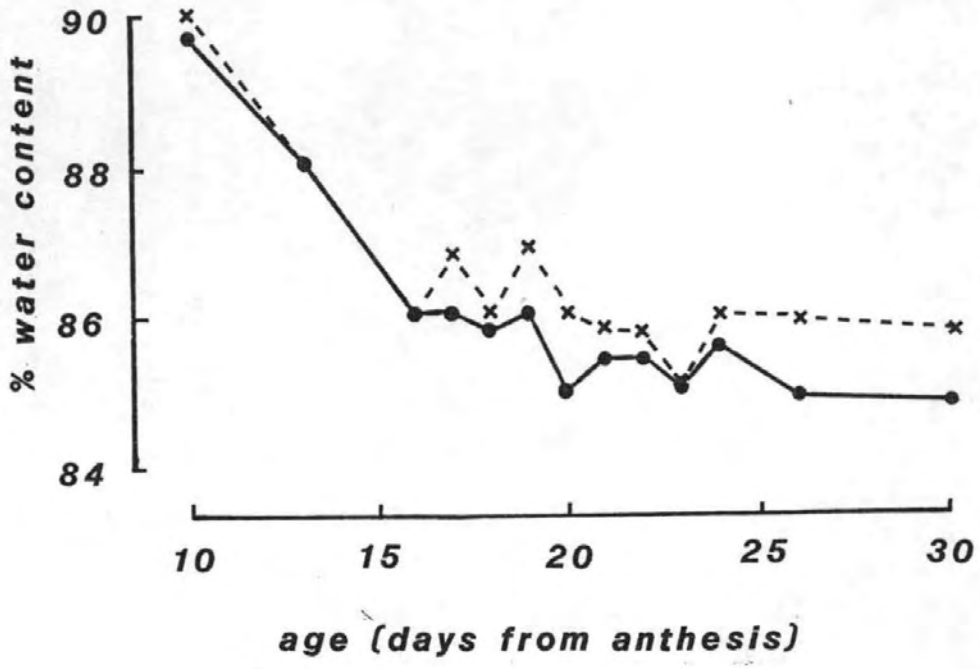
FIGURE 2.2

Comparison of the percent water content of pods of the two lines of Pisum sativum L. near-isogenic except for the r-locus

o——o round-seeded BC/R genotype (RR)

x - x wrinkled-seeded BC/r genotype (rr)

Each point is the mean of 45 samples


90
88
86
84
% water content
10
15
20
25
30
age (days from anthesis)

(Fig. 2.1). There was no significant difference between genotypes at any stage examined.

The greater variation in the fresh weight data may have been due to the greater vulnerability of the fresh pod when being weighed as it would have been losing water.

There appeared to be no difference between the round- and the wrinkled-seeded genotypes in the fresh weight/ dry weight ratio (Fig. 2.3).

Pod wall thickness of the homozygous round- (RR) and wrinkled-seeded fruits was the same ($P < 5\%$) at the stage examined (Table 2.1). It was 1.3 mm in both genotypes at the fully expanded stage (stage III). There was no significant difference ($p < 1\%$) between stomatal frequency of homozygous round and wrinkled pods at the stage examined (Table 2.2). It was approximately 6 stomata per square mm of pod epidermis.

ii) Seed Growth

There was an initial exponential phase of growth to day 16. At days 17-18 there was a lag in wrinkled seed growth which was not evident in the round seed (Fig. 2.4). The round seed however exhibited a lag in growth between day 21-22, which was not apparent in the wrinkled seed.

Analysis of variance also showed that there was no difference in seed growth between genotypes prior to day 18, but that after this there was a significant difference, the wrinkled seed having a consistently greater fresh weight than the round.

Starch grains of mature dried seed differed; RR being simple, round and larger than the rr grains which were composite (Plate 2.4).

FIGURE 2.3

Relationship between fresh weight and dry weight of pods of the two lines of *Pisum sativum* L. near-isogenic except for the *r* locus

- round-seeded BC/R genotype (*RR*)

x wrinkled-seeded BC/r genotype (*rr*)

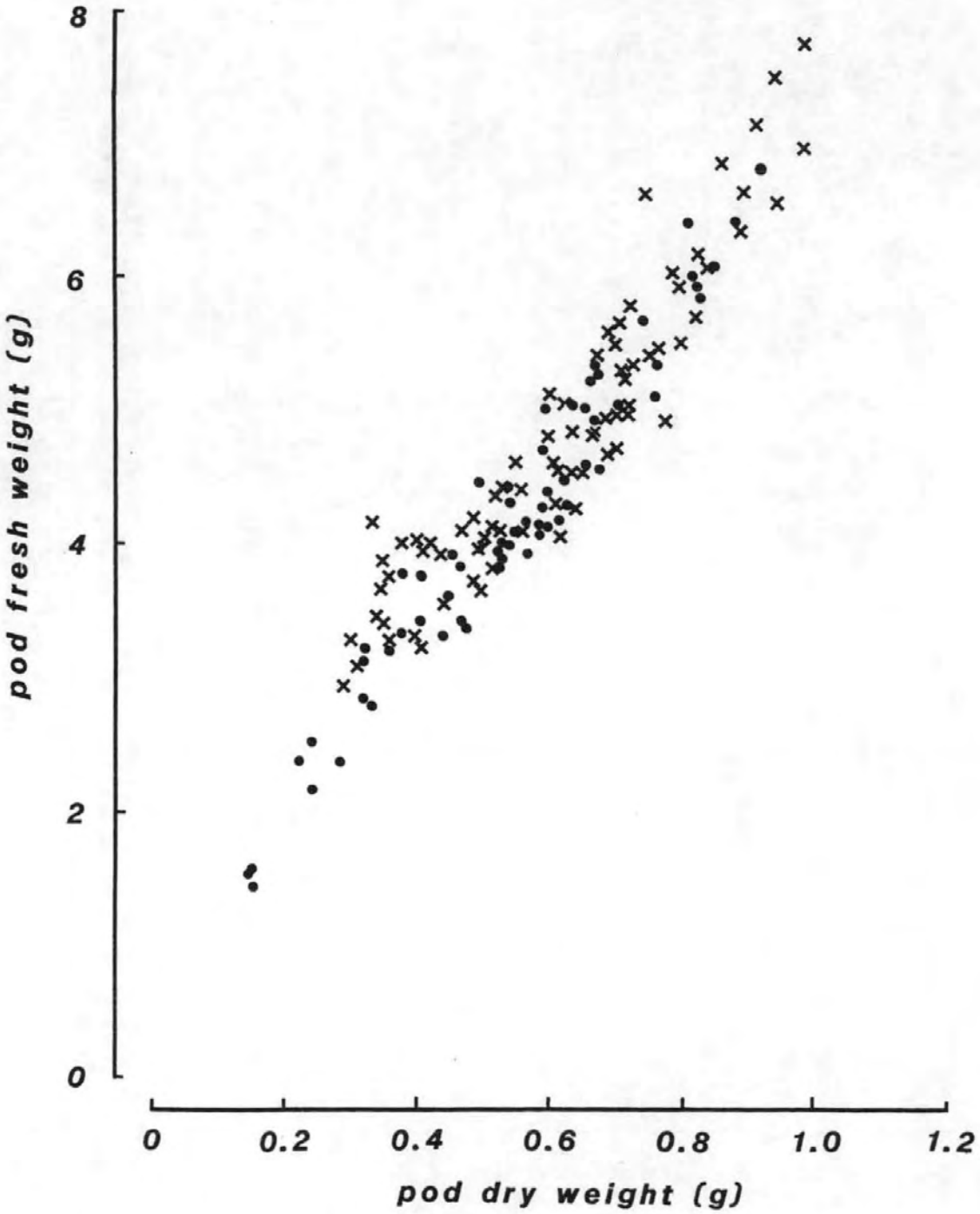

8
6
4
2
0
pod fresh weight (g)
0
0.2
0.4
0.6
0.8
1.0
1.2
pod dry weight (g)

TABLE 2.1

Comparison of pod wall thickness of two lines of Pisum sativum L., BC/R and BC/r, near-isogenic except for the r-locus at a single developmental stage (V)

	mean* thickness (mm) of pod walls:	
Genotype:	RR	rr
	1.44	1.23
	1.22	1.27
	1.35	1.37
	1.30	1.38
	1.42	1.28
Mean of means:	1.35 ±0.09	1.31 ±0.07

*means were derived from five samples each of 10 pods

TABLE 2.2

Comparison of stomatal density of pods of two lines of *Pisum sativum* L. near-isogenic except for the *r*-locus at a single developmental stage (stage III)

genotype:	mean stomatal density: (2) (no. stomata/0.1mm^2)
BC/R (RR)	11.0 + 0.9
BC/r (*rr*)	10.2 ± 1.37

Means were derived from 5 samples each from 10 different pods of the same age

FIGURE 2.4

Comparison of the change in seed fresh weight with time of the two lines of Pisum sativum L. near-isogenic except for the r-locus

o——o round-seeded BC/R genotype (RR)

x- - x wrinkled-seeded BC/r genotype (rr)

Each point is the mean of 45 samples

Bar lines represent the standard error of the mean

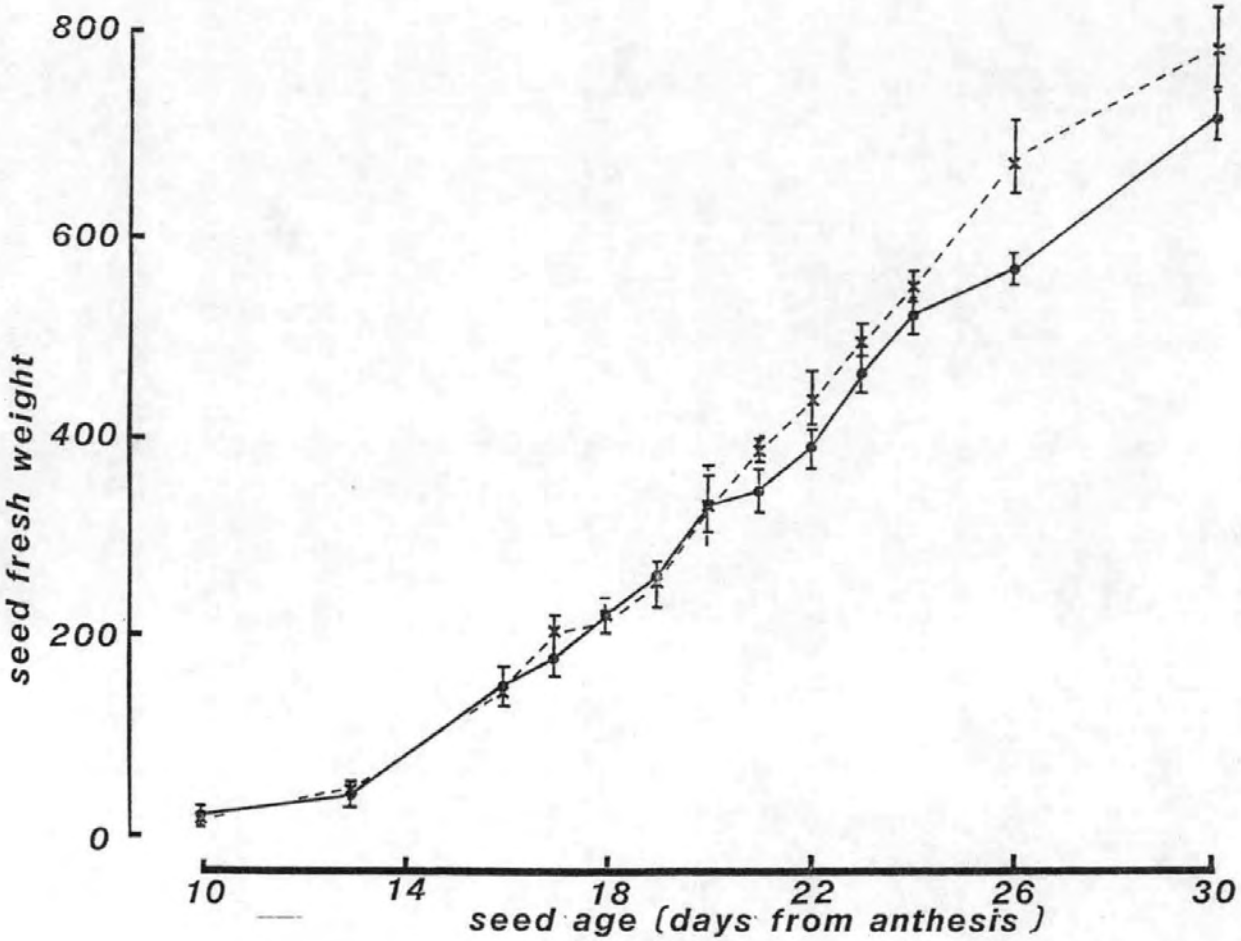

800
600
400
200
0
seed fresh weight
10
14
18
22
26
30
seed age (days from anthesis)

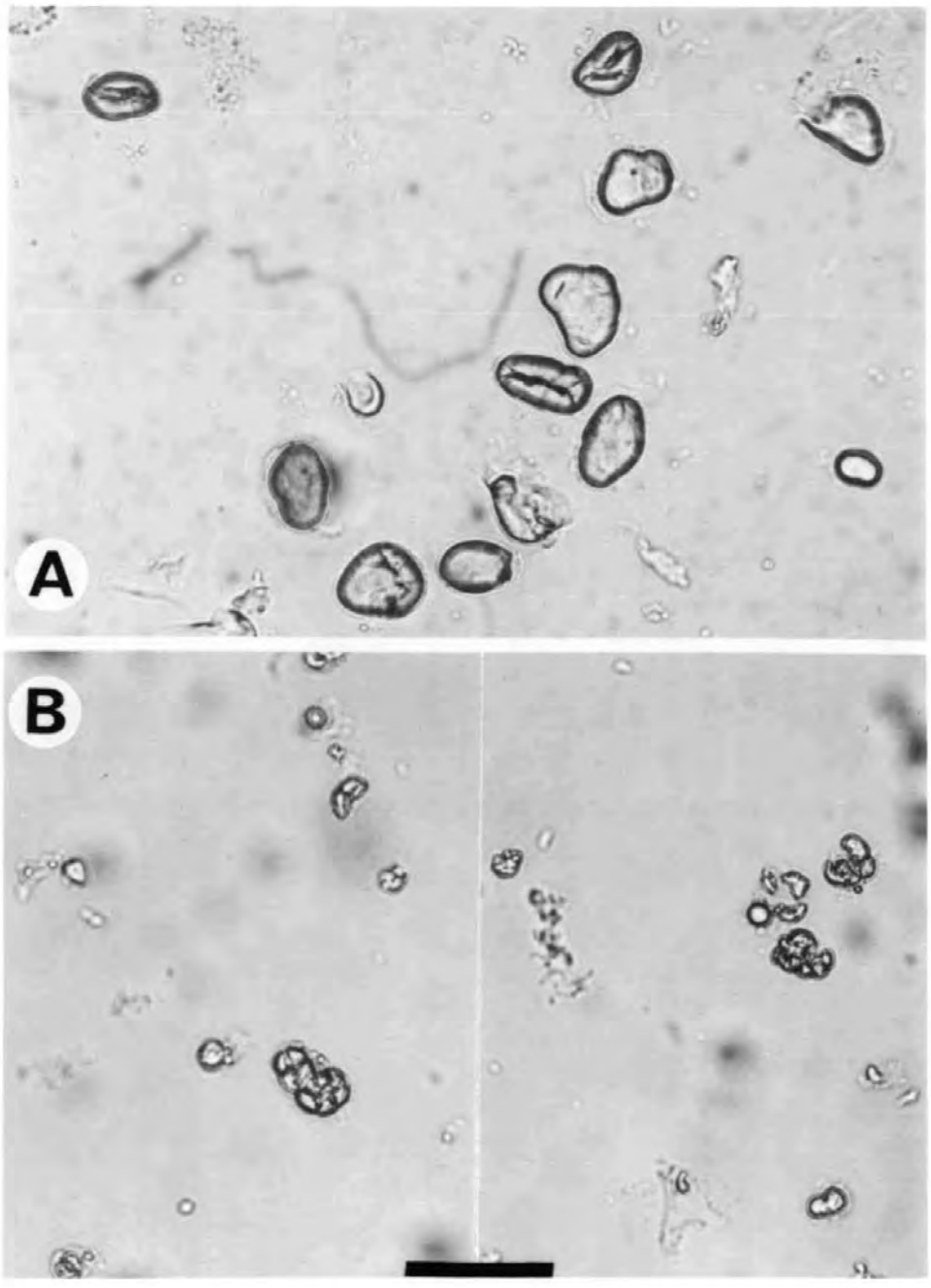
PLATE 2.4

Comparison of light micrographs of starch grains from mature dried seeds of two lines of Pisum sativum L. near-isogenic except for the r-locus

A. Simple starch grains characteristic of the BC/R round-seeded genotype (RR)

B. Compound starch grains characteristic of the BC/r wrinkled-seeded genotype (rr)

bar line 20 µm


A
B

iii) Testa Growth

There was an initial exponential phase between days 10-17, followed by a linear phase up to day 30 (Fig. 2.5). Slight lags in development occurred in both genotypes about days 18 and 21 (120 mg and 150 mg respectively) and a final decline in rate of increase occurred after day 23, resulting in a maximum dry weight at day 30 of 39.5 mg.

There was no difference between genotypes in the ratio between fresh and dry weights (Fig. 2.6).

iv) Embryo Growth

A multiple t-test analysis of variance on regression coefficients also showed that there was no significant difference between genotypes prior to day 18 but that there was a significant difference in fresh weight and dry weight of the two genotypes after day 19 (Table 2.3).

Fresh weight increased exponentially up to day 19 (Fig. 2.7A) and linearly thereafter. Beyond day 19 the wrinkled-seeded (rr) genotype had a greater fresh weight than the round-seeded (RR) genotype at all ages. It should be noted that day 19 coincided with the final presence of liquid endosperm.

There appeared to be a slight lag phase between days 20-22, which was more evident in the round- than in the wrinkled-seeded genotype and by day 30 the wrinkled-seeded embryos were 15% heavier than the corresponding round-seeded embryos.

There was an initial exponential phase for increase in dry weight until about day 20 after which the increase was linear (Fig. 2.7B). The largest difference occurred at the oldest stage, day 30,

FIGURE 2.5

Comparison of the change in testa fresh weight (A) and testa dry weight (B) with time of the two lines of *Pisum sativum* L. near-isogenic except for the *r*-locus

o——o round-seeded BC/R genotype (*RR*)

x- - x wrinkled-seeded BC/r genotype (*rr*)

Each point is the mean of 45 samples

Bar lines represent the standard error of the mean

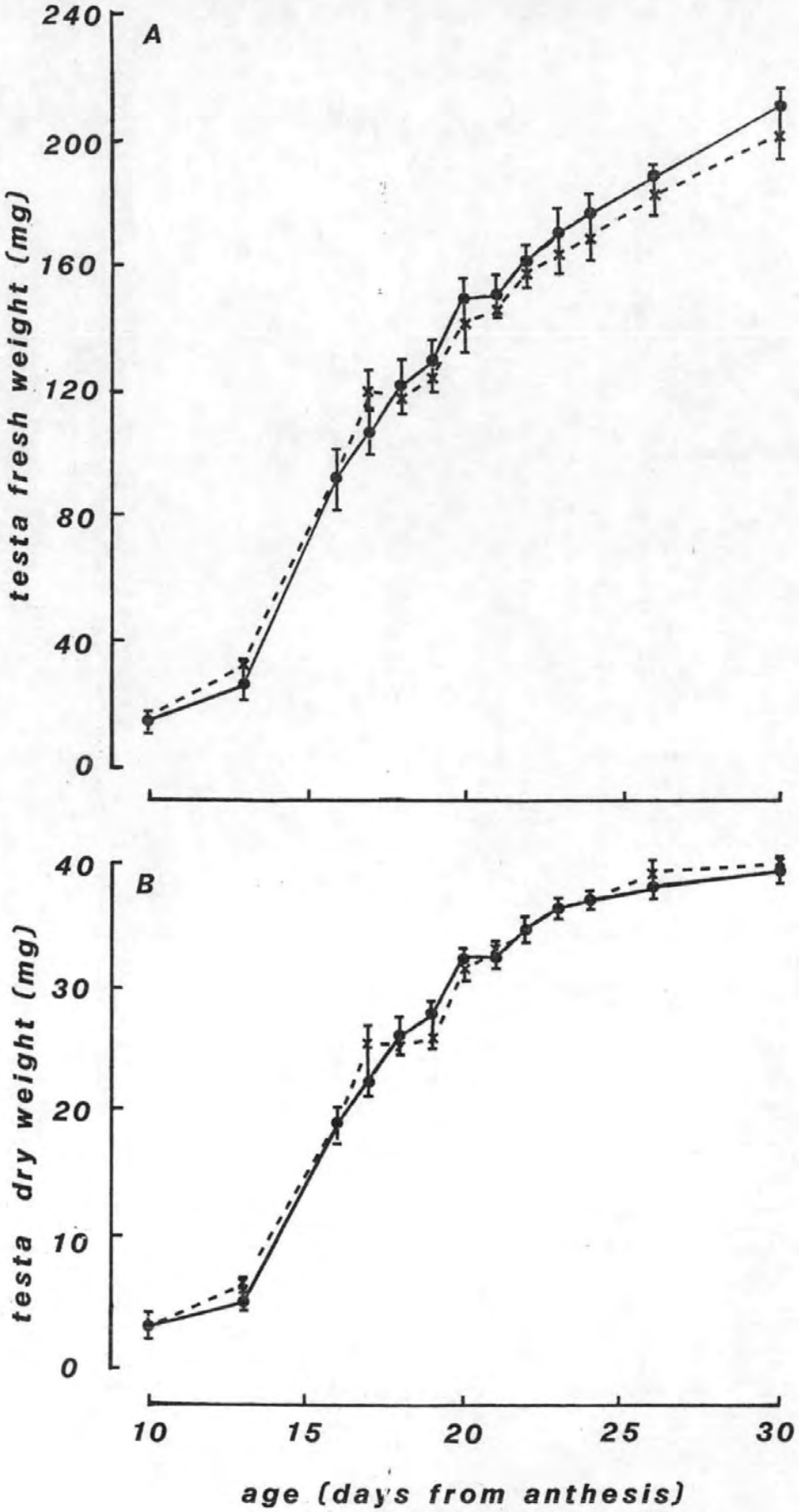

A
240
200
160
120
80
40
0
testa fresh weight (mg)
B
40
30
20
10
0
testa dry weight (mg)
10
15
20
25
30
age (days from anthesis)

FIGURE 2.6

Relationship between fresh weight and dry weight of developing testas of the two lines of Pisum sativum L. near-isogenic except for the r-locus

• round-seeded BC/R genotype (RR)

x wrinkled-seeded BC/r genotype (rr)

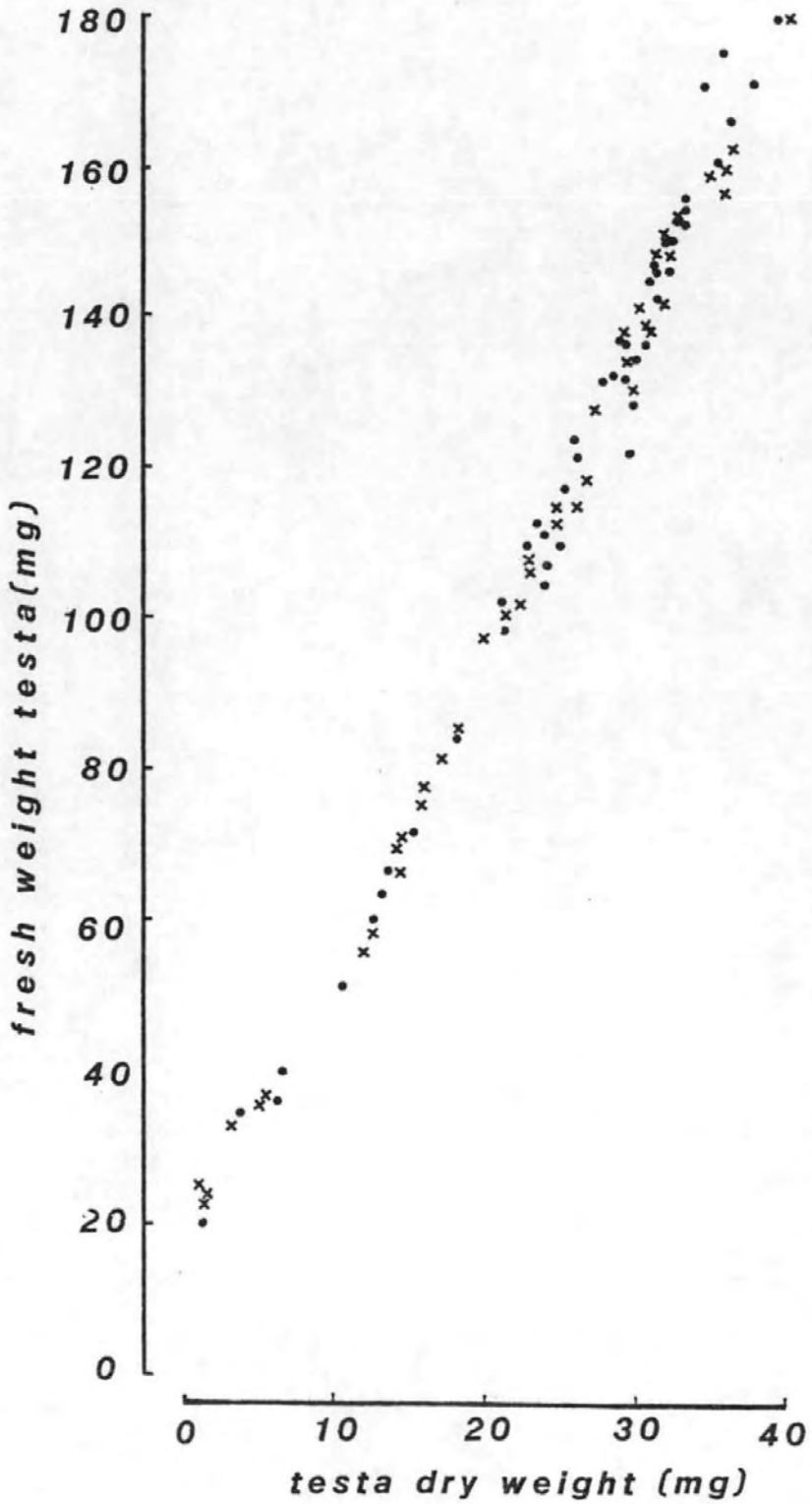

180
160
140
120
100
80
60
40
20
0
fresh weight testa(mg)
0
10
20
30
40
testa dry weight (mg)

TABLE 2.3

Multiple t-test on Regression Coefficients of growth data of embryos from pods of 3 different nodes of the two lines of Pisum sativum L. near-isogenic except for the r-locus (Fig. 2.7)

a) Embryo fresh weight days 10-18

Degrees of freedom = 6

	R1	R2	R3	r1	r2	r3
R1	-	2.8731*	3.795*	3.474*	2.655*	3.369*
R2		-	2.373	1.4157	0.7163	1.6371
R3			-	1.5076	2.6587	0.8854
r1				-	1.9892	0.6093
r2					-	1.9947
r3						-

b) Embryo dry weights days 10-18

Degrees of freedom = 6

	R1	R2	R3	r1	r2	r3
R1	-	0.2446	1.6165	1.0750	0.4341	1.1238
R2		-	2.0025	1.8447	0.3064	1.5889
R3			-	0.9810	2.1912	0.6605
r1				-	2.3751	0.2950
r2					-	1.8357
r3						-

See page 115 for key

TABLE 2.3 continued

c) Embryo fresh weights days 19-30

Degrees of freedom = 12

	R1	R2	R3	r1	r2	r3
R1	-	0.3108	0.1856	2.7533*	2.1948*	2.7478*
R2		-	0.4842	2.5324*	1.9450	2.4900*
R3			-	2.8161*	2.2763*	2.8012*
r1				-	0.7247	0.4616
r2					-	0.4616
r3						-

d) Embryo dry weight days 19-30

Degrees of freedom = 12

	R1	R2	R3	r1	r2	r3
R1	-	0.3138	0.0171	3.8999**	3.733**	3.0834**
R2		-	0.3138	4.241***	4.067***	3.4185**
R3			-	4.259***	4.051***	3.334***
r1				-	0.0327	0.8666
r2					-	0.7746
r3						-

* significant at the 1-5 % level

** significant at the 0.1-1 % level

*** significant at < 0.1 %

Values not followed by asterisks are not significantly different from each other

---

R = BC/R genotype (RR), r = BC/r genotype (rr)

Nos. 1-3 after the genotype letter indicate which node the sample is from (1 being the first node from the bottom)

FIGURE 2.7

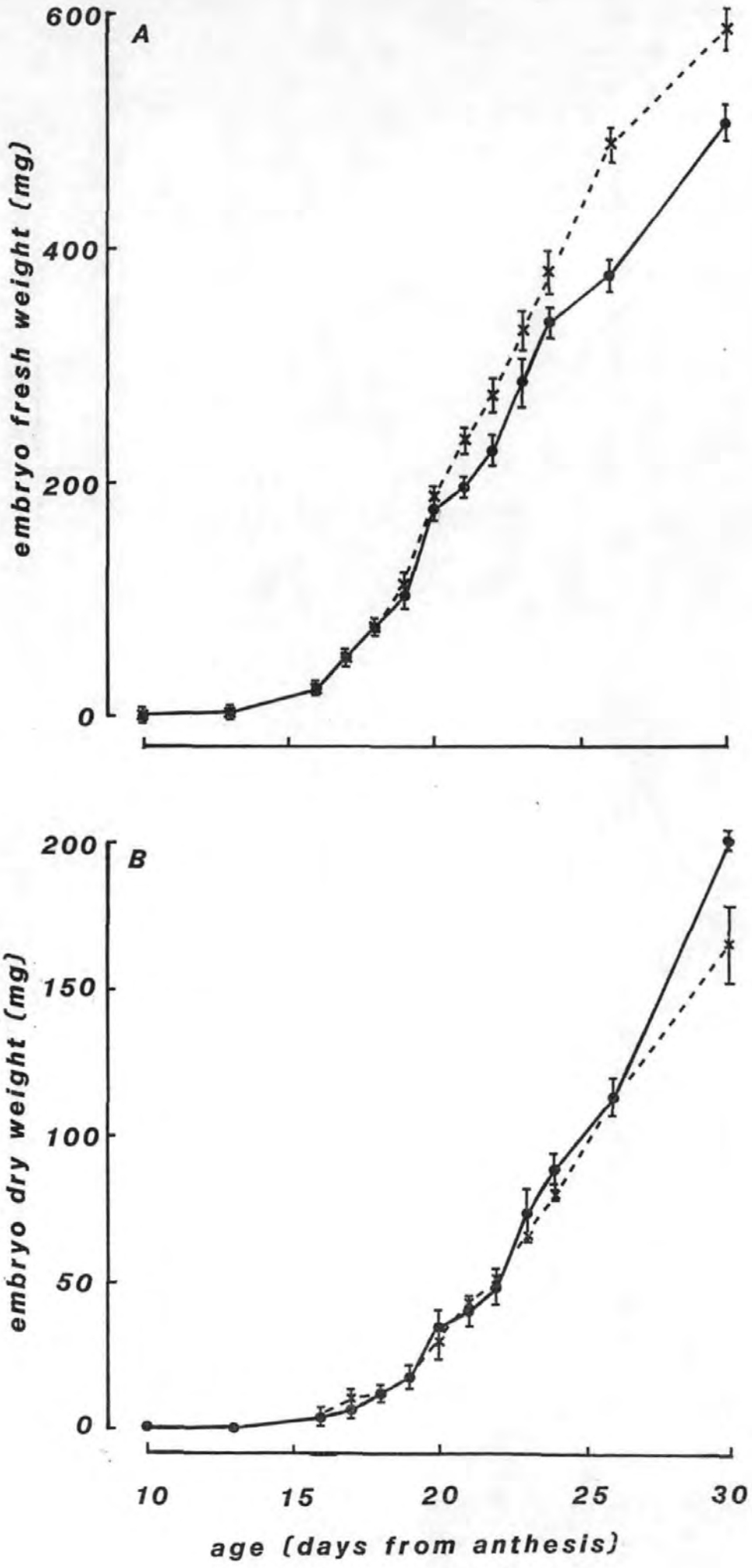
Comparison of the change in embryo fresh weight (A) and embryo dry weight (B) with time of the two lines of Pisum sativum L. near-isogenic except for the r-locus

round-seeded BC/R genotype (RR)

x- - x wrinkled-seeded BC/r genotype (rr)

Each point is the mean of 45 samples

Bar lines represent the standard error of the mean


A
600
400
200
0
embryo fresh weight (mg)
B
200
150
100
50
0
embryo dry weight (mg)
10
15
20
25
30
age (days from anthesis)

when the round embryo had a dry weight 25 % greater than the wrinkled embryo.

Prior to 70 mg fresh weight there was virtually no difference between the 2 genotypes in the fresh weight/ dry weight ratio (Fig. 2.8). Quadratic curves were fitted to the data, giving the best fit and most meaningful curves of several types tried. The equations for the curves were different for each genotype (Fig. 2.8 and Table 2.4). A range of data points evenly spaced over the time examined gave similar results (Table 2.4) and only these points are shown on the fitted curve (Fig. 2.9). Thus although the difference between round- and wrinkled-seeded genotypes was most apparent after 70 mg fresh weight, it was also different prior to 60mg fresh weight although to all intents and purposes it may be considered to be the same. Thereafter it was constantly changing, the wrinkled embryo always having a greater water content than the round for any given fresh weight. This difference increased during development, wrinkled embryos having 50mg more fresh weight than round embryos at 60 mg dry weight which is 20% of their total fresh weight.

v) Endosperm Growth

Endosperm volume increased from day 10 to a maximum at day 16/17 of 33µl (Fig. 2.10 A). After this it declined rapidly to zero by day 20 when the embryo had a fresh weight of 190 mg.

There was no difference between genotypes in the rate of decline of the endosperm, but at day 13 the wrinkled seeds contained more endosperm than the round (Fig. 2.10 A). However, overall variation was much greater at these ages and the seed was growing exponentially.

FIGURE 2.8

Relationship between fresh weight and dry weight of developing embryos of the two lines of Pisum sativum L. near-isogenic except for the r locus

• round-seeded BC/R genotype (RR)

x wrinkled-seeded BC/r genotype (rr)

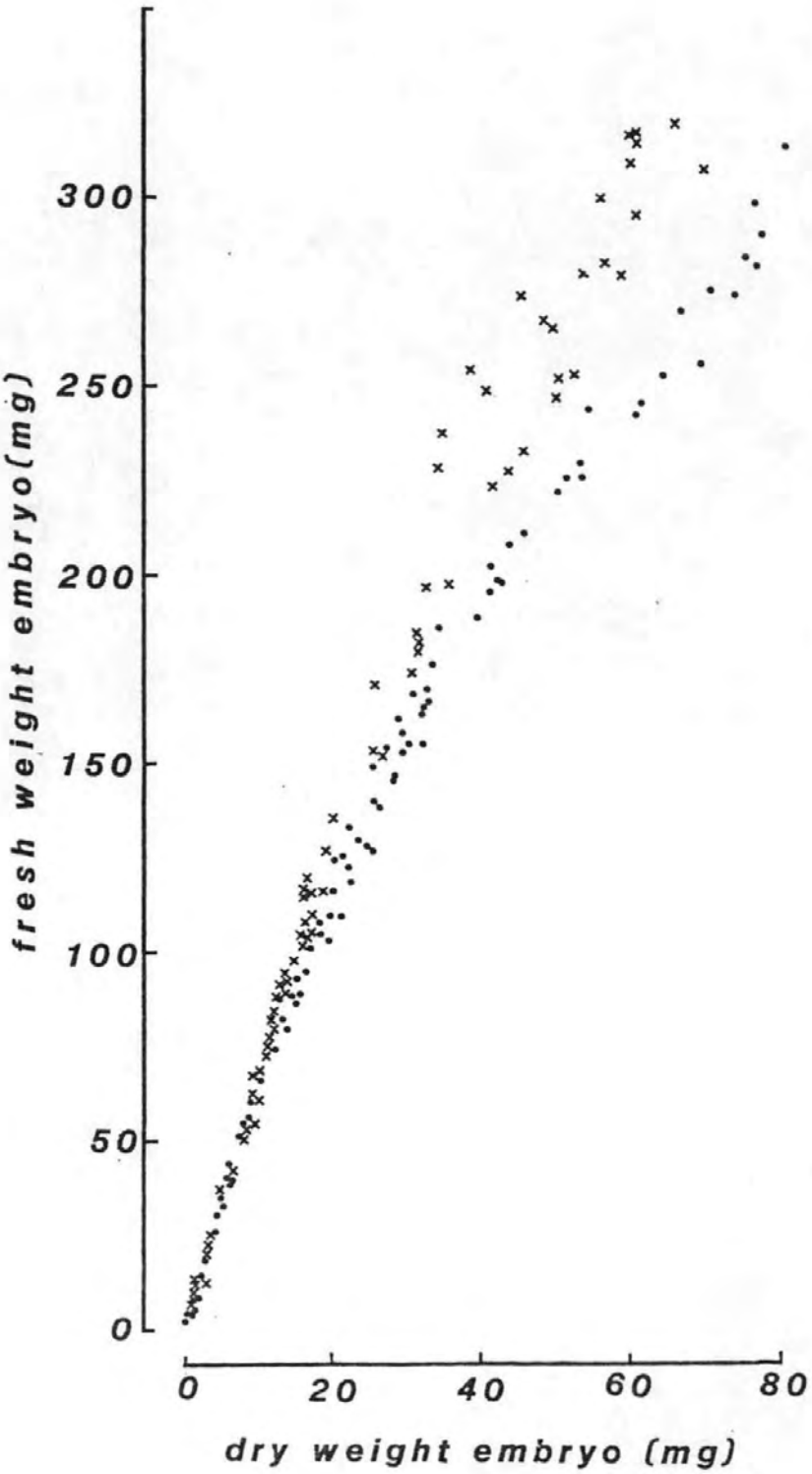

fresh weight embryo(mg)
300
250
200
150
100
50
0
0
20
40
60
80
dry weight embryo (mg)

TABLE 2.4

Comparison of quadratic curves fitted to the graphs (Figure 2.9) of dry weight against fresh weight of developing embryos of two lines of Pisum sativum L. near-isogenic except for the r-locus

Equation of quadratic curve: $y = a + bx + cx^2$

where a is the intercept
b is the initial slope of the graph
c is the degree of curvature

	Parameter	RR	rr
A	a	1.71	1.01
	b	0.0574	0.101
	c	0.000644	0.000268
B	a	1.79	2.08
	b	0.0552	0.082
	c	0.00065	0.000341
C	a	1.58	1.84
	b	0.0567	0.0834
	c	0.00065	0.00034

RR: round-seeded BC/R genotype (RR)

rr: wrinkled-seeded BC/r genotype (rr)

A : curve for all the data, n = 561
B : curve for sample of data, n = 32 evenly spaced over the time measured
C : curve for sample data (above, n=32) but including the point (0,0)

FIGURE 2.9

Comparison of the relationship between dry weight and fresh weight of developing embryos of two lines of *Pisum sativum* L. near-isogenic except for the *r*-locus

- round-seeded BC/R genotype (*RR*)

x wrinkled-seeded BC/r genotype (*rr*)

curve RR : quadratic curve fitted (from Table 2.4, n=561)

curve rr : quadratic curve fitted (from Table 2.4, n=561))

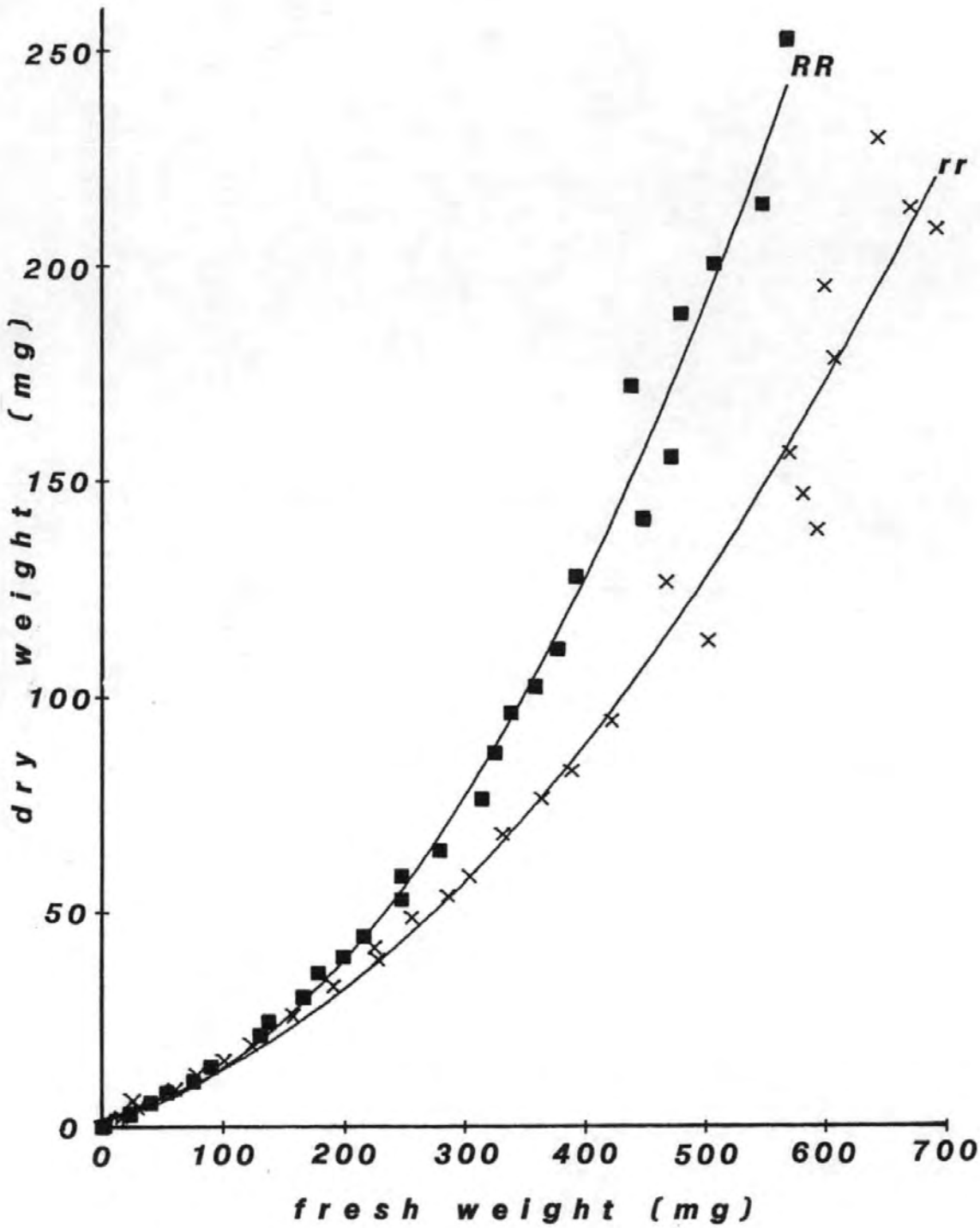

250
200
150
100
50
0
dry weight (mg)
RR
rr
0
100
200
300
400
500
600
700
fresh weight (mg)

FIGURE 2.10

Comparison of endosperm volume during development of seeds of the two lines of Pisum sativum L. near-isogenic except for the r-locus

A. Change in endosperm volume with age of embryo

 round-seeded BC/R genotype (RR)

 wrinkled-seeded BC/r genotype (rr)

Number above each bar is the number in the size class

Bar lines represent one side of the standard error of the mean

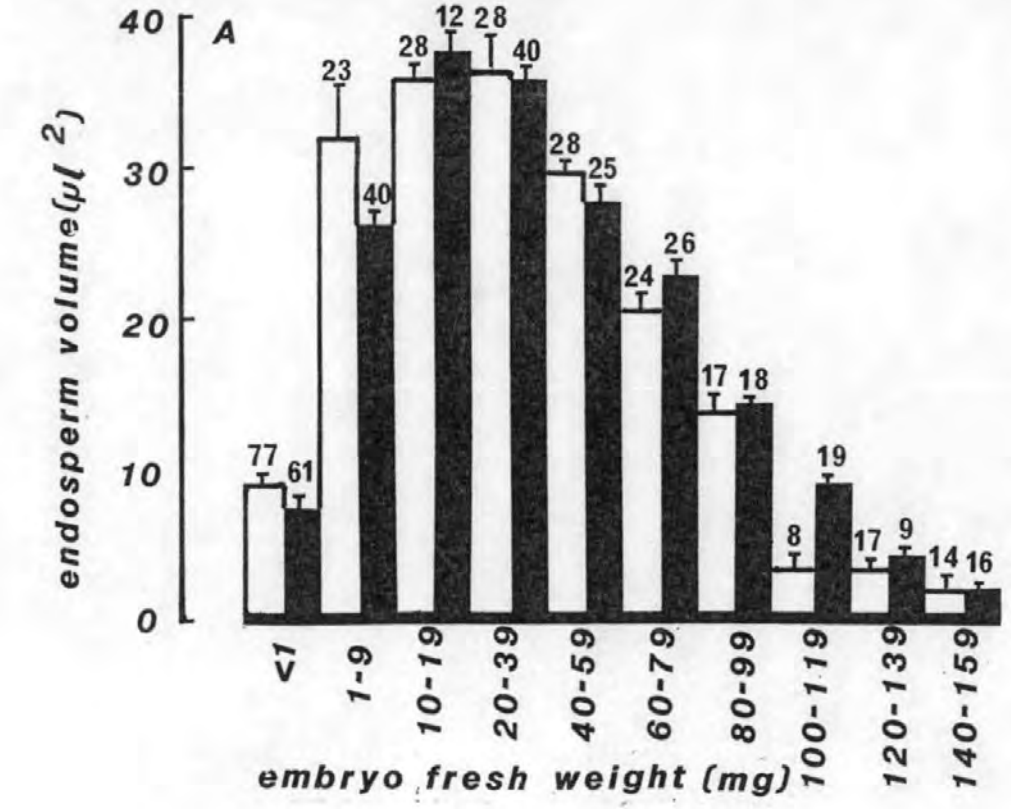
B. Change in endosperm volume with size of embryo

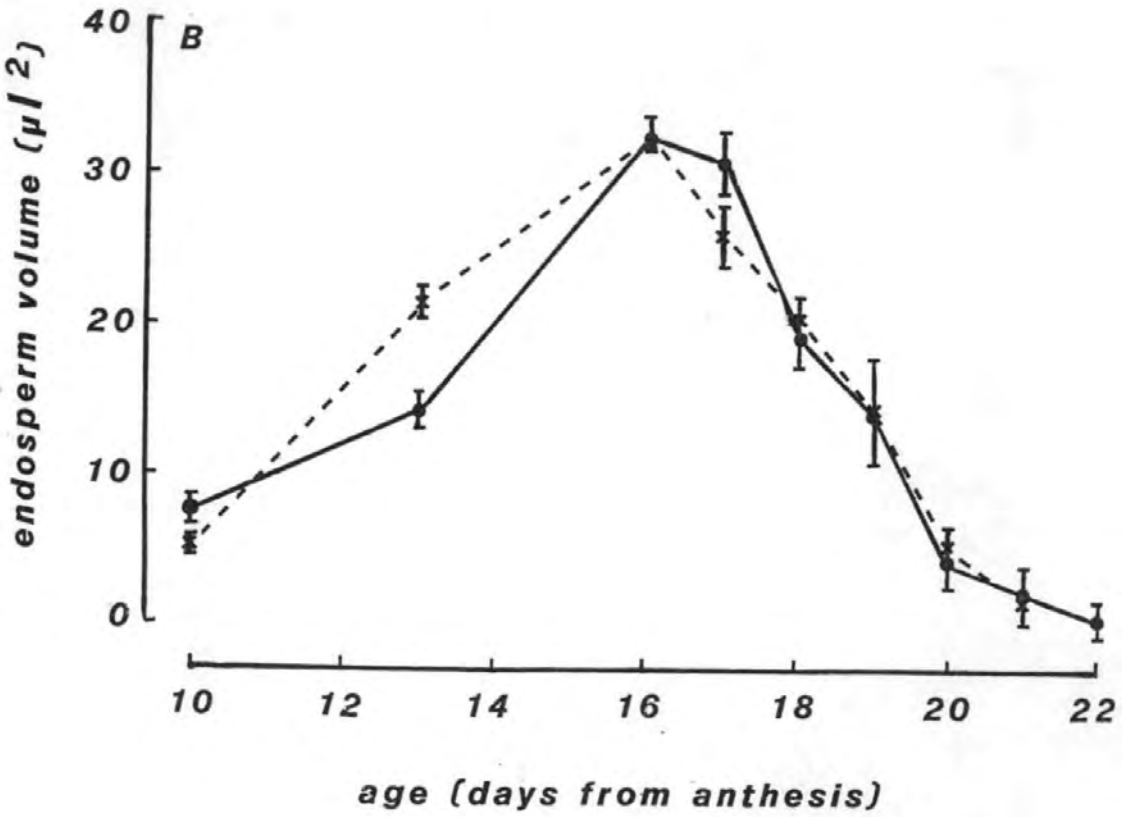
 round-seeded BC/R genotype (RR)

x- - x wrinkled-seeded BC/r genotype (rr)

Each point is the mean of 45 samples

Bar lines represent the standard error of the mean


A
endosperm volume(μl 2)
40
30
20
10
0
77
61
23
40
28
12
28
40
28
25
24
26
17
18
8
19
17
9
14
16
<1
1-9
10-19
20-39
40-59
60-79
80-99
100-119
120-139
140-159
embryo fresh weight (mg)


B
endosperm volume (μl 2)
40
30
20
10
0
10
12
14
16
18
20
22
age (days from anthesis)

Expressed on an embryo fresh weight basis (Fig. 2.10 B), the volume increased rapidly to a maximum of 36-37 µl by 10 mg fresh weight. The difference between this value and that above is a result of the two different methods of describing the data; the absolute maximum is about 39 µl, 10 mg fresh weight which would have occurred just prior to day 16. Since no embryos were measured between day 13-16, this result was obscured by the general variation in the embryos. The endosperm volume declined after 40mg fresh weight to virtually zero by 120 mg fresh weight (contact point). Two differences between the genotypes were observed; between 1-9mg fresh weight, the wrinkled rr embryos were surrounded by more endosperm than the round RR embryos, although the error at this stage was slightly larger due to there being fewer wrinkled embryos between 1-9mg and hence no statistical weight may be given to this result. The other difference was observed between 100-119 mg fresh weight, when the round embryo was surrounded by more than twice as much endosperm as the wrinkled embryo. Although only a few seeds of this size contained any endosperm, the error terms were very small and it may be assumed that this difference is significant.

C. OSMOTIC STUDIES DURING FRUIT DEVELOPMENT

In order to aid comparison between genotypes and tissues, water potential is expressed according to the fresh weight of the embryo, except for pods where it is expressed according to the age of the pod as the weight of embryos within a given pod was quite variable. However, using the growth analysis studies it is possible to estimate embryo size at a given pod age.

i) Pods

The general trend of water potential during development was the same for both genotypes (Fig. 2.11). At day 12, the water potential was at a maximum of -0.98 MPa. It then decreased to a minimum of c.-1.27 MPa before increasing again. The point of minimum water potential was different in the two genotypes; in the wrinkled genotypes it occurred at day 20, while in the round genotypes it occurred later, at day 24. Up to day 16 there was no difference between the two genotypes. Between day 16-18 the water potential of the round-seeded pods decreased, which may indicate a differential lag in growth of the pods, and after day 18 there was a significant difference between the genotypes of 0.1 MPa (Fig. 2.11).

ii) Testa

The general trend of water potential was the same for both genotypes (Fig. 2.12). Early in development it was at its most negative, -1.85 MPa. This increased during development to a maximum of -1.40 MPa by the final stage examined, embryo fresh weight 250-299 mg. By this time the water content of the testa was decreasing and no further stages were able to be assessed.

iii) Embryo

The pattern of changes in water potential of the embryos was different for the two genotypes (Fig. 2.13). The round (RR) embryo had a minimum (most negative) water potential of -1.65 MPa early in development (0-49 mg). This increased to -1.46MPa by 150 mg fresh weight and remained at approximately this level for the remaining period.

FIGURE 2.11

Changes in water potential during development of pods of the two lines of Pisum sativum L. near-isogenic except for the r-locus

 round-seeded BC/R genotype (RR)

 wrinkled-seeded BC/r genotype (rr)

Each column is the mean of 5 samples. Error bars represent the standard deviation from the mean.

* maximum endosperm

^ loss of endosperm

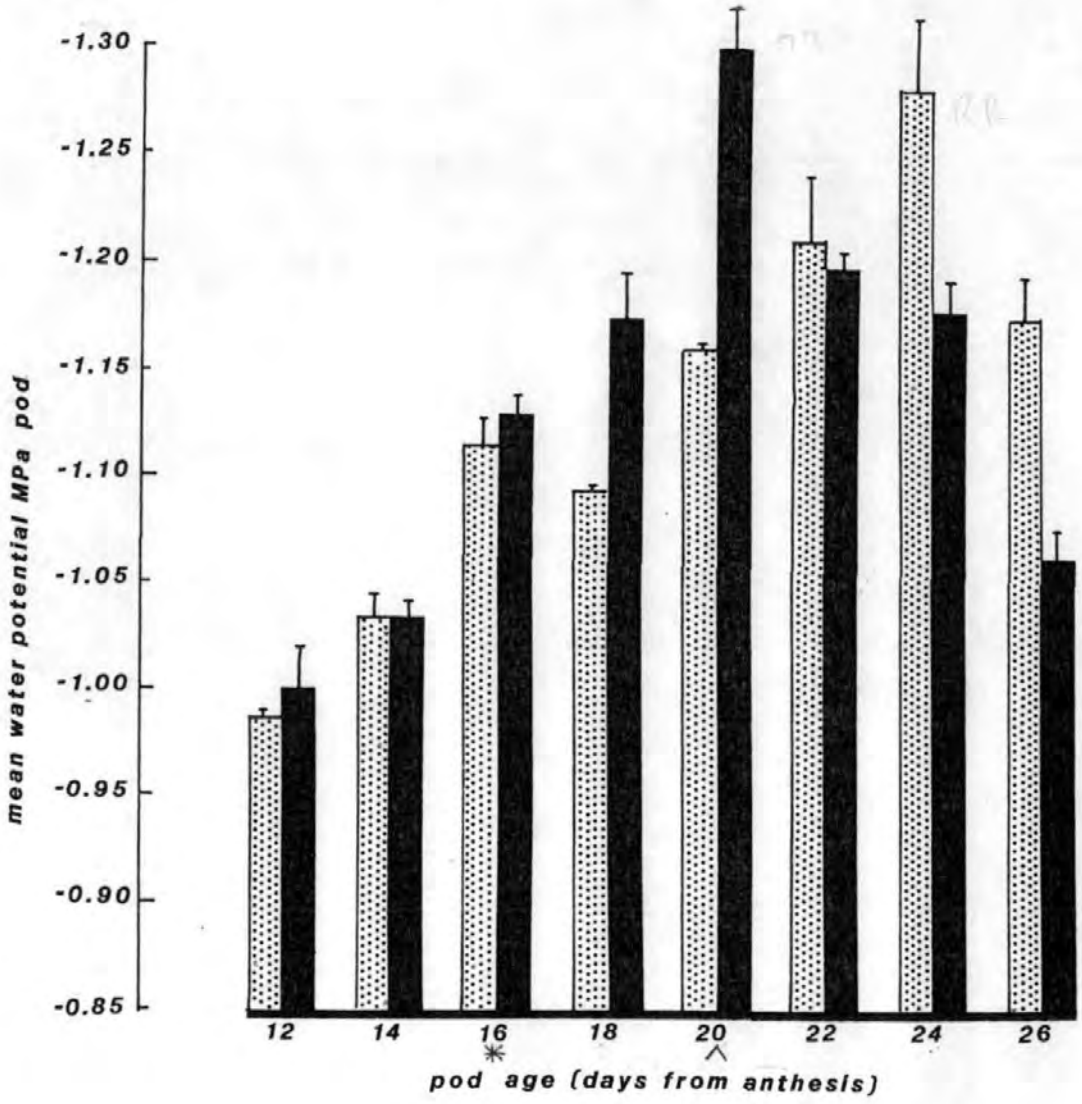

-1.30
-1.25
-1.20
-1.15
-1.10
-1.05
-1.00
-0.95
-0.90
-0.85
mean water potential MPa pod
12
14
16
18
20
22
24
26
pod age (days from anthesis)

FIGURE 2.12

Changes in water potential during development of testas of the two lines of Pisum sativum L. near-isogenic except for the r-locus

 round-seeded BC/R genotype (RR)

 wrinkled-seeded BC/r genotype (rr)

Each column is the mean of 50 samples. Bar lines represent the standard deviation from the mean.

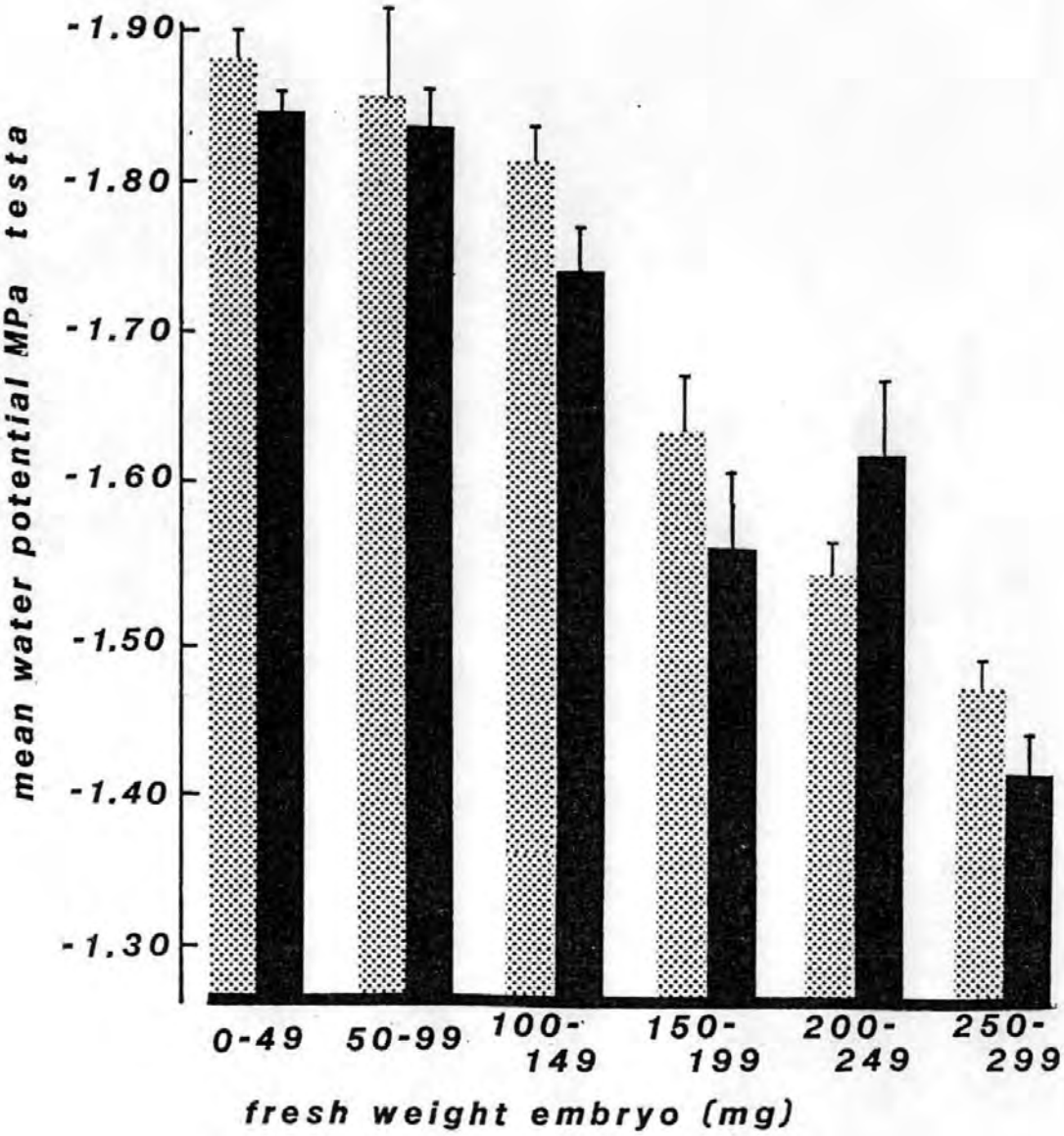

-1.90
-1.80
-1.70
-1.60
-1.50
-1.40
-1.30
mean water potential MPa testa
0-49
50-99
100-149
150-199
200-249
250-299
fresh weight embryo (mg)

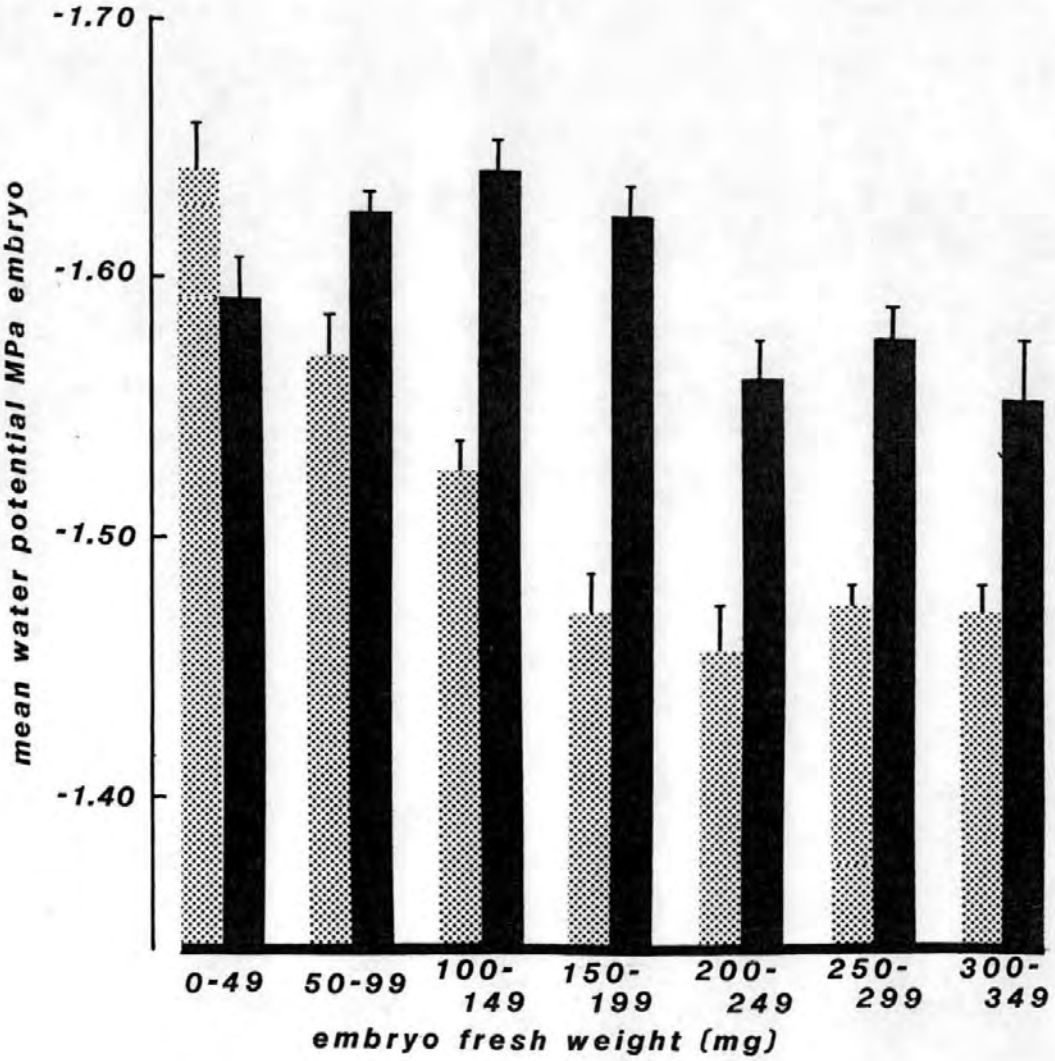
FIGURE 2.13

Changes in water potential during development of embryos of the two lines of Pisum sativum L. near-isogenic except for the r-locus

 round-seeded BC/R genotype (RR)

 wrinkled-seeded BC/r genotype (rr)

Each column is the mean of 50 samples
Bar lines represent one side of the standard deviation from the mean


-1.70
-1.60
-1.50
-1.40
mean water potential MPa embryo
0-49
50-99
100-149
150-199
200-249
250-299
300-349
embryo fresh weight (mg)

The wrinkled (rr) embryos had a water potential of -1.59 MPa initially (0-49 mg) which decreased to a minimum of -1.6 MPa by 100-149 mg and then increased to a maximum of about -1.56 MPa by 200 mg fresh weight. It stayed at this level for the remainder of the developmental period examined.

The round embryo always had a higher (less negative) water potential than the wrinkled embryo, except below 50 mg fresh weight when the round embryo had a lower (more negative) water potential than the wrinkled embryo.

The water potential of the embryo was always lower (more negative) than that of the endosperm and higher (less negative) than that of the testa (Fig. 2.14).

iv) Endosperm

The water potential of the endosperm was somewhat variable, between -1.44 MPa and -1.57 MPa, and there did not appear to be any particular trend (Fig. 2.15). The water potential was always less negative than the testa but more negative than the embryo (Fig. 2.14).

Differences occurred between the two genotypes; at 20-39 mg embryo fresh weight, the round endosperm had a more negative potential than the wrinkled, while at 80-99 mg embryo fresh weight the wrinkled endosperm had a more negative potential than the round (Fig. 2.15).

D. CELLULAR DEVELOPMENT OF THE EMBRYO

No difference in cell number per mg fresh weight of cotyledon minus the embryonic axis was found between the round and wrinkled

FIGURE 2.14

Relationship between water potentials of various tissues during development of the fruit of the two lines of Pisum sativum L. near-isogenic except for the r-locus

RR : round-seeded BC/R genotype (RR)

rr : wrinkled-seeded BC/r genotype (rr)

e : liquid endosperm

Each column is the mean of 50 samples
Bar lines represent one side of the standard deviation from the mean

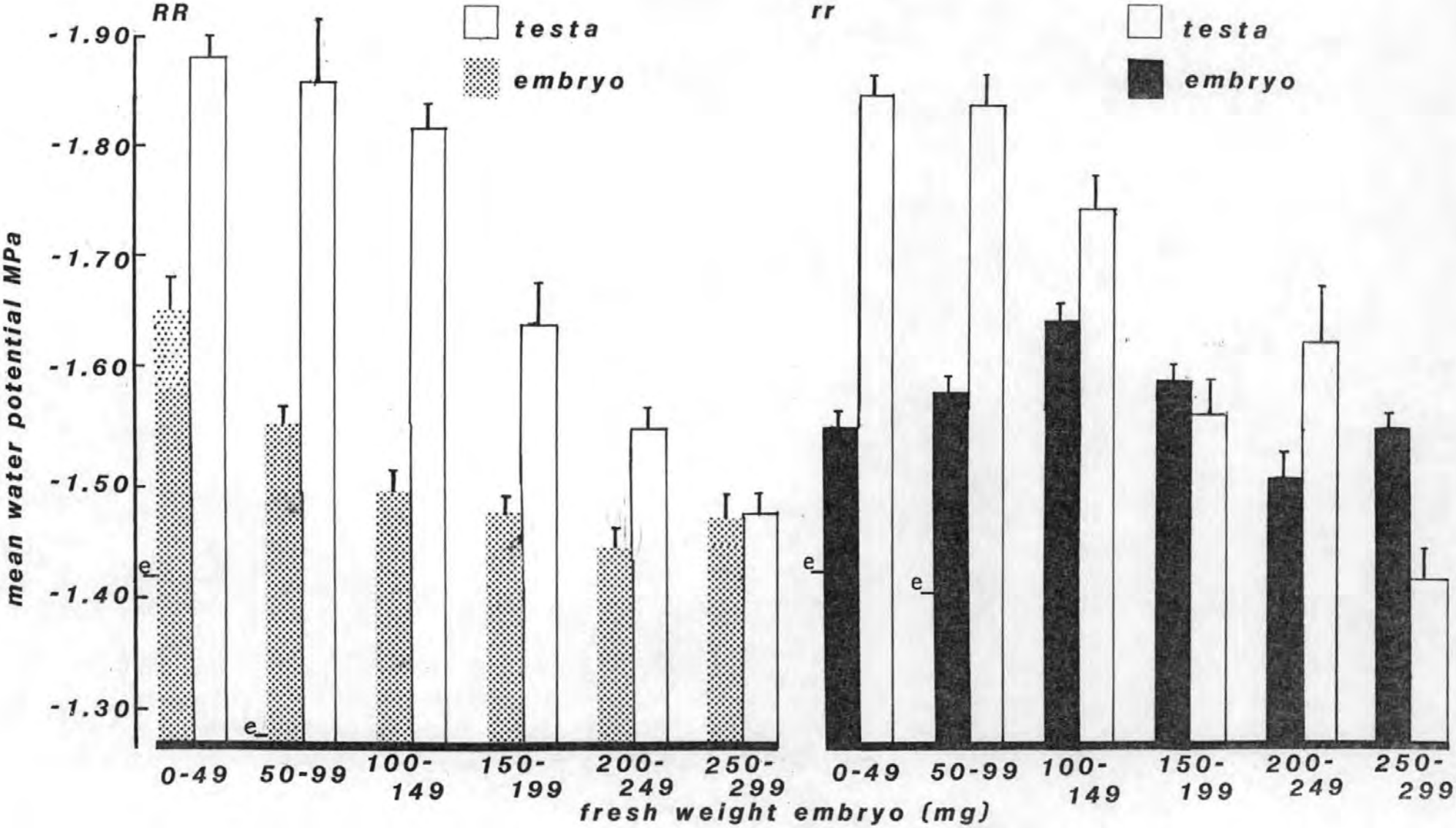

RR
testa
embryo
rr
testa
embryo
-1.90
-1.80
-1.70
-1.60
-1.50
-1.40
-1.30
mean water potential MPa
e
e
e
e
0-49
50-99
100-149
150-199
200-249
250-299
0-49
50-99
100-149
150-199
200-249
250-299
fresh weight embryo (mg)

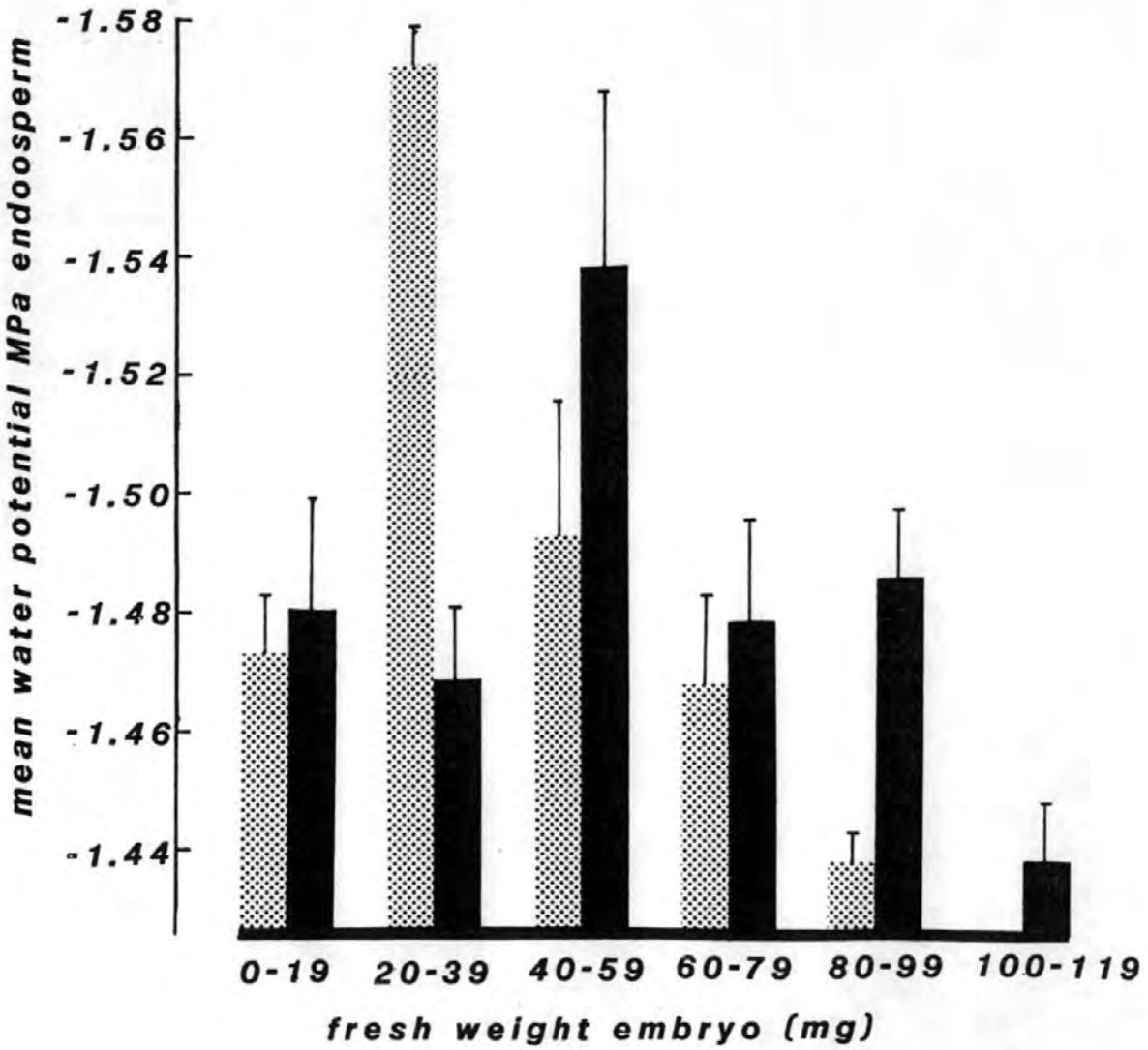
FIGURE 2.15

Changes in the water potential of the liquid endosperm during development of the two lines of Pisum sativum L. near-isogenic except for the r-locus


round-seeded BC/R genotype (RR)


wrinkled-seeded BC/r genotype (rr)

Each column is the mean of 20 samples
Bar lines represent one side of the deviation from the mean


-1.58
-1.56
-1.54
-1.52
-1.50
-1.48
-1.46
-1.44
mean water potential MPa endoosperm
0-19
20-39
40-59
60-79
80-99
100-119
fresh weight embryo (mg)

embryos (Fig. 2.16). There was an initial phase of cell division up to fresh weight of approximately 30 mg, having about 600,000 cells per embryo. This was followed by a phase of both cell division and cell expansion between 30-120 mg, which became a phase of mainly cell expansion after 140 mg fresh weight. The initial rate of cell expansion was 35,000 cells per mg increase in fresh weight. During the cell expansion / cell division phase this rate declined to c. 4750 cells per mg fresh weight increase and above 170 mg there was virtually no increase in cell number.

There was little difference in the % frequency distribution of cell sizes between the two genotypes (Fig. 2.17). These were the cross-sectional areas determined, not the surface areas. At the stage prior to contact point, most cells were of the order 12-18 μm^2, which is a size similar to that of dividing cells although a proportion of the cells was larger, up to 60 μm^2. Although only the areas were measured, it is possible to derive a volume for these cells by assuming the cells are spherical. Thus the volume of these cells would be 31.1 and 349.6 μm^3 respectively. At the later stage, there were less small cells and larger numbers of much larger cells, indicating that the embryos were growing mainly by cell expansion (Fig. 2.17).

From observations, cells at stage II contained less starch and were smaller than those at stage III (Plate 2.5). The starch grains of the rr embryos were larger than those of the RR embryos and they tended to aggregate in the centre of the cells (Plate 2.5).

FIGURE 2.16

Relationship between the logarithm (ln) of cell no./f.wt. and fresh weight during development of embryos of the two lines of Pisum sativum L. near-isogenic except for the r-locus

- ● round-seeded BC/R genotype (RR)
- x wrinkled-seeded BC/r genotype (rr)

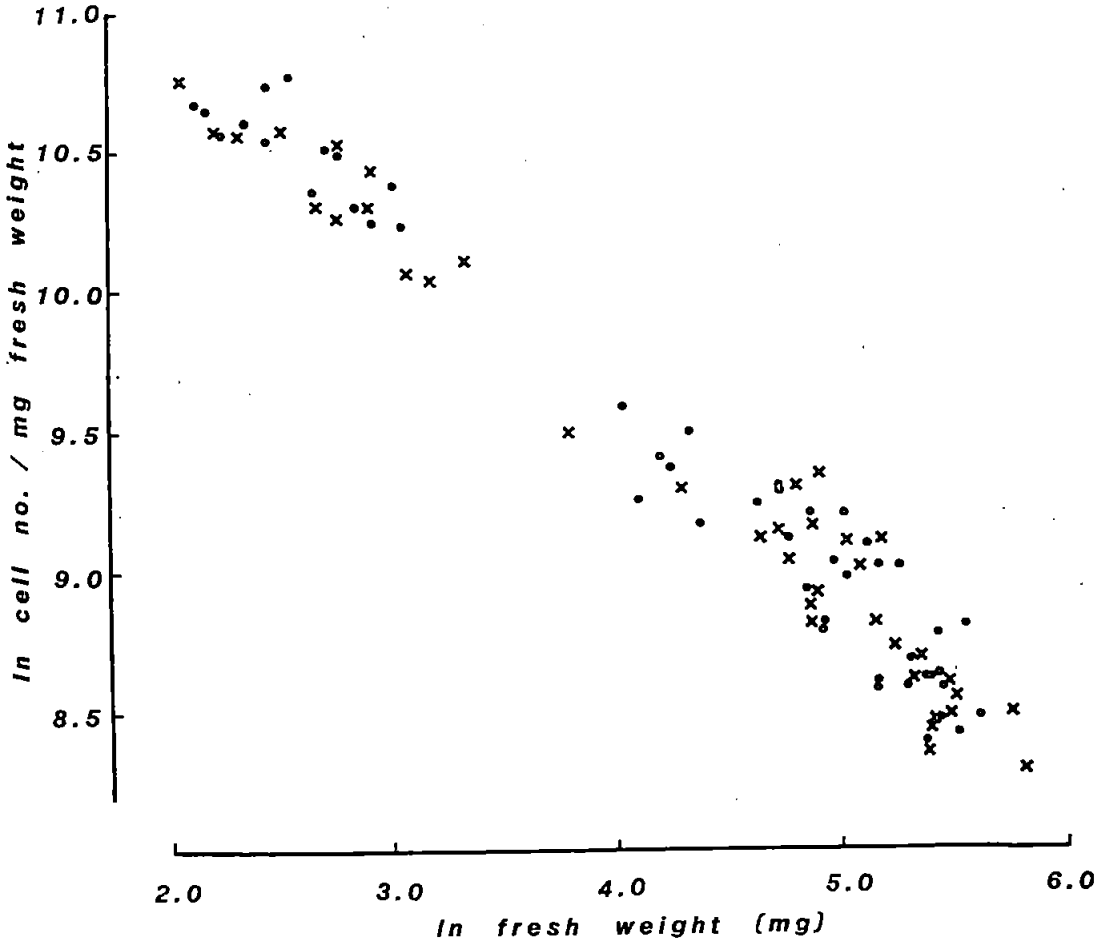

11.0
10.5
10.0
9.5
9.0
8.5
ln cell no. / mg fresh weight
2.0
3.0
4.0
5.0
6.0
ln fresh weight (mg)

FIGURE 2.17

Frequency histograms of cotyledon cell cross-sectional area of embryos of the two lines of Pisum sativum L. near-isogenic except for the r-locus, at two stages of development; 100mg (± 2mg) and 240mg (± 5mg) fresh weight

rr : wrinkled-seeded BC/r genotype (rr)

RR : round-seeded BC/R genotype (RR)

Cell areas are grouped in 6μm^2 size classes, the upper limit of which is indicated along the abscissa

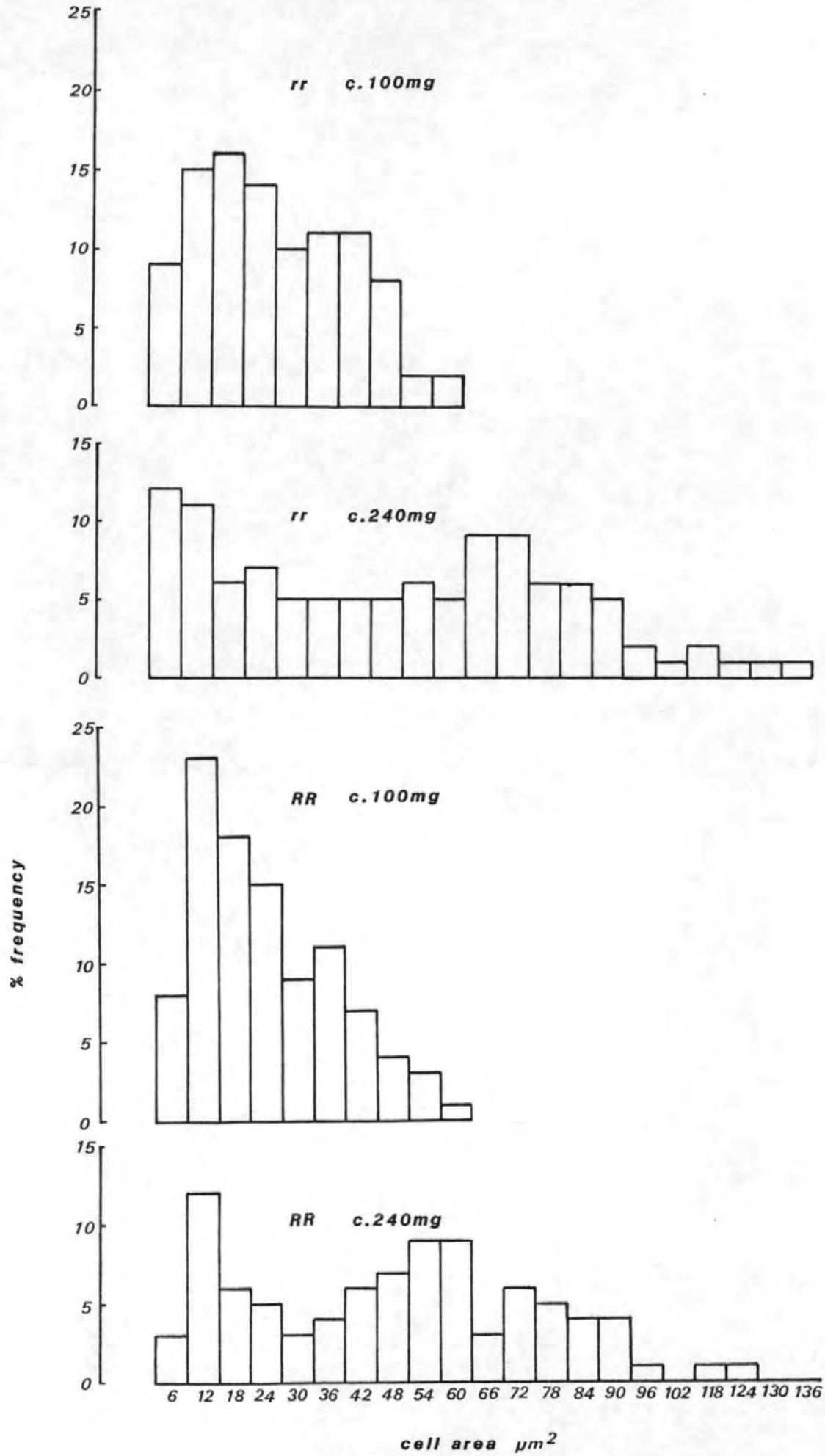

rr c.100mg
rr c.240mg
RR c.100mg
RR c.240mg
% frequency
cell area μm²
0
5
10
15
20
25
6 12 18 24 30 36 42 48 54 60 66 72 78 84 90 96 102 118 124 130 136

PLATE 2.5

Comparison betweeen characteristic cells of embryos from the two lines of Pisum sativum L. near-isogenic except for the r-locus at three developmental stages

A. wrinkled-seeded BC/r genotype (rr), 10 mg fresh weight

B. round-seeded BC/R genotype (RR), 10 mg fresh weight

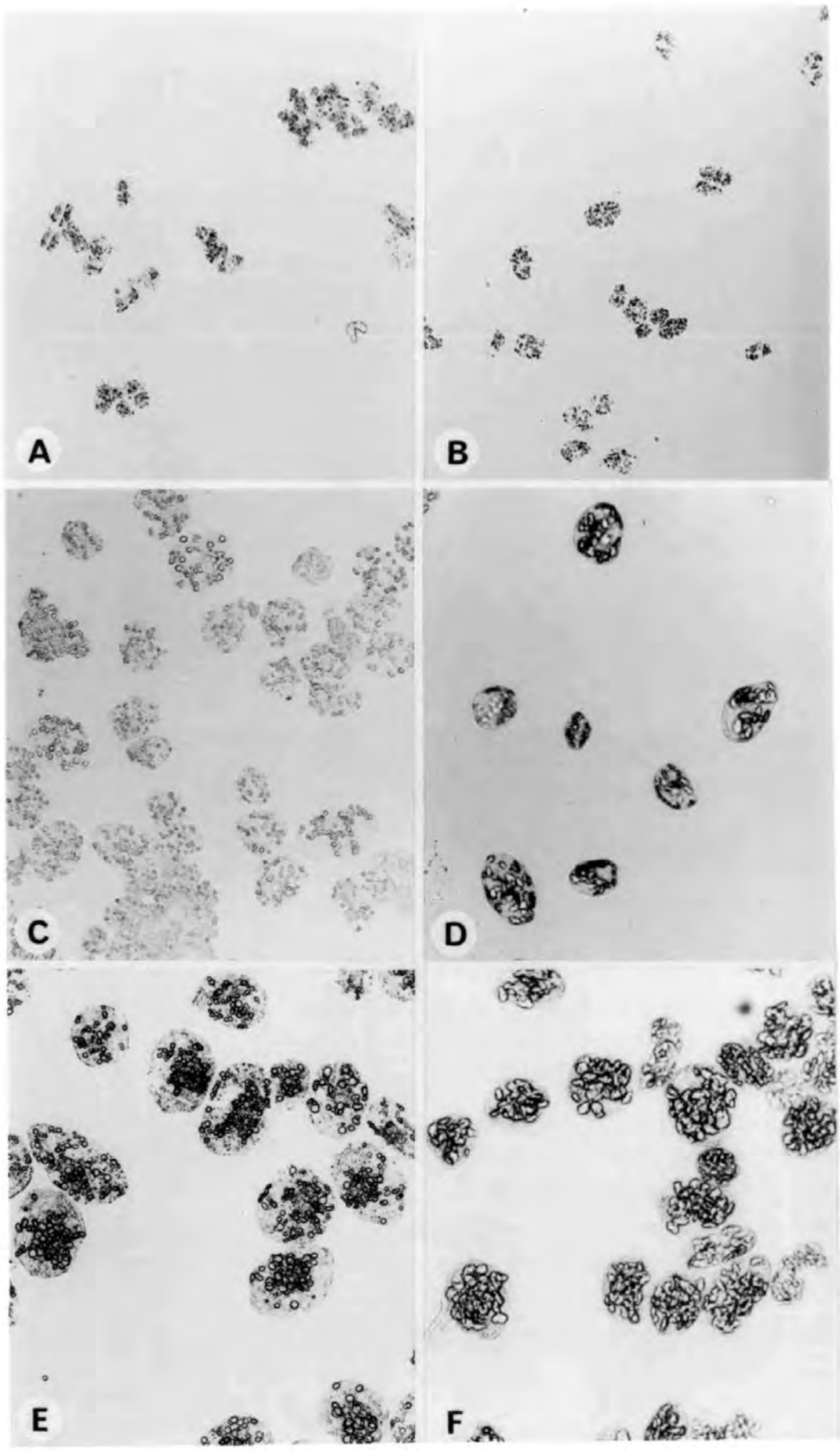
C. wrinkled-seeded BC/r genotype(rr), 100 mg fresh weight

D. round-seeded BC/R genotype (RR), 100 mg fresh weight

E. wrinkled-seeded BC/r genotype (rr), 200 mg fresh weight

F. round-seeded BC/R genotype (RR), 200 mg fresh weight

magnification: x


A
B
C
D
E
F

E. DEVELOPMENT OF SEEDS FROM RECIPROCAL CROSSES

There was no significant difference ($p < 1\%$) in mature dried seed weight between seeds which were either homozygous or heterozygous for the R gene. There was a significant ($p < 1\%$) decrease in seed weight of the rr genotype of c. 50 mg, 17% (Table 2.5). Both the heterozygote seeds have a round appearance characteristic of the RR genotype (Plate 2.6).

No difference was observed in the fresh / dry weight ratio of heterozygous R♀ r♂ and r♀ R♂ embryos resulting from reciprocal crosses of the homozygous (round RR and wrinkled rr) parents (Fig. 2.18), although they developed in testas which were genetically different, i.e. RR or rr testas. The ratio fell on the curve corresponding to the homozygous RR embryos (Fig. 2.5). To confirm this result more samples would need to be taken.

No difference was observed in the fresh / dry weight ratio of testas resulting from the reciprocal crosses (Fig. 2.19). They showed the same growth as corresponding testas from the homozygous parents, RR or rr (Fig. 2.6).

There was little or no difference in water potential between the RRr endosperm and the rrR endosperm (Fig. 2.15), it was of the order -1.44 MPa to -1.50 MPa in both types, compared with -1.44 to -1.58 for the RR genotypes..

There was some difference between the reciprocal crosses and the homozygous types; endosperm of the 20-39 mg embryos had a lower (more negative) water potential in the dominant (RRr) state than in the heterozygous recessive (rrR) state, but at the next size class this was reversed with the homozygous recessive type having a higher (less negative) water potential than the heterozygous or dominant

TABLE 2.5

Comparison between weights of mature dried seed of 4 lines of Pisum sativum L. near-isogenic except for the r-locus; homozygous dominant (RR), homozygous recessive (rr), heterozygous (R♀ r♂ and r♀ R♂)

Genotype	Phenotype	mean* dried seed weight (mg)	standard deviation
R R	round	351.8	15.6
R r	round	361.6	8.8
r R	round	364.7	14.9
r r	wrinkled	303.3	12.0

* means were derived from ten samples, each of 10 seeds

From t-tests of pairs of samples, it was found that the dried seed weight of the homozygous recessive genotype was significantly different at the 1% level from the homozygous dominant genotype, which was not significantly different from the two heterozygotes.

PLATE 2.6

Comparison of the external morphology of mature dried seed of the two lines of Pisum sativum L. near-isogenic except for the r-locus and the two heterozygous lines resulting from crossing a rr parent with a RR parent reciprocally

RR : round-seeded BC/R genotype (RR)

rr : wrinkled-seeded BC/r genotype (rr)

R♀ r♂: round-seeded (RR) maternal parent

r♀ R♂ : wrinkled-seeded (rr) maternal parent

bar line 8 mm

Seeds are illuminated from the side to highlight their topography

Note that both offspring are similar in appearance to the dominant parent, RR.

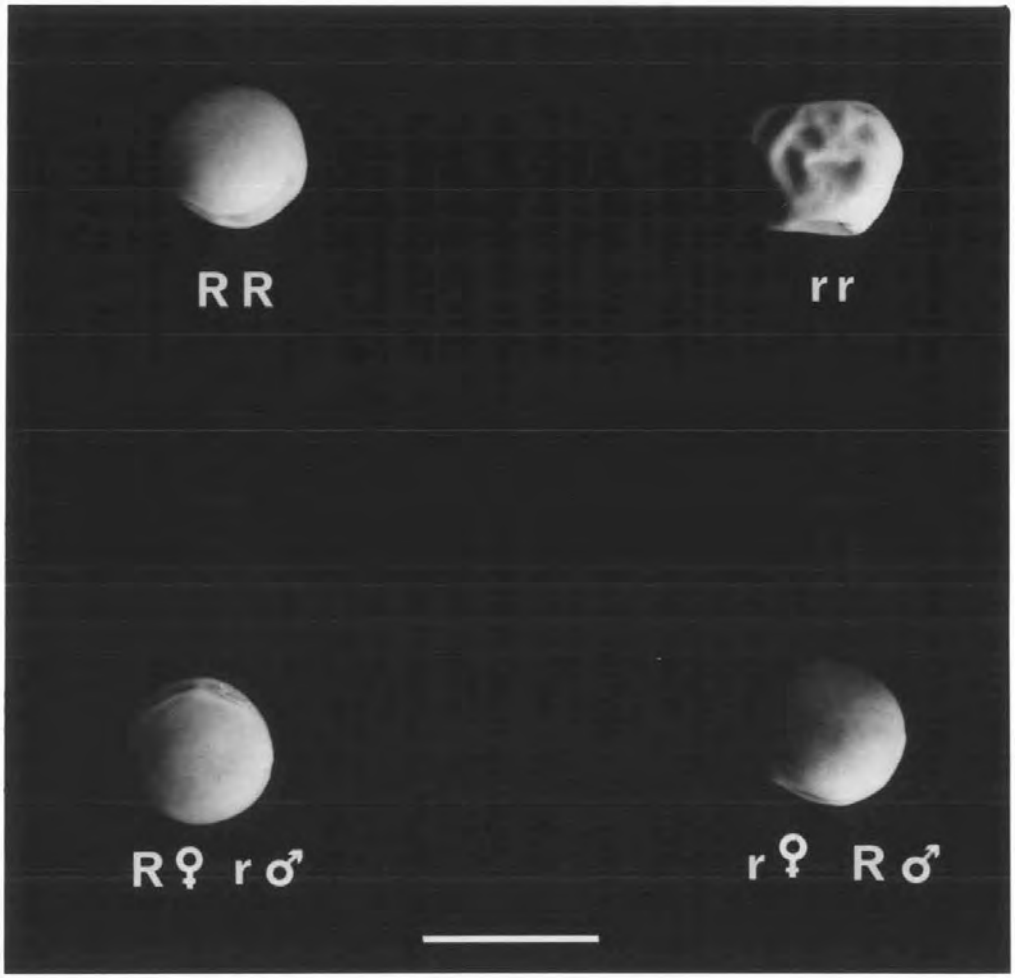

RR
rr
R♀ r♂
r♀ R♂

FIGURE 2.18

Relationship between the fresh weight and dry weight of embryos of Pisum sativum L. resulting from reciprocal crosses of the two lines near-isogenic except for the r-locus.

R♀ r♂ maternal parent BC/R genotype (RR)

r♀ R♂ maternal parent BC/r genotype (rr)

RR curve represents in vivo data for the BC/R genotype (RR) (see Table 2.4)

rr curve represents in vivo data for the BC/r genotype (rr) (see Table 2.4)

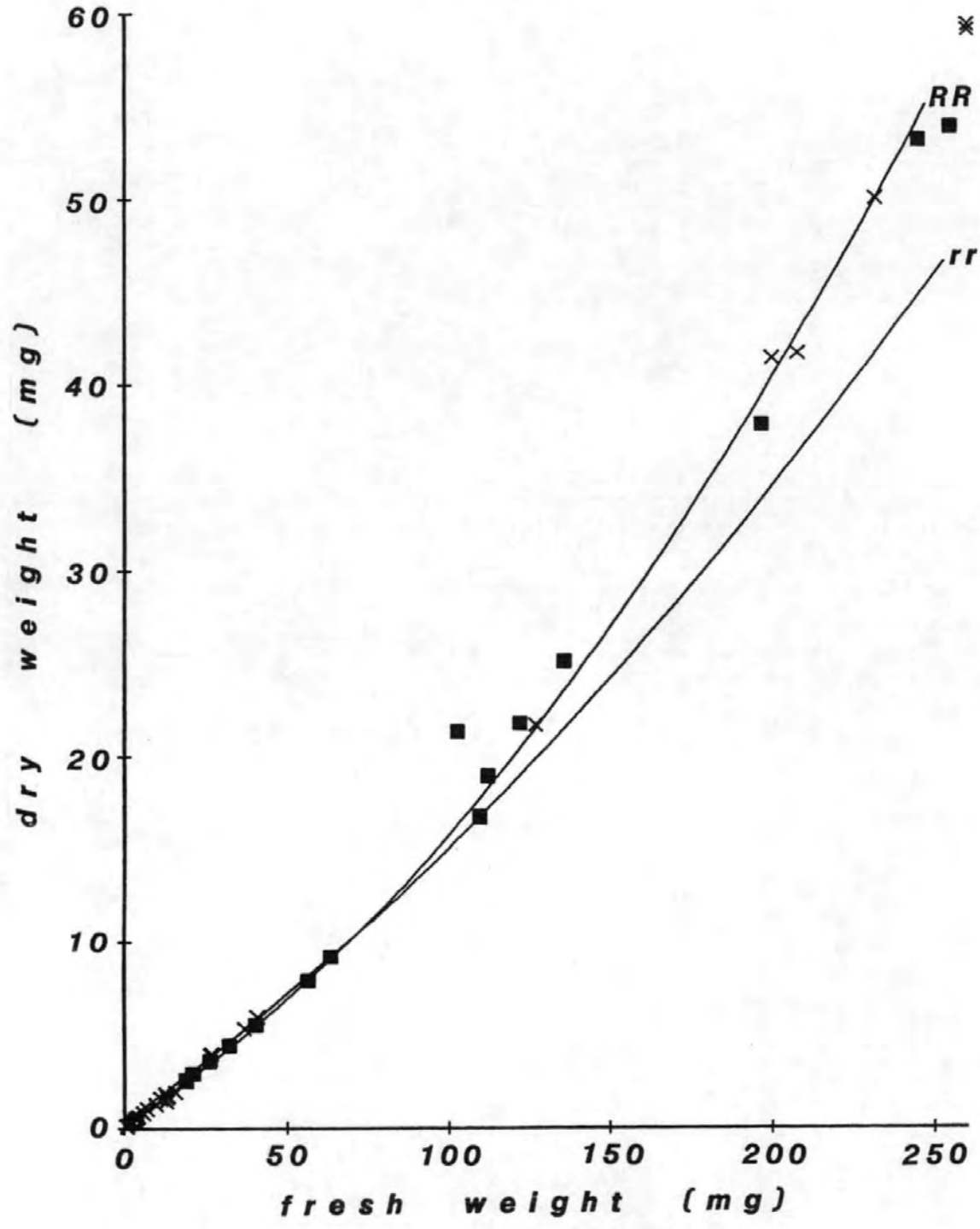
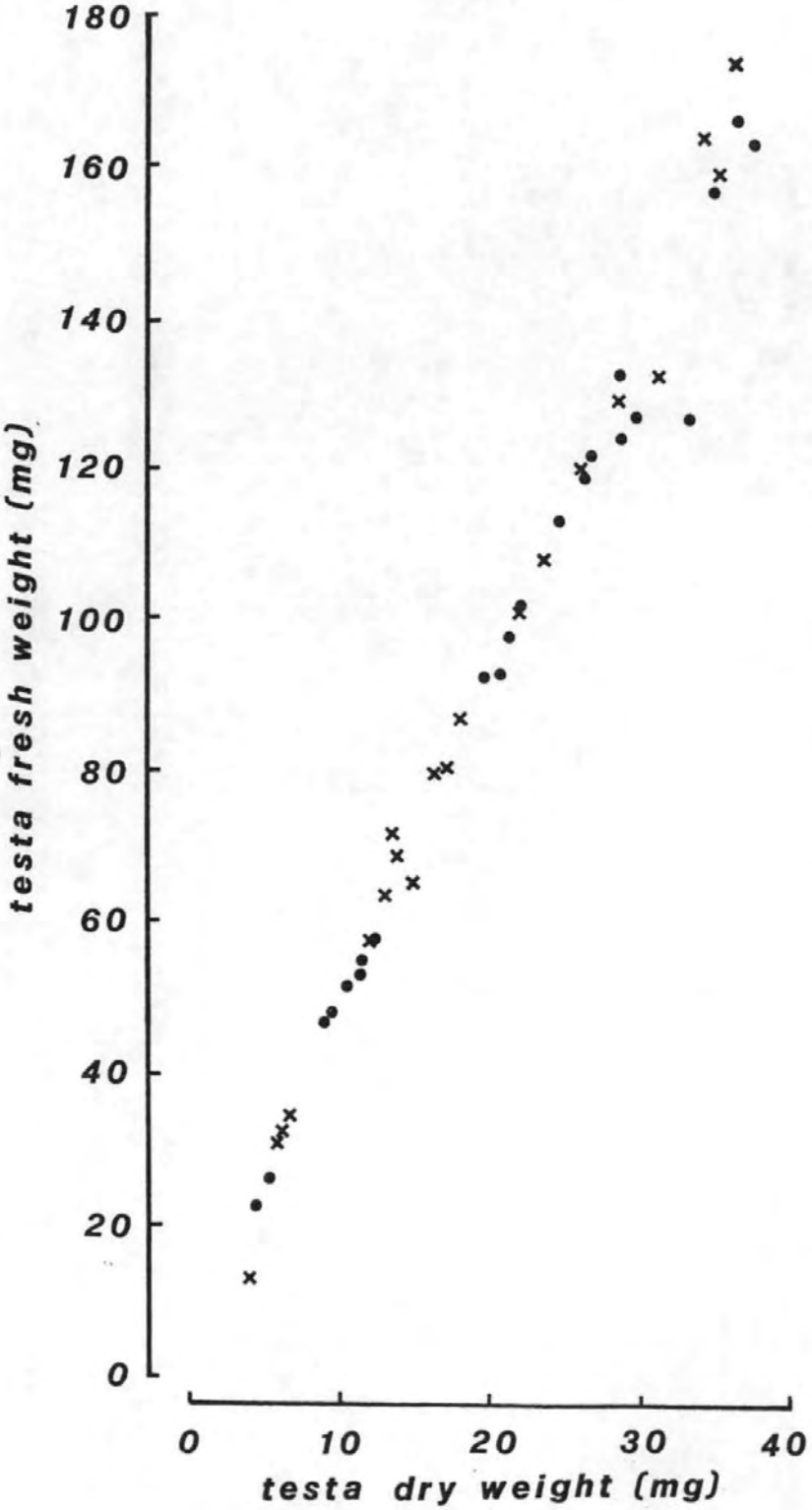

60
50
40
30
20
10
0
dry weight (mg)
0
50
100
150
200
250
fresh weight (mg)
RR
rr

FIGURE 2.19

Relationship between the fresh weight and dry weight of testas of Pisum sativum L. resulting from reciprocal crosses of the two lines near-isogenic except for the r-locus

• maternal parent BC/R genotype (RR)
(i.e. testa genotype is RR)

x maternal parent BC/r genotype (rr)
(i.e. testa genotype is rr)


180
160
140
120
100
80
60
40
20
0
testa fresh weight (mg)
0
10
20
30
40
testa dry weight (mg)

state.

Overall the water potential of the endosperm from any genotype was variable but within the limit -1.44 MPa to -1.57 MPa.

F. EMBRYO GROWTH IN VITRO

The aim of the following experiments was to determine whether embryos of the round- and wrinkled-seeded near-isolines could be grown in the absence of maternal tissues and still maintain the differences in fresh and dry weight observed *in vivo*. In order to aid this comparison, final fresh and dry weights are plotted on the "standard" curves obtained by fitting a quadratic curve (Table 2.6) through *in vivo* fresh weight and dry weight data to a maximum fresh weight of 255 mg.

Solute potential of sucrose concentrations are expressed in MPa. To aid comparison with other work, the equivalent osmotic potentials are listed in Table 2.7.

i) Experiment to determine the effect of 4 different sucrose concentrations on embryo growth in vitro.

Twelve-day old embryos of initial fresh weight 5 mg, cultured for 9 days on 5%, 10%, 15% and 20% sucrose, grew different amounts (Fig. 2.20). Maximum fresh and dry weight was obtained in both the *RR* (round-seeded) and *rr* (wrinkled-seeded) embryos in media containing 10 % sucrose (Fig. 20 A and B respectively). In media containing 15% sucrose, the *RR* embryos also increased substantially, having a final fresh weight of *ca.* 110 mg, as compared with a final weight of 13-14 mg in media having 5 % and 20 % sucrose (Fig. 2.20 A and B).

TABLE 2.6

Comparison of the equations of quadratic curves fitted to the graphs of fresh weight against dry weight (Fig. 2.9) of developing embryos (up to 255 mg fresh weight), of two lines of Pisum sativum L. near-isogenic except for the r-locus

Equation of quadratic : $y = a + bx + cx^2$

where a is the intercept
b is the initial slope of the graph
c is the degree of curvature

Parameter	RR	rr
a	0.261	0.416
b	0.110	0.123
c	0.000456	0.000238

n = 19 All units are mg

TABLE 2.7

Relationship between sucrose concentration, water potential and osmotic pressure of media

Added Sucrose % in media	water potential at that % sucrose	measured osmotic pressure of media mOsM/kg	theoretical % sucrose equivalent value
0.0	-0.018	70	2.4*
3.5	-0.507	208	7.1
5.0	-0.706	290	9.9
7.0	-0.833	342	11.7
10.0	-1.46	446	15.3
15.0	-1.88	650	22.2
20.0	-2.346	852	29.1

* Osmometer readings in this low range are inaccurate

The equivalent sucrose % value is not a constant above the measured readings, probably due to some break down of the sucrose into monomers

FIGURE 2.20

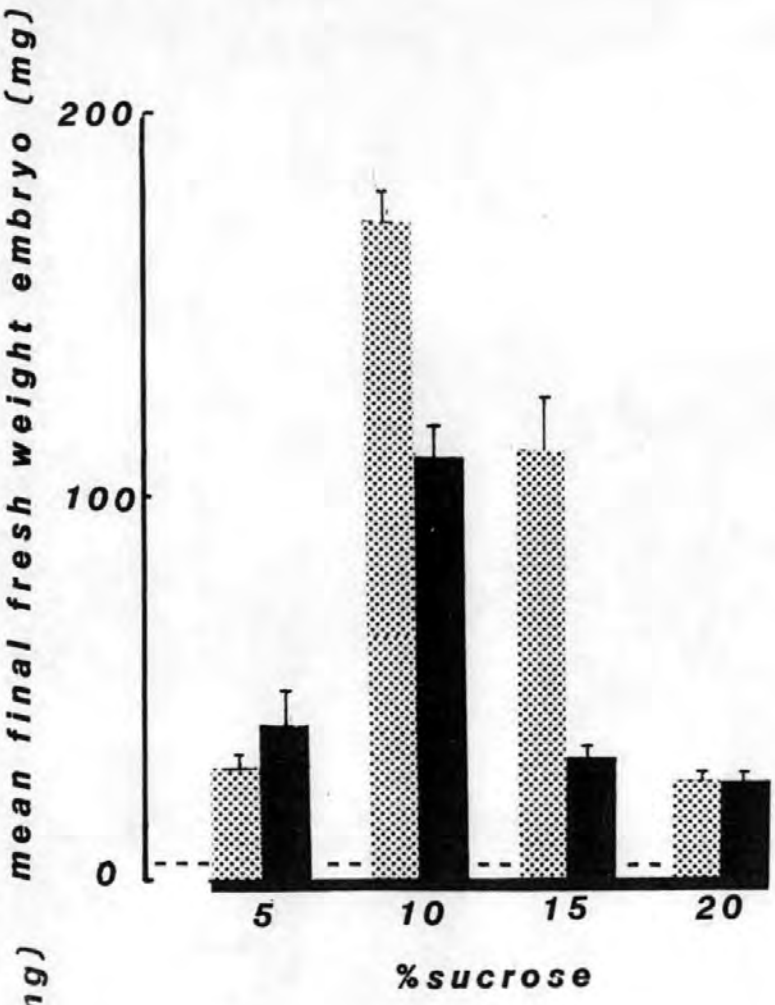
Comparison of mean final fresh weight (A) and mean final dry weight (B) of developing embryos of the two lines of Pisum sativum L., near-isogenic except for the r-locus, cultured in vitro for 9 days on liquid media of 4 different sucrose concentrations; 5 %, 10 %, 15 % and 20 %

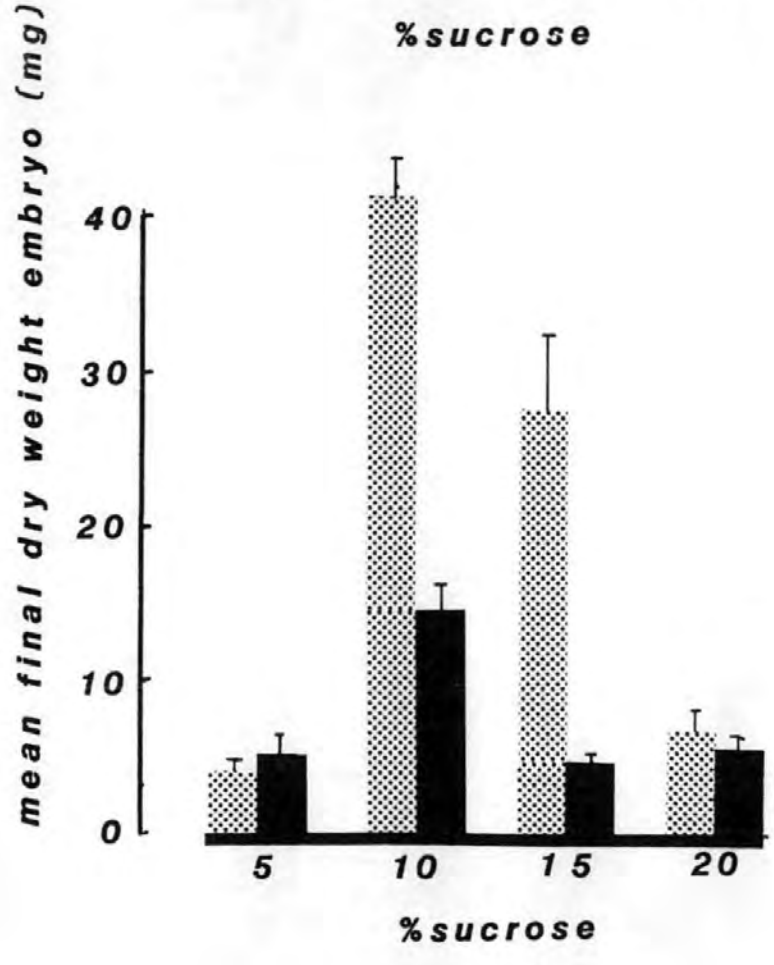
round-seeded BC/R genotype (RR)

wrinkled-seeded BC/r genotype (rr)

Bar lines represent one side of the standard deviation of the mean of 6 samples

Dashed line represents the initial fresh weight of embryos


mean final fresh weight embryo (mg)
200
100
0
5
10
15
20
%sucrose


mean final dry weight embryo (mg)
40
30
20
10
0
5
10
15
20
%sucrose

Hierarchical analysis of variance (see Techniques) indicated that there was no difference between dishes of embryos within treatments but that there was significant difference between the 2 genotypes, between treatments and that there was an interaction between genotype and treatment (Table 2.8).

Embryos grown in 5% sucrose had final fresh and dry weights similar to the *in vivo* embryos (Fig. 2.21). Embryos grown at the other sucrose concentrations had a greater dry weight than *in vivo* embryos of corresponding fresh weight (Fig. 2.21). This was particularly apparent at 10 % and 15 % sucrose when the round *in vitro* embryos had greatest dry weight.

The fresh weight / dry weight ratio of the wrinkled genotypes declined with increasing sucrose concentration (Fig. 2.22). At 5% and 20% sucrose there was no difference between the two genotypes but at 10% and 15% sucrose the wrinkled embryos had a greater proportion of fresh weight (i.e. contained more water) than the round embryos. The fresh weight / dry weight ratio of the round embryos did not vary significantly between 10%, 15% and 20% sucrose (Fig. 2.22). In addition, the embryos in the 5% sucrose produced shoots from the embryonic axis in both round and wrinkled genotypes. None of the embryos became albino.

The water potential of these cultured embryos increased with increasing sucrose concentration (Fig.2.22a). Hierarchical analysis of variance indicated that there was no significant difference between dishes of embryos within treatments nor any interaction between genotype and treatment. There was a significant difference in water potential between embryos in the 4 sucrose concentrations and a slight difference between genotypes (Table 2.9).

TABLE 2.8

Hierarchical Analysis of Variance of dry weights of embryos of *Pisum sativum* L., near-isogenic except at the r-locus, cultured *in vitro* for 12 days on media containing 5 %, 10 %, 15 % and 20 % sucrose

	d.f.	MS	p
between genotypes	1	1729	***
between treatments	3	1424	***
genotype x treatment	3	644	***
between replicates	16	33	n.s.
between dishes within treatments	24	27	--

* 5-1%
** 1-0.5%
*** < 0.5%
n.s. not significant at any level

d.f. degrees of freedom

MS mean squares

FIGURE 2.21

Relationship between fresh weight and dry weight of embryos of two lines of Pisum sativum L., near-isogenic except for the r-locus, cultured in vitro for 9 days

Curves represent in vivo data fitted to the quadratic equations of Table 2.6

□ round-seeded BC/R genotype (RR)

\+ wrinkled-seeded BC/r genotype (rr)

A Medium + 5 % sucrose

B Medium + 10 % sucrose

C Medium + 15 % sucrose

D Medium + 20 % sucrose

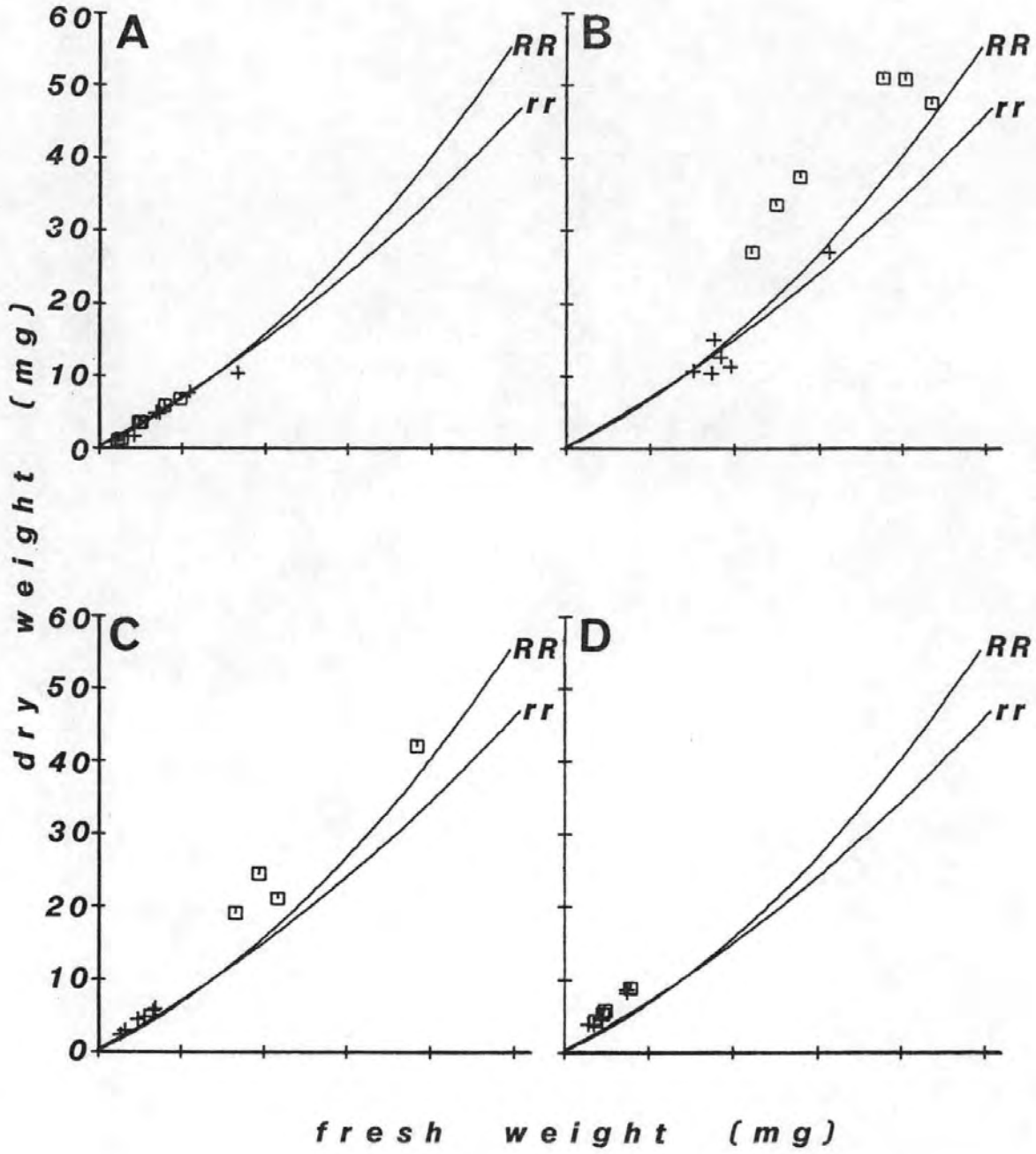

A
B
C
D
RR
rr
60
50
40
30
20
10
0
dry weight (mg)
fresh weight (mg)

FIGURE 2.22

A Relationship between fresh weight and dry weight of embryos near-isogenic except at the r-locus cultured for 9 days in vitro in 4 different sucrose concentrations; 5 %, 10 %, 15 %, 20 %

B Water potentials of embryos cultured in A. above

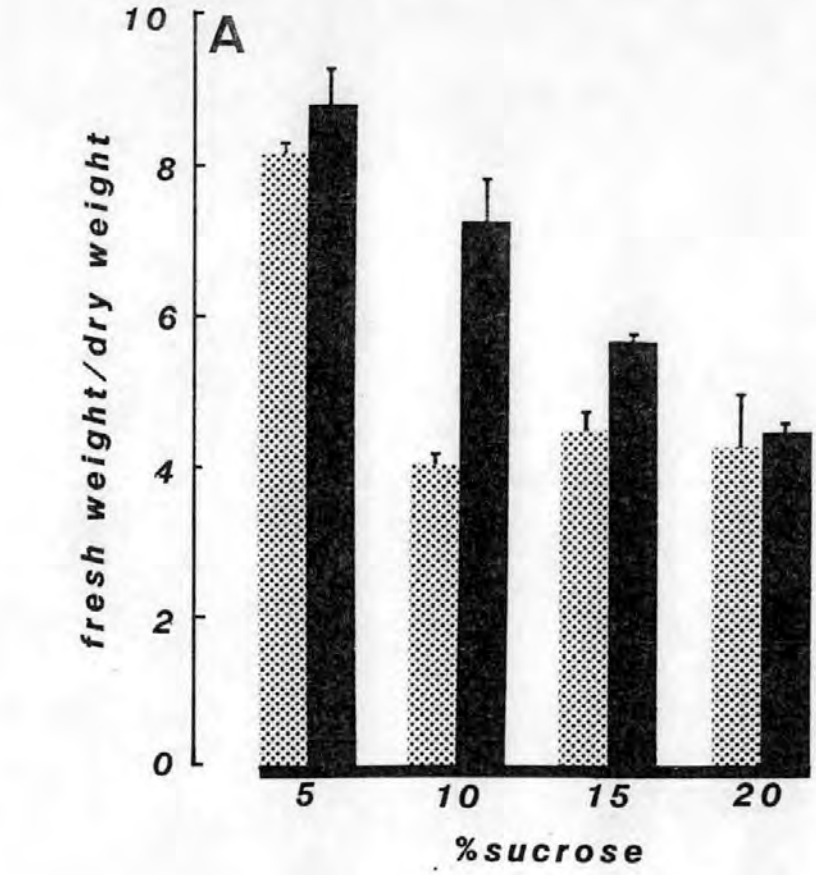
▨ round-seeded BC/R genotype (RR)

■ wrinkled-seeded BC/r genotype (rr)

m water potential of the medium

Each column is the mean of 6 samples

Bar lines represent one side of the error from the mean


A
10
8
6
4
2
0
fresh weight/dry weight
5
10
15
20
%sucrose

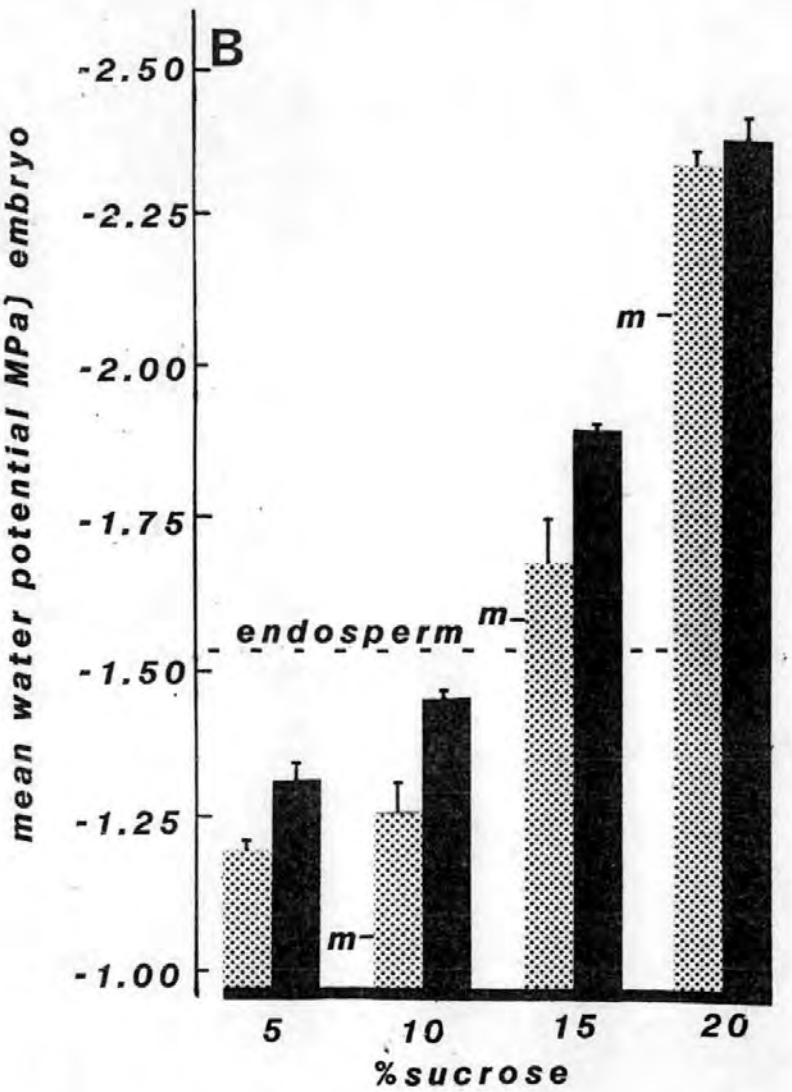

B
-2.50
-2.25
-2.00
-1.75
-1.50
-1.25
-1.00
mean water potential MPa) embryo
endosperm
m
m
m
5
10
15
20
%sucrose

TABLE 2.9

Hierarchical Analysis of Variance of water potentials of embryos of 2 lines of Pisum sativum L., near-isogenic except at the r-locus, cultured in vitro for 9 days in media containing 5 %, 10 %, 15 % and 20 % sucrose

	d.f.	MS	p
between genotypes	1	39045	**
between treatments	3	470447	***
genotype x treatment	3	3223	n.s.
between replicates	16	1992	n.s.
between dishes within treatments	24	1017645	-

d.f. degrees of freedom

MS mean square

* 5-1%
** 1-0.5%
*** < 0.5%
n.s. not significant at any level

ii) Experiment to determine the effect of low sucrose concentrations on embryo growth in vitro.

At 3% - 7% sucrose neither RR nor rr embryos aged 12 days of 2 mg initial fresh weight and cultured for 9 days grew substantially (Fig. 2.23). At 3% sucrose both round and wrinkled embryos became albino and did not increase in fresh weight or dry weight during the culture period (Table 2.10). The final fresh and dry weights of these embryos were very variable (Fig. 2.23). No shoots developed from the embryonic axis of embryos in any of the sucrose concentrations. There was no difference between genotypes, but there was a difference between treatments and also a genotype x treatment interaction (Table 2.10).

When compared with the expected fresh weights of similar-aged in vivo embryos, the final fresh weights of the in vitro embryos were much lower. However, when compared with the fresh and dry weights of in vivo embryos, they were similar (Fig. 2.23). Since they had grown very little they were all at the base of the curve where there was little or no difference between the genotypes (Fig. 2.23).

Since the embryos in this experiment did not grow substantially, it was not possible to determine their osmotic potential.

From their fresh and dry weights, it appears that 7 % sucrose produced the best growth of the 3 sucrose concentrations, but growth was not equivalent to that produced in vivo or in 10 % sucrose (i above).

FIGURE 2.23

Relationship between fresh and dry weight of embryos cultured in vitro for 9 days in media containing sucrose of 3 %, 5 % and 7 %.

Curves represent in vivo data, fitted to the quadratic equations in Table 2.6

RR : round-seeded BC/R genotype (RR)
rr : wrinkled-seeded BC/r genotype (rr)

A. Medium + 3 % sucrose

B. Medium + 5 % sucrose

C. Medium + 7 % sucrose

⊡ round-seeded BC/R genotype (RR)

\+ wrinkled-seeded BC/r genotype (rr)

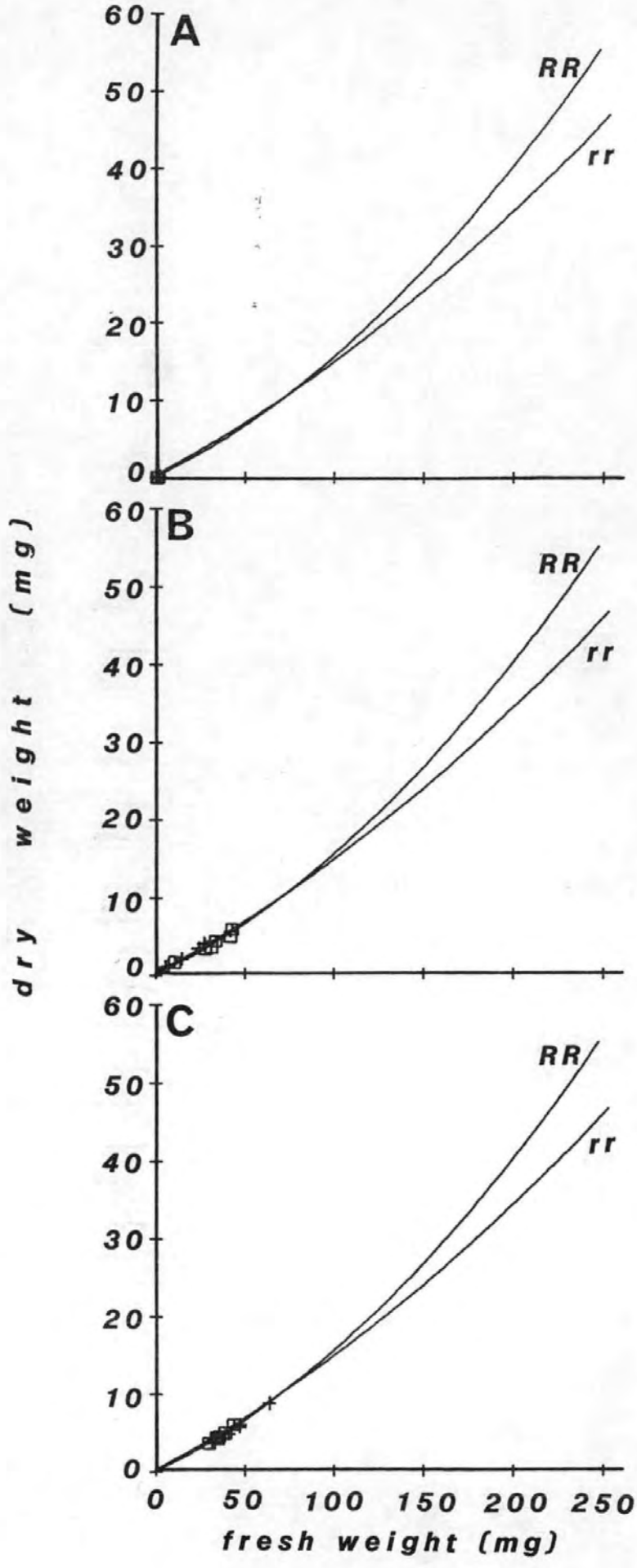

A
B
C
RR
rr
60
50
40
30
20
10
0
dry weight (mg)
0
50
100
150
200
250
fresh weight (mg)

FIGURE 2.24

Relationship between fresh and dry weight of embryos of 2 lines of Pisum sativum L., near-isogenic except for the r-locus, cultured in vitro for 9 days in media containing sucrose of 9 %, 11 % and 13 %.

Curves represent in vivo data, fitted to the quadratic equations in Table 2.6

RR : round-seeded BC/R genotype (RR)
rr : wrinkled-seeded BC/r genotype (rr)

A. Medium + 9 % sucrose

B. Medium + 11 % sucrose

C. Medium + 13 % sucrose

□ round-seeded BC/R genotype (RR)

\+ wrinkled-seeded BC/r genotype (rr)

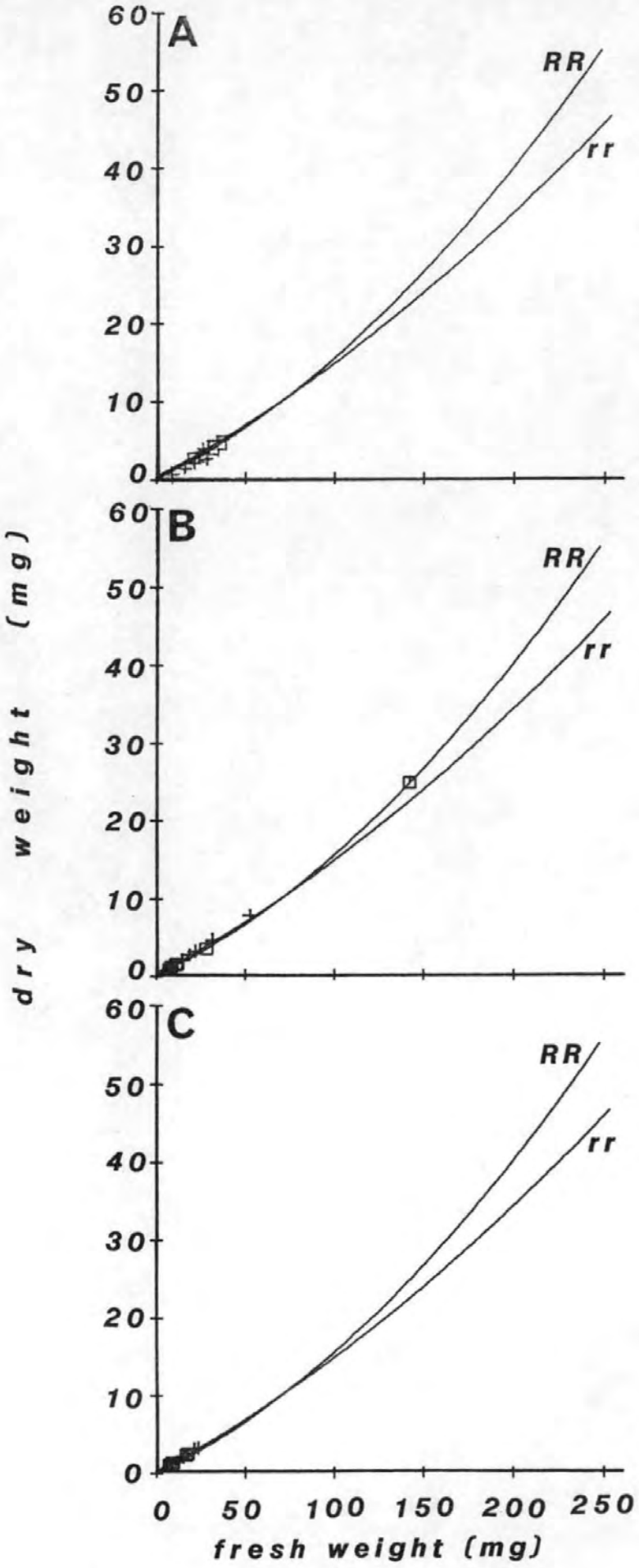

A
B
C
RR
rr
60
50
40
30
20
10
0
0
50
100
150
200
250
dry weight (mg)
fresh weight (mg)

TABLE 2.10

Final fresh and dry weights and water potential of embryos of 2 lines of Pisum sativum L., near-isogenic except for the r-locus, cultured in vitro for 9 days in media containing 3 %, 5 % and 7 % sucrose

% sucrose		water potential MPa embryo	media	mean final fresh wt. embryo (mg)	mean final dry wt. embryo(mg)
3	a	-0.523	-1.18	1.02±0.3	0.08±0.03
	b	-0.524	-1.19	1.35±0.4	0.11±0.03
5	a	-0.71	-1.25	31.2±11	4.06±0.4
	b	-0.79	-1.25	14.35±5	1.32±0.3
7	a	-0.81	-1.34	35.9±5	4.81±0.4
	b	-0.79	-1.45	55.3±10	7.6 ±0.4

a round-seeded BC/R genotype (RR)
b wrinkled-seeded BC/r genotype (rr)

Each weight is the mean of 6 samples ± standard deviation from the mean

Hierarchical Analysis of Variance of fresh weights of the embryos above

	d.f.	MS	p
between genotypes	1	16	n.s.
between treatments	3	6073	***
genotype x treatment	3	10879	***
between replicates	16	62	n.s.
between dishes within treatments	24	76	--

d.f. degrees of freedom
MS mean square

* 5-1 %
** 1-0.5 %
*** < 0.5 %
n.s. not significant

iii) Experiment to determine the optimum sucrose concentration for embryo growth in vitro.

Since 10 % sucrose produced the best growth, sucrose concentrations of 7 %, 9 % and 11 % were used as culture media to determine how critical sucrose concentration was. Embryos of 5 mg initial fresh weight were cultured for 9 days. The mean final fresh weight was greater in the cultured round (RR) embryos than in the cultured wrinkled (rr) embryos although all were of the same order, having grown between 7-and 8-fold. When compared with in vivo growth (Fig. 2.24), embryos which had grown in 11 % sucrose were most consistent with the in vivo growth curves. The 9 % and 13 % sucrose concentrations produced embryos which did not increase in fresh weight to the same extent. The optimum sucrose thus appeared to be 11 %.

iv) Experiment to determine the effect of mannitol as an osmoticum

In order to assess whether it was the water potential or the sucrose concentration which determined embryo growth, sucrose was replaced with mannitol to produce high water potentials but without providing any extra nutrients. Due to the lack of adequately-sized embryos no strict control for each sucrose concentration was carried out. Instead the 10 % sucrose concentration was compared with 10 % sucrose plus mannitol equivalent to 15 % sucrose and with 10 % sucrose plus mannitol equivalent to 20 % sucrose.

Replacing sucrose with mannitol resulted in very little growth of any of the embryos (Table 2.11) with no significant difference

TABLE 2.11

Final fresh weights and dry weights and water potential of embryos of 2 lines of Pisum sativum L., near-isogenic except for the r-locus, cultured in vitro for 9 days in media containing 10 % sucrose + varying amounts of mannitol equivalent to 15 % and 20 % sucrose

% sucrose equivalent		media MPa	mean final fresh wt. embryo	mean final dry wt. embryo
10	a	-1.13	14.46	1.92
	b	-1.12	20.5	2.0
15	a	-1.50	17.8	1.97
	b	-1.49	13.1	1.73
20	a	-1.93	11.4	1.54
	b	-1.93	8.1	1.31

a round-seeded BC/R genotype (RR)
b wrinkled-seeded BC/r genotype (rr)

Hierarchical Analysis of Variance of fresh weight of embryos from above

	d.f.	MS	p
between genotypes	1	0.000044	n.s
between treatments	2	0.034	***
genotype x treatment	2	0.025	n.s
between replicates	12	0.041	***
between dishes within treatments	18	0.0022	___

d.f. degrees of freedom
MS mean squares

* 5-1 %
** 1-0.5 %
*** < 0.5 %

n.s. not significant at any level

between treatments or a genotype x treatment interaction. When compared with *in vivo* growth, all the embryos had similar fresh weights with correspondingly similar dry weights to the *in vivo* embryos (Fig. 2.25) although they were all at the base of the graph. These embryos all remained green during culture and did not appear abnormal (Plate 2.7).

FIGURE 2.25

Relationship between fresh and dry weight of embryos of 2 lines of Pisum sativum L., near-isogenic except for the r-locus, cultured in vitro for 9 days in media containing sucrose of 10 %, plus mannitol to give conditions equivalent to that of 15 % and 20 % sucrose

Curves represent in vivo data, fitted to the quadratic equations in Table 2.6

RR round-seeded BC/R (RR) genotype in vivo

rr wrinkled-seeded BC/r genotype (rr) in vivo

A. 10 % sucrose

B. 10 % sucrose + mannitol (15 % equivalent)

C. 10 % sucrose + mannitol (20 % equivalent)

□ round-seeded genotype in vitro

\+ wrinkled-seeded genotype in vitro

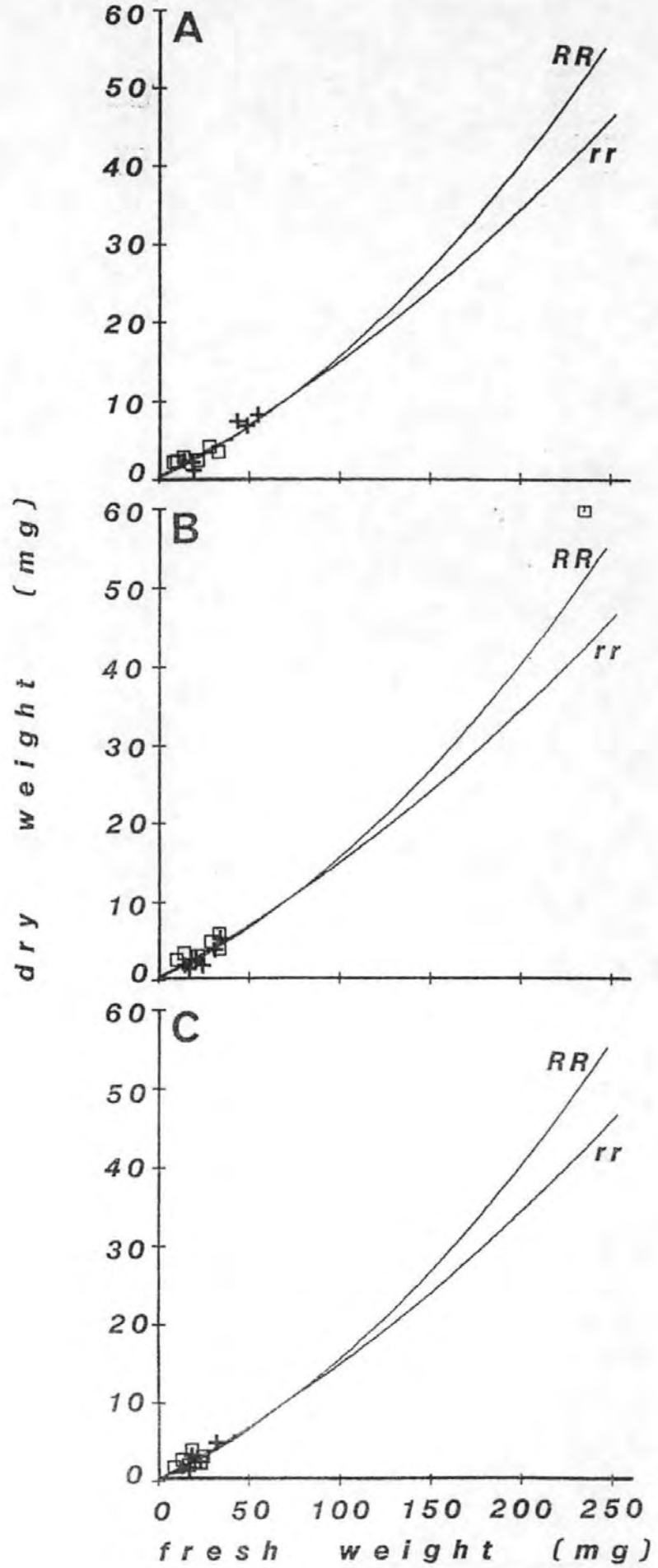

A
B
C
RR
rr
60
50
40
30
20
10
0
0
50
100
150
200
250
dry weight (mg)
fresh weight (mg)

PLATE 2.7

Embryos of 2 lines of Pisum sativum L., near-isogenic except for the r-locus, cultured in vitro for 9 days, in media containing 10 % sucrose plus mannitol to give equivalent of 15 % and 20 % sucrose

rr wrinkled-seeded BC/r genotype

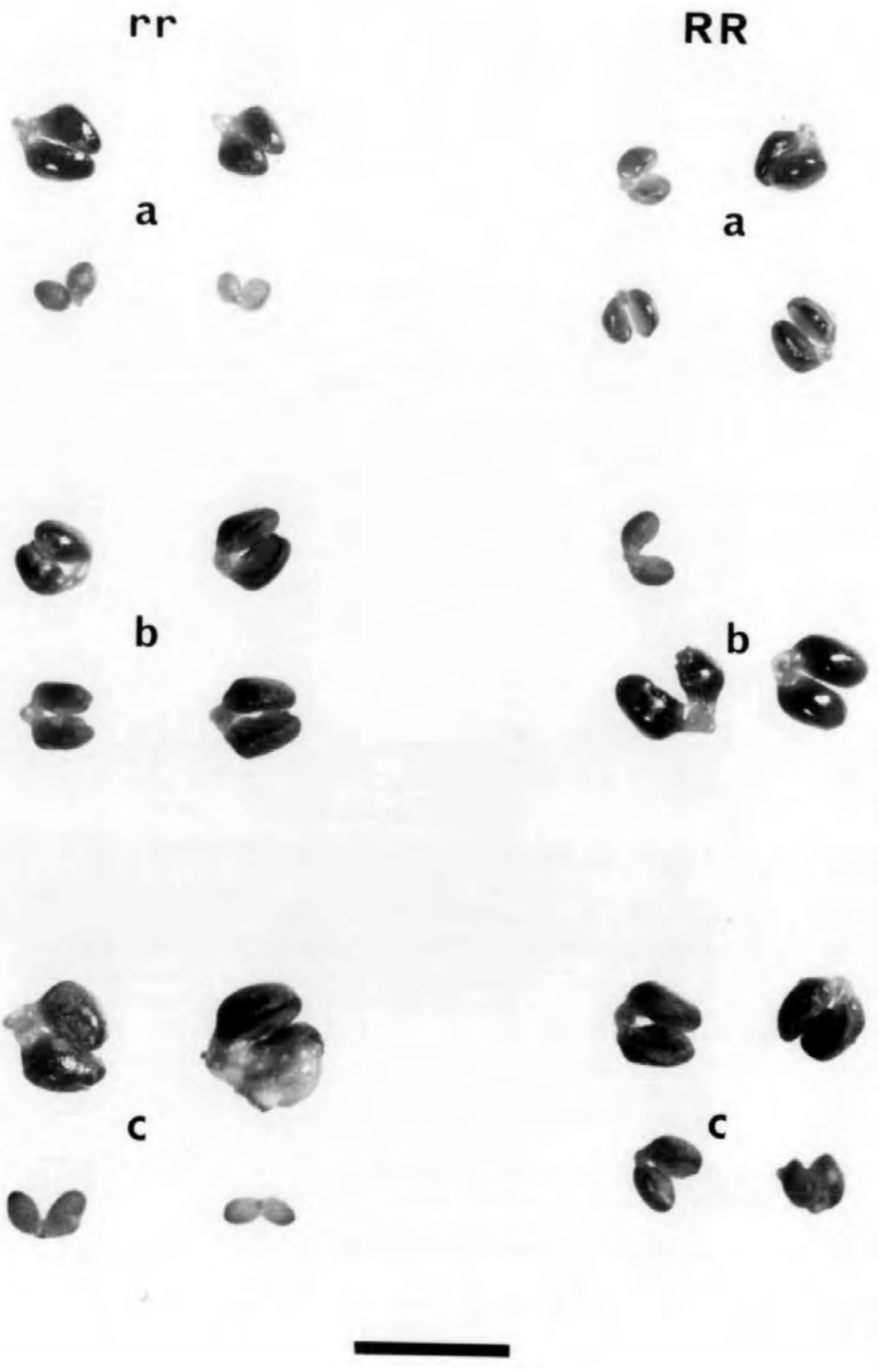
RR round-seeded BC/R genotype

a 10 % sucrose

b 10 % sucrose + mannitol (15 % equivalent)

c 10 % sucrose + mannitol (20 % equivalent)

bar line 1 cm


rr
RR
a
a
b
b
c
c

DISCUSSION

The general pattern of growth of pods, testas and seeds of the two genotypes near-isogenic except at the r-locus was similar to that of other genotypes described previously (Bisson and Jones, 1932; McKee, Robertson and Lee, 1955: Bain and Mercer, 1966; Hedley and Ambrose, 1980). Variation in the pod growth was quite high, with larger differences in fresh weight than in dry weight, suggesting that the pod is highly susceptible to water loss once it is removed from the plant. Pod fresh weight did not continue to increase after day 25 but fresh weight and dry weight of the seeds did increase, reflecting the lag phases described by Flinn and Pate (1968), Eeuwens and Schwabe (1975) and Hedley and Ambrose (1980). This indicates that the pod still acts as a source of nutrient either directly by itself losing dry weight or indirectly by acting as a transport route. That there was no significant variation in growth rate of pods and seeds from different nodes on the plants suggests that all nodes are fed equally by the plant. This is similar to Flinn and Pate (1970) who found that pods at different nodes contributed similar amounts to seed development. Final seed weight varied acccording to node; seeds from the lower nodes had smaller final seed weights than those from higher nodes and according to their position within the pod; seeds at either end tended to be smaller than those in the centre (personal observations). In the current instance, the comparable good growth of all 3 nodes was probably a consequence of there being only one pod growing at each node. Thus the total demand on the plant was reduced and therefore not truely comparable with Flinn's system.

Since no difference was found in growth of the pods and testas of the two lines, while differences were observed in the growth of the embryo, it appears that the r gene does not affect maternal (pod and testa) growth but is a gene which specifically affects the embryo. Dried seed weight was found to be affected only when the r gene was present in the homozygous double recessive (rr) state. Dried seed weight of the homozygous dominant (RR) and reciprocal heterozygotes (Rr and rR) were all found to be the same. This indicates that the R gene has complete dominance over the r gene for this character. This result contrasts with data of Davies (1975) who reported that dried seed weight of reciprocal crosses was determined by the maternal parent irrespective of whether it was round (RR) or wrinkled (rr). This suggests that in the various seeds examined by Davies (1975), which were not near-isogenic except for the r-locus, other genes carried by the maternal parent were affecting final seed size.

The dominance of the R gene was also reflected during development of the reciprocal crosses (as well as of the homozygotes). The R♀r♂ and r♀R♂ embryos had fresh weight and dry weight curves similar to that of the RR homozygote which was different to that of the rr homozygote. Since both Rr and rR embryos were genetically identical, had the same growth curves, final seed shape and starch grain shape as each other and as the homozygous (RR) parent, despite having developed inside genetically dissimilar testas, it appears that the maternal genotype had no effect on these characters. Kooistra (1962) suggests that wrinkling of the mature seed is associated with the increased water loss during ripening. This is consistent with the reciprocal cross data, where the R♀ r♂

and $\underline{r}^{\varphi}$ $\underline{R}^{\sigma}$ seeds lost less water than the rr seeds.

A major observed effect of the r gene was the difference in fresh and dry weight increase of the embryo during development. After day 20, 160 mg fresh weight, the wrinkled (rr) embryo had a greater fresh weight but lower dry weight than corresponding round (RR) embryos. Thus the water content of the wrinkled embryos was greater, which is consistent with data of Kooistra (1962) from non-isogenic lines. Reduction of water content within cells of Phaseolus is mainly due to the substitution of solid storage materials for water in the cells (Walbot, Clutter and Sussex, 1972). However, in the rr peas, although more water is lost it does result in a correspondingly high increase in dry weight, indicating that more than a straight substitution is involved.

According to Hsu (1979) there is an increase in endogenous ABA in bean embryos coincident with changes in water and osmotic potentials and the decline in water content. ABA has the ability to stimulate storage protein synthesis in cultured bean embryos (Sussex and Dale, 1979). If this occurred in developing peas then it may be that the differences in final protein content was due to an increase in the amount of ABA present. A close correlation has been established between water potential of sap and endogenous ABA content of bean plants (Walton, Galson and Harrison, 1977) and bean cotyledons (Dumbroff, Brown and Thompson, 1977). Hsu (1979) demonstrated that water stress can induce ABA formation in developing embryos, but it is at present unclear whether or not this is a causal relationship. It is interesting to speculate that an increase in water stress in rr pea embryos resulted in an increase in ABA which in turn caused an increase in cell size.

The attempts in this thesis to determine whether the cell size or number in the two different genotypes was responsible for their differences in final size were unfortunately inconclusive. There did appear to be some difference in cell size at the two stages examined but not significantly enough to be able to pinpoint their relevance. The wrinkled rr embryos had larger cells than the corresponding RR embryos, but the RR embryos at 240 mg still had quite a high proportion of small, probably dividing, cells. In order to determine definitively any differences in cell size and number between the two genotypes, it would be necessary to measure cell size and number over many more days of development. This work is currently being continued by Hedley and Ambrose at the John Innes Institute. Further investigations could include computer-assisted analysis of embryo volumes in relation to cell areas. The technique employed in this thesis was to derive an area from a 2-dimensional image assuming it was circular and to let this be an estimate of the cross-sectional area of the cell. In reality cells are 3-dimensional and are of various shapes. It would be interesting to determine cell volumes from the areas and assign them various shapes; for example spheres, cubes, pyramids, hexagons, and determine the effect of cell shape on final embryo volume.

It would also be informative if an analysis of ABA and other growth regulators were carried out in tandem, as Yeung and Brown (1982) suggest that changes in water stress, inducing ABA production, may affect m-RNA synthesis and indirectly trigger a switch in the developmental process. Another group of growth regulators which are relevant when discussing growth are the cytokinins. These are generally considered either to stimulate cell division or to be

produced as a consequence of this process. They have been postulated as being triggers of mitosis (Hall, 1973) which implies that a critical amount of cytokinin is essential for initiation of cell division (MacKenzie and Street, 1972). Thus if it were found that there was a difference in the final number of cells in the RR and rr embryos, it may be a reflection of differences in cytokinin content. Cytokinins have been detected in developing seeds of Pisum sativum (Burrows and Carr, 1970; Hahn, De Zacks and Kende, 1974; Krechting, Varga and Bruinsma, 1978; Van Staden and Button, 1978). In particular after fertilisation and during rapid seed or fruit growth when cell division and cell enlargement was occurring, cytokinin levels were increasing greatly, but nothing is known of the effect of genotype.

The fresh weight / dry weight ratio of the embryo highlights the difference in water content which occurred initially just after all the endosperm had been absorbed. The relevance of this observation to subsequent growth may be important; gibberellins and auxins, which affect cell growth, have been found to be abundant in endosperm during pea development (Eeuwens and Schwabe, 1975). Gibberellins in particular were abundant immediately prior to the endosperm being absorbed and may play a part in subsequent embryo development. Although these workers were using different genotypes and environments, it is possible that the differences in subsequent growth of the two near-isogenic lines reported here may be related to differences in production of growth regulators.

In none of the in vitro experiments reported here did there appear to be a difference between the round and wrinkled genotypes in the fresh weight to dry weight ratio. When small embryos were

cultured very little growth resulted, suggesting that there may be a threshold below which the embryos require something to sustain their growth which is lacking from the medium. The older embryos grew, but the fresh weight to dry weight ratio was not the same as that in vivo and it did not differ for the two genotypes. These embryos always had a greater dry weight than in vivo embryos of corresponding fresh weight. This suggests that the sucrose may be entering the cells to a greater extent than happens in vivo, resulting in greater dry weight. Further experiments on this system could include weighing individual embryos prior to culturing them in isolated compartments, so that the growth of each embryo could be monitored more precisely.

A further difference between the rr and RR genotypes was in the solute (osmotic) potential of the embryos during development. No difference was found in the potential of the developing pods or testa, suggesting that solute potential and thus sucrose / amino acid content of the embryo is controlled by the r gene. The solute potential should be considered in respect of the water contents. If similar absolute amounts of sugars and amino acids are present but in different quantities of water then the solute potential will be less negative with increasing water content. If we consider the solute potential of the embryo in relation to its environment, i.e. endosperm, testa and pod, it may be possible to elucidate the regulation of water transport and the differences between round (RR) and wrinkled (rr) embryos. Water tends to flow from a low solute concentration (high osmotic potential) to a high solute concentration (low osmotic potential). Thus water will flow from the pod to the testa at all stages examined in both round and wrinkled pea fruits, since the pod always had a higher (less negative) solute potential.

The relationship between the solute potentials of the different fruit parts of the two genotypes during development is illustrated diagrammatically in Fig. 2.26 and will be used in the subsequent discussion. The relationship between the testa - endosperm - embryo is more complicated than that for the pod and is dependant upon the developmental stage of the fruit. When endosperm is present, there is a gradient of solute potential from the endosperm to the embryo and to the testa in both genotypes.

A similar gradation of solute potentials was found in developing fruits of Phaseolus vulgaris L. (Yeung and Brown, 1982). They suggested that the embryonic axis maintains the most negative solute and osmotic potentials but there is no evidence to confirm this. However, the embryo is thus subjected to an osmotic gradient; one part is surrounded by the liquid endosperm with low solute potential, the other part is adjacent to the seed coat via the suspensor or the embryonic axis with higher solute potential. Such gradients are known to affect the differentiation of the embryos of macro-alga Fucus (Wareing and Phillips, 1978). However, in higher plants this osmotic gradient may have a more basic regulatory role in the differentiation process during early embryogenesis. The solute potential of the endosperm was variable over the period examined, suggesting that it acts as a buffer for incoming solutes and amino acids. This is supported by data of Murray (1979) who found that the endosperm of peas was actually capable of modifying compounds imported from the plant. Ryczkowski (1969) found that the osmotic potential of endosperm fluid from a variety of species, both mono- and di-cotyledonous, undergoes a steady decline during embryo development.

FIGURE 2.26

Diagrammatic representation of the relationship between the water potential of different tissues of developing fruits of 2 lines of Pisum sativum L., near-isogenic except for the r-locus

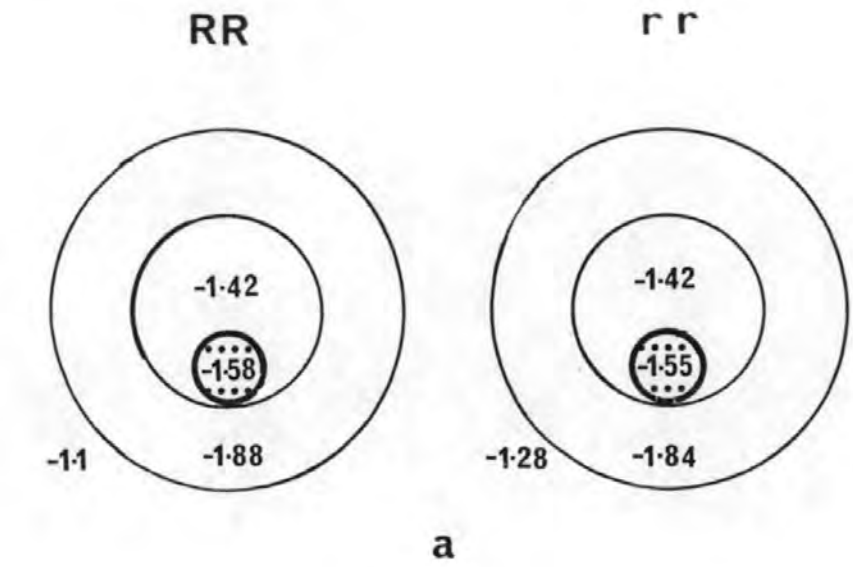
RR round-seeded BC/R genotype (RR)

rr wrinkled-seeded BC/r genotype (rr)

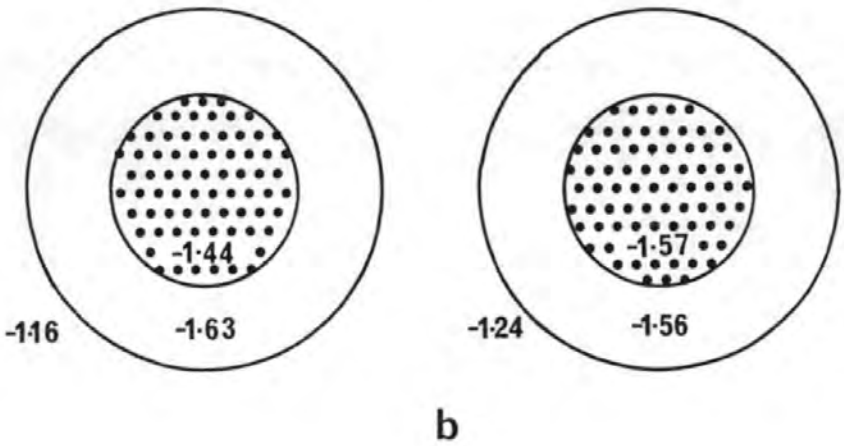
Negative numbers are the mean values of the water potential for each tissue;

shaded area	: embryo
value outside circles	: pod
value in outer circle	: testa
value in centre (a only)	: endosperm

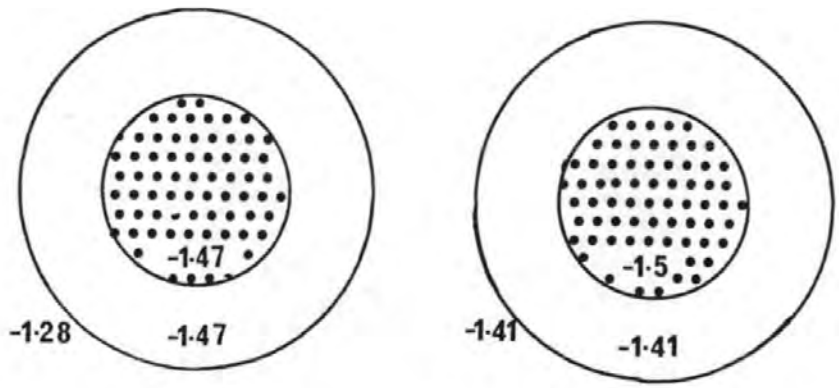
a 0-49 mg fresh weight embryo
b 150-199 mg fresh weight embryo
c 250-299 mg fresh weight embryo


RR
r r
-1·42
-1·58
-1·1
-1·88
-1·42
-1·55
-1·28
-1·84

a


-1·44
-1·16
-1·63
-1·57
-1·24
-1·56

b


-1·47
-1·28
-1·47
-1·5
-1·41
-1·41

c

Perhaps more important is the observation that although the absolute gradient of solute concentration is the same for the two genotypes early in development, it differs later on. The solute concentration of the embryo and testa was much higher in the round (RR) embryos than in the wrinkled (rr) embryos once the endosperm had been absorbed, suggesting that water had been taken up by both tissues. In the rr genotype, however, the solute potential of the embryo had not altered substantially, whereas that of the testa had increased. Since the sugar content of the rr embryos is much higher than in the RR embryos (Stickland and Wilson, 1983), it is probably an increase in sugar content which accounts for the maintenance of solute potential while the water content is increasing.

At the later stage of development there was no difference between the solute potential of the testa and embryo of the RR seed; both had a lower solute potential than the pod and were therefore probably taking up water from the pod which at this stage is just beginning to senesce. The solute potential of the rr embryo at this stage was slightly lower than that of the testa and therefore seems likely to be taking up water from both the testa and the pod. The difference in solute potential of the embryo and pod was much greater in the rr wrinkled-seeded fruit than in the RR round-seeded fruit. This suggests that water movement is favoured from the pod to the embryo in the wrinkled type more than in the round type. This theory is supported by the growth data (Fig. 2.7) which indicates that the wrinkled embryo contained more water than the corresponding aged round embryo.

Patrick (1983) established that photosynthate unloading from seed coats of developing ovules of Phaseolus vulgaris L. was

facilitated by and depended upon an energised membrane. His data suggest an osmoregulatory mechanism for transfer of K^+, sucrose and amino acids from the testa to the embryo (via the liquid endosperm). Analysis of extracts from seed-coat protoplasts and cotyledons demonstrated that K^+ and sugars (principally sucrose; Patrick and MacDonald, 1980) were the main osmotic components for these cellular compartments (Patrick, 1984). The osmotic potential of the seed coat (protoplast) was comparable to that of leaves (Cram, 1976) and was in the order of 600 mM (equivalent to -1.46 MPa). This is comparable with that found in pea embryos (-1.46 to -1.63 MPa according to genotype; Fig. 2.13), although the value for the cotyledons was found to be much lower, in the order of 330 mM (-0.804 MPa) (Patrick, 1984). He suggests that the influence of sucrose on photosynthetic transfer from plant to embryo was mediated by the osmolality (solute potential) of the apoplast solution and he showed that photosynthetic unloading was specifically sensitive to cell turgor potential. He further suggests that a turgor homeostat in the seed coats may be significant in terms of long-term assimilate transport. Thus the maintenance of constant turgor would minimise fluctuations in the turgor potential at the sink (embryo) end of the phloem path and hence ensure a constant driving force for pressure-driven flow. In this context, control of turgor within sink regions may contribute to their relative competitive ability to receive transported assimilates.

Thus the difference in solute potential of the embryos is an effect of the r gene and is not an effect of the surrounding solute potentials, since they were essentially the same in the two genotypes. Further investigations of the control of the solute potentials within the different genotypes was carried out using in vitro techniques.

Thus genetically different embryos were cultured under identical controlled conditions. Although none of the cultured embryos grew to sizes comparable with *in vivo* embryos, the optimum sucrose concentration was found to be 10 %, which was equivalent to a solute potential of -1.086 MPa. This is higher than that of the endosperm *in vivo*; -1.44 to -1.57 MPa (Fig. 2.16) and further suggests that the sucrose concentration is critical in determining embryo growth. The *RR* round embryos always increased in fresh weight to a greater extent than corresponding *rr* wrinkled embryos.

The media had a higher solute potential than the embryos, agreeing with that found *in vivo*, suggesting that water uptake was occurring. Since the embryos also increased in final dry weight at this sucrose concentration, it suggests that the embryos are synthesising protein and storage products from the surrounding media. This result contrasts with that of Stafford and Davies (1979), who found that the optimum sucrose concentration for pea growth was 18 %. However, upon closer examination their data shows no difference in fresh weight accumulation between embryos below 100 mg fresh weight grown on 4 %, 10 %, and 18% sucrose and no difference in dry weight accumulation between embryos above 100 mg fresh weight. The smallest embryos they cultured were 47.8 mg, which is substantially larger than most of those cultured here and the results are therefore not comparable. The larger (30 mg fresh weight) embryos which were cultured here also showed improved growth on all sucrose concentrations and for both genotypes. It thus appears that there is a critical time in development, before which the embryo is reliant upon external signals from the parent plant. These may be in the form of essential amino acids necessary to synthesise storage products,

proteins, etc., or they may be growth regulators which define subsequent embryo development. The foregoing discussion on the role of hormones in cellular and nucleolar development is thus equally relevant here. It would be very interesting to continue this project and grow a range of different sized embryos of the two genotypes in culture and then determine subsequent starch, protein and lipid contents. Attempts have been made at this (Casey and Domoney, 1983) but they were using high sucrose concentrations and relatively large embryos. It would be particularly useful to use the near-isogenic lines to determine definitively the effect of the r gene.

Several workers have examined the in vitro growth of other species which may provide some insight into subsequent growth of peas. Chang (1963) found that cultured barley embryos grew more quickly than corresponding in vivo embryos, becoming fully differentiated mature embryos while the in vivo embryos were only just at the early differentiation stage. However, he found that cultured embryos were larger in size at any given stage than in vivo embryos and that cell size differences accounted for the discrepancy. It was apparent that his culture conditions were responsible for inducing this growth and cell division. Clearly, for any meaningful results to be obtained by an examination of in vitro embryo growth it is necessary to determine the correct media for producing growth in vitro comparable to that in vivo.

Poor growth of small embryos of Capsella (< 50μm) indicates that they could not be successfully cultivated (Monnier, 1984). He suggests that either the media did not contain sufficient nutrient or that separation of the embryo from the plant causes a wound at the suspensor which prevents subsequent growth by preventing nutrient

uptake through the opening. He also found that the optimum sucrose concentration for embryo culture was 120 gl^{-1}, while a lower concentration of 80 gl^{-1} was most suitable for ovules. This difference probably reflects a difference in solute potential in the seed coat and the pod in a similar manner to that observed for peas. The optimum sucrose concentrations are in the same order as that for peas and beans, suggesting that there is a range of potentials which control fruit development.

It should be remembered that the testa is generally accepted as determining the final size of the embryo and hence of the seed as it imposes a physical constraint. In embryo culture this constraint will have been removed. Thus in theory it may be possible to grow larger embryos in culture than on the plant, particularly if the nutrient source is not limiting. In culture, it has been found in some species (periwinkle: Komamine, Morigaki and Fujimura, 1978; Acer pseudoplatanus: Gould and Street, 1975; Gould, Everett, Wang and Street, 1981) that nutrient deficiency can produce cell cycle synchrony.

Thus it appears that the control of embryo development both in vivo and in vitro is very complex. The r gene clearly plays a very important role in determining protein, starch, lipid and final size of the embryo. It would therefore be advantageous in trying to improve the nutritional quality of the pea seed to further investigate the mechanism of this gene for subsequent utilisation.

ENVOI

"Where shall I begin, please your Majesty?"

"Begin at the beginning" replied the King "and go on till you come to the end: then stop."

Alice in Wonderland

The science of genetics has grown from stength to strength since Batesons' initial founding of it. The unit of genetic information, the "cistron", has been identified and investigated extensively, resulting in a better understanding of how characters are controlled. The discovery of the structure and function of DNA (Watson and Crick, 1953) marked the beginning of a revolution in science and "molecular genetics" was born. Most of the work on DNA, cistrons, studies of mutations and recombinations has been carried out on E.coli, and by the late 1950s bacteria and bacteriophages had become model systems for all genetics. It opened many doors to elucidating the inheritance and control of higher organisms and was considered only a small step to go from E.coli to the rest of the living world. The French Nobel Prize winner, Jacques Monod, is reported to have tried to extend this knowledge of bacterial systems by saying that "what is true for E.coli is true for the elephant" (Judson, 1979) and at present there is much interest by molecular biologists in extending this knowledge to the "genetic engineering" of higher organisms.

However, it should be remembered that the study of bacteria has been going on for some 250 years and much information has been gathered regarding their life cycle, mutations, development, etc., to which molecular studies may be related. In order to achieve the sorts of genetic developments in higher organisms as have been produced in bacterial systems (for example insertion and reorganisation of genes) by molecular biologists, requires an extensive knowledge of that organism.

Not only are molecular biologists lacking that extensive knowledge of whole plants (and animals) but the system is further

confounded by the fact that while bacteria are haploid, higher plants are diploid. This means that although a mutation will be immediately manifest in the next generation of bacteria, a similar mutation in a plant may take several generations before it is observable.

Thus this thesis has gone back one step and examined the gross effects of two genes known to affect fruit development and described these effects further. When more information of this nature is obtained, it is anticipated that it may be used by molecular biologists to improve the quality of the pea crop. It may even be used as a model system for higher plants which could ultimately be extended to other plants.

It was apparent that only a limited amount of information could be usefully gained from the studies on the effect of the gp gene, since the green- and yellow-podded lines used for the comparative study were not near-isogenic except for this locus. Much more could be deduced from the studies of the effect of the r gene since near-isogenic lines were available. This is the more important gene from a plant breeding point of view, since it affects starch and lipid content and qualitatively affects storage proteins. However, it may be that the gp gene also has an effect on cell biochemistry; light has been shown to inhibit synthesis of DNA, RNA and protein in germinating hypocotyls of Lupinus angustifolius L. (Van Oostveldt, Van Goethem and Van Parijs, 1976). Since the pod modifies the quality and quantity of radiation reaching the seed (Section I), the gp gene may thus also (indirectly) affect these factors in the developing seed. It would therefore be useful if near-isogenic lines were available to follow up this theory and in understanding how chlorophyll production is regulated.

Another gene which is believed to affect storage products of the developing seed but about which little else is known, is the rb gene. Very few RR rbrb or rr rbrb genotypes exist, but there is a good case for introducing this locus into the near-isogenic lines used in this thesis. Growth analysis and osmotic studies could be carried out similar to those reported here for the r locus. Near-isogenic lines of these would also be useful for further study of its effects not only on the synthesis of storage products but also on its interactions with the r locus.

Since the r, rb and gp genes are all located on different chromosomes, it should not be too difficult to obtain near-isogenic lines. If such lines were desired for loci which were present on the same chromosome, difficulties may arise in separating them. However, such results would in themselves be useful in providing information about linkage.

The culturing experiments investigated in this thesis would provide much valuable information about the growth of fruits if they were extended using more near-isogenic lines. In particular they may be used to determine the effect of genes on pod development in isolation from the seed, just as the embryo was cultured in isolation from the plant to determine the effect of genes on embryo development. A combination of in vitro techniques with near-isogenic lines could open up a whole area of developmental biology so far not investigated.

The scope of the variation which exists in the garden pea makes it a particularly useful species to use in an investigation of gene action. Of course, to obtain such lines takes several generations which in the case of peas means several years work even if we asssume

two generations may be obtained each year. It is perhaps this long-term investment from which molecular biologists shy away and try to incorporate fragments of DNA into plants without adequate understanding. They may do well to heed, and plant physiologists may draw reassurance from, the response of Barbara McClintock (Keller, 1983) to the question of why she seemed to have the insight that many of her contemporaries lacked:

"...one must have a feeling for the organism, ...understand how it grows, understand its parts, understand when something is going wrong with it. [An organism] isn't just a piece of plastic, it's something that is constantly being affected by the environment, constantly showing attributes or disabilities in its growth. You have to be aware of all that..."

TECHNIQUES

"But you've no idea what a difference it makes,
mixing it with other things-
like gunpowder and sealing-wax."

Through the Looking Glass

A. GROWTH ANALYSIS STUDIES

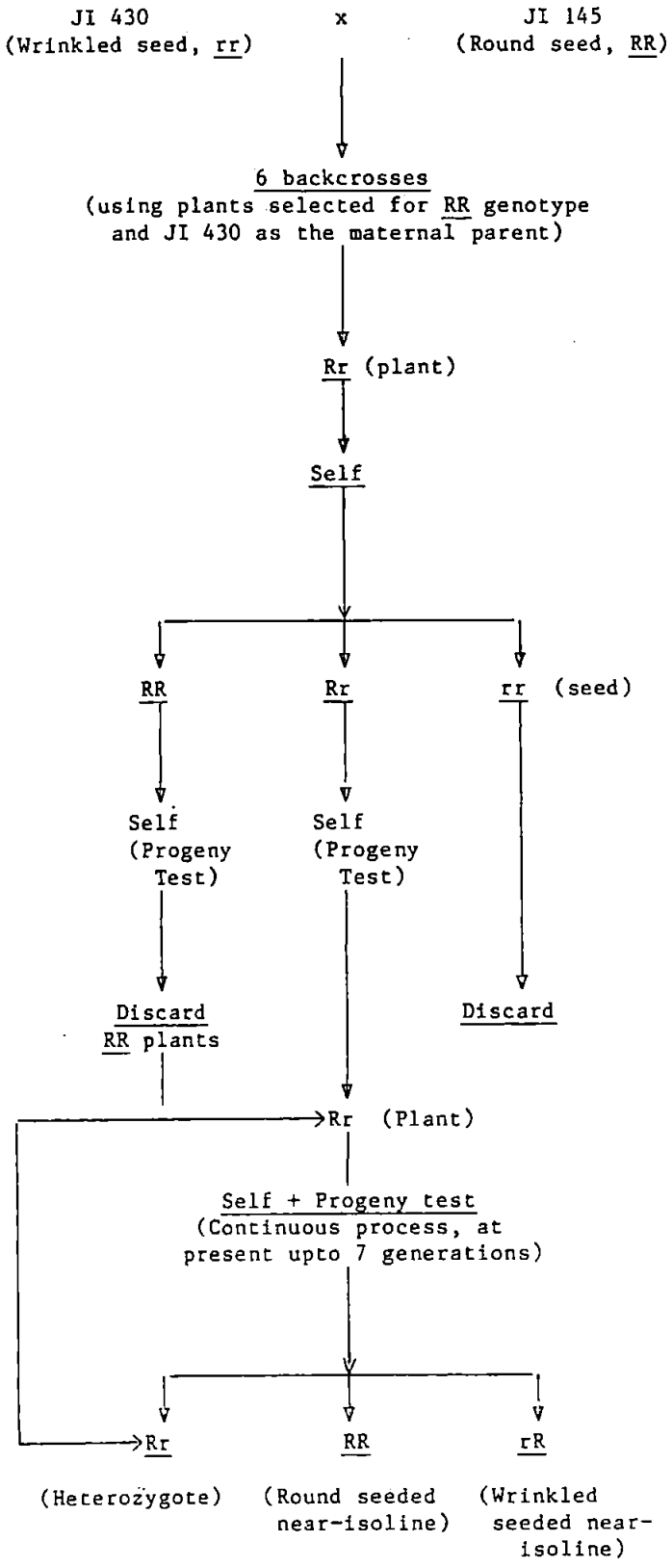
i) Plant Material

Green podded genotypes near-isogenic for the r-locus were used throughout this study. These were developed by Dr C.L.Hedley at the John Innes Institute, from a series of 6 back-crosses using JI 430, a wrinkled line, as the maternal parent and JI 145 as the round seeded parent. A further 5 generations of selfing the heterozygote (Rr) and progeny testing the round seeded segregants resulted in the round and wrinkled seeded near-isolines which were used in this thesis (Fig. 3.1; Hedley and Smith 1985).

The resulting round (RR) and wrinkled (rr) lines had very similar morphological characteristics (Table 1.1). In addition this similarity has been found to extend to the biochemical level. Comparisons of legumin and vicilin banding patterns from 2-dimensional O'Farrell gels have shown that the polypeptide patterns for both lines are very similar (S. Turner, personal communication). Using a number of storage protein cDNA probes, it has been shown from DNA digests using 3 different restriction enzymes that the DNA hybridisation patterns of the 2 lines are identical (Domoney and Casey, 1985). Also the original parents of the near-isolines (JI 430 and JI 145) have been compared for copy number and restriction enzyme digest pattern for a number of repetitive sequences including the rRNA genes, the 5S RNA genes and 4 other uncharacterised repetitive sequences. The parental lines could be distinguished from one another on the basis of copy number and patterns for rDNA. However, the comparison of the near-isolines showed no differences and were the same as the back-cross parent

FIGURE 3.1

Diagram of the breeding programme undertaken to obtain two lines of Pisum sativum L., near-isogenic except for the r-locus, using JI 430 genotype (rr) as the maternal parent and JI 145 genotype (RR) as the paternal parent


JI 430
(Wrinkled seed, rr)
x
JI 145
(Round seed, RR)
6 backcrosses
(using plants selected for RR genotype
and JI 430 as the maternal parent)
Rr (plant)
Self
RR
Rr
rr (seed)
Self
(Progeny
Test)
Self
(Progeny
Test)
Discard
RR plants
Discard
Rr (Plant)
Self + Progeny test
(Continuous process, at
present upto 7 generations)
Rr
RR
rR
(Heterozygote)
(Round seeded
near-isoline)
(Wrinkled
seeded near-
isoline)

(JI 430) for all probes including the rDNA (C. Cullis, pers. comm.).

Only seed from a single batch of plants were used, in order to prevent any possibility of genetic drift following multiplication.

A yellow-podded genotype (gpgp) JI 13, was selected from the John Innes pea germ-plasm collection to compare with the green-podded (GpGp) type. It was chosen for its similarity in other respects to the near-isogenic lines. These plants are described in Table 1.1 of Section 1.

ii) Growth of plants

Seeds were sown at 15 mm depth in 14 cm diameter pots in John Innes No 1 potting compost plus an additional 30% chick grit. Plants were kept in a glasshouse until flower initiation and were then transferred to a controlled environment room at $15^{o}C \pm 2^{o}C$ with an 18 hour photoperiod. Photon flux density at pod height was 400 $\mu molm^{-2}s^{-1}$, from 360W high pressure sodium lamps (GEC Solarcolour).

In order for pod age to be determined, flowers were date-tagged at anthesis (Plate 1.1), corresponding to when the corolla of the flower had fully opened. This time was designated day 1 and all references to the number of days from anthesis refer back to this day. Side shoots, basal branches and the main apex were removed from all plants leaving only a single main stem per plant with 3 flowereing nodes. Flower buds were also removed to allow only one flower to open at each flowering node. Only one pod was allowed to develop per node and three pods per plant, to minimise within plant variation and reduce the risk of seed abortion.

Plants were fed weekly with a low N fertiliser (Fisons, Solinure) and a systemic fungicide (Duponte, Benlate) to prevent powdery mildew.

iii) Reciprocal crosses

The near-isogenic lines were crossed reciprocally to produce seeds with genetically similar embryos within a testa with either the rr or the RR genotype. This was achieved by emasculating flowers of RR and rr genotypes prior to pollen production and fertilising the stigmas with pollen from rr and RR flowers respectively.

iv) Harvest of material

Thirteen batches of fruits were harvested for measurement between the 10th and 30th days after anthesis. Each harvest consisted of 5 pods. The centre 3 seeds from each pod were taken for subsequent analysis (fresh weight, dry weight, cell numbers, cell size determination), the remaining seeds being discarded. It has been shown previously that this method of plant growth and seed selection reduces environmental variation and increases the chance of identifying genetic differences between genotypes (Hedley and Ambrose, 1980).

v) Seed weight distribution

The seed size distribution and the mean seed weight was determined by weighing 100 seeds individually of each genotype and grouping the weights into 10 mg $\pm$ 1 mg classes.

vi) Fresh weight

Pods were harvested by cutting at the peduncle. Whole fruits, pods and seeds greater than 200 mg were weighed on a top-pan balance accurate to ±1 mg. Testas and embryos less than 200 mg were weighed on a Cahn balance, accurate to ± 0.01 mg. Prior to weighing, testas and embryos were blotted on tissue paper to remove any endosperm. Endosperm fresh weight was estimated as the difference in fresh weight between the whole seed and the testa + embryo (assuming the density of endosperm to be similar to that of water).

vii) Dry weight

Whole organs were placed on pre-weighed aluminium foil and dried in an oven at 80°C to constant dry-weight.

viii) Osmotic studies

Pod sections and whole testas and embryos of known fresh weight were ground individually in 1 ml Eppendorf vials and centrifuged at 17,000 r.p.m. for 15 min. The osmotic pressure of the resulting supernatants and of pure endosperm was then measured using a Wescor 5100B Vapour Pressure Osmometer.

In simple systems at constant temperatures, the water potential of a system results from the combined but opposing actions of pressure and osmotic potential according to the equation

$$\Psi = \Psi_p + \Psi_s + \Psi_m$$

water potential	pressure potential	osmotic potential	matric potential

The matric potential is a measure of the tendancy for the matrix to absorb water molecules. These effects can be included in the term for water potential (Milburn, 1979) and so may be ignored for this purpose.

The pressure potential is caused by pressure, for example from the cell walls. Since in the system examined in this thesis the tissue has been macerated, the pressure potential must be zero. Thus

$$\psi = \psi_s \quad \text{and} \quad -\pi = \psi \quad \text{(Milburn, 1979)}$$

water potential	osmotic potential	osmotic pressure	water potential

Hence water potential was calculated from the measured osmotic pressure according to the van't Hoff equation

$$\text{osmotic pressure} = -m \times \frac{RTMw}{1000Vw}$$

where R is the gas constant, T is the absolute temperature, m is the osmolality as measured, Mw is the molecular weight of the solvent, Vw is the partial vapour pressure of the solvent.

Thus in this instance,

osmotic pressure = -m x 24.36 bar

= -m x 2.436 MPa at $20^{o}C$.

Mean water potentials were expressed for pod, according to pod size and for testa, endosperm and embryo according to the fresh weight of the embryo (50 mg size classes).

ix) Cell number determination

Cell numbers were determined by digesting whole cotyledons in 5% chromic acid at 50°C for different times according to embryo size. The chromic acid was then replaced with a known volume of distilled water and the cells were separated by being sucked gently up and down a Pasteur pipette. Suspensions were pipetted onto a haemocytometer and cells counted. The mean of 5 replicates was taken as representative for each digest.

x) Cell area determinations

Cells were separated as for cell number determination. Areas were measured by taking random samples of cell suspension and photographing under a Zeiss photomicroscope II. The resultant negatives were then used to project an image onto a graphics tablet connected to an Apple micro-computer and areas determined by outlining the perimeter of the cell with a light pen. These cross-sectional areas are expressed as percent frequency histograms for each size class of embryo (50 mg classes).

xi) Statistical Analysis

The results of the above fresh and dry weight determinations were analysed using regression analysis to determine whether there was any difference between nodes within each genotype and between genotypes. Where significant differences were found, further analyses on the regression slopes were carried out using t-tests to determine more precisely the significance and basis of the differences (Steele and Torrie, 1980).

B. MICROSCOPY STUDIES

i) Light microscopy

Sections were immersed in approximately 5 cm^3 of freshly prepared fixative: 1 part formalin, 1 part glacial acetic acid, 18 parts 60% ethanol (Jensen 1962) and vacuum infiltrated. The sections were transferred to fresh fixative and were kept in specimen vials at $4^{\circ}C$ for a minimum of two weeks. The fixative was then discarded, replaced by 70% alcohol and left at room temperature for 12 hours. The sections then underwent the following embedding procedure in a Shandon Elliott automatic tissue processor:

90% ethanol	24 hours
absolute ethanol	2 hours
absolute ethanol	30 minutes
xylene	30 minutes
fresh xylene	15 minutes

The sections were individually immersed in liquid paraffin wax and left under vacuum for 24 hours to allow full infiltration. The sections were then set in individual wax blocks (3 cm x 2 cm x 1 cm) and left in cold water for 1/2 hour to harden. The blocks were sectioned to a thickness of 15 μm using a Leitz Rotary Microtome. Sections were mounted on glass microscope slides, using egg albumin as mountant and left to dry overnight.

The slides underwent the following procedure to stain the lignin in the cell walls, the nucleoli and the chromosomes with safrannin and the cytoplasm and cellulose with fast green (Jensen, 1962):

xylene	2 min.
xylene	2 min.
absolute ethanol	2 min.
absolute ethanol	1 min.
90% ethanol	1 min.
70% ethanol	1 min.
50% ethanol	1 min.
30% ethanol	1 min.
distilled water	1 min.
1% safrannin	30 min.
1% fast green in 2 % glacial acetic acid	10 min. (leaf) 10 sec. (pod, testa) 30 sec. (cotyledon)
distilled water	10 sec.
70% ethanol	1 min.
abs. ethanol	1 min.
abs. ethanol	1 min.
abs. ethanol	1 min.
xylene	1 min.
xylene	1 min.
xylene	1 min.

Slides were covered with no.1 coverslips using D.P.X. mountant (Kirkpatrick and Lendrum, 1939) and left to dry overnight.

Photographs were taken on black and white Pan F 35 mm film using a Zeiss photomicroscope II.

To stain specifically for lignin, fresh sections were cut by hand and stained with saturated phloroglucinol in 20% HCl for 5 min. Lignified tissue stained bright pink.

ii) Electron microscopy

Tissue was fixed using the double fixation method of Juniper, Cox, Gilchrist and Williams, (1970). This results in better fixation than either one alone since the glutaraldehyde penetrates faster than osmium teroxide and thus cellular activity stops more quickly, resulting in better preservation of the fine structure.

Fresh tissue sections were fixed by placing them in 3%

glutaraldehyde in cacodylate buffer (100 cm^3 0.1M sodium cacodylate + 8.3 cm^3 0.1M hydrochloric acid, diluted to 200 cm^3 with distilled water; Sabatini, Bencsh and Barrnett, 1963), pH 7.2 and vacuum infiltrated for 2 hours at room temperature. The fixative was replaced with a fresh solution and sections were left on an automatic rotator overnight. Sections were washed 3 times, 15 minutes each wash, with cacodylate buffer. The buffer was replaced by 2% osmium tetroxide in cacodylate buffer and left on an automatic rotator for 2 hours. Sections were washed as above, dehydrated and infiltrated with Spurr's (1969) resin using the following modification of Juniper et al., (1970):

30% ethanol	1 hour
50% ethanol	1 hour
70% ethanol	overnight
90% ethanol	15 min.
absolute ethanol	15 min.
absolute ethanol	15 min.
2:1 ethanol: resin	2 hours
1:1 ethanol: resin	2 hours
1:2 ethanol: resin	3 hours
pure resin	overnight

Spurr's resin:

ERL-4206	10.0g
DER 736	6.0g
nonenyl succinic anhydride, NSA	26.0g
s-1 accelerator	0.4g

Sections were embedded individually in pure resin in silicone rubber moulds and cured at 60°C for 8 hours. These blocks were trimmed and sectioned on an L.K.B. II microtome using freshly cut glass knives. The large amounts of starch present, particularly in the testas and cotyledons, made sectioning difficult. Diamond knives were used but produced no better results, so glass knives were used and replaced frequently.

Gold-coloured sections were floated on water, expanded in

chloroform vapour and mounted on naked copper grids, mesh-size 200. These sections were stained as follows:

saturated uranyl acetate	30 min. in a CO_2-free atmosphere to prevent precipitate forming
Reynold's (1963) lead citrate	10 min. in the dark

The lead citrate enhances the contrast of nucleic acids, membranes and particularly glycogen (Juniper et al., 1970).

Sections were viewed under a Phillips 300 or a Siemens electron microscope. Micrographs were taken at 80kV on black and white EMscope film.

C. MISCELLANEOUS STUDIES

i) Measurement of transmission of radiation

Pod, testa or leaf sections were placed over the sample hole of the reflectance sphere attachment of a Pye Unicam SP 8-100 double-beam spectrophotometer. Light transmitted through tissue was measured between 350nm-750nm. Four pods of each genotype were measured at each of 4 growth stages, 2 replicates per pod. Leaves and testas were measured at a single, fully developed stage.

Percent transmission measurements thus obtained were converted to typical absolute amounts of radiation by multiplying the amount of radiation from the sun at any given wavelength (Smith, 1981) by the % transmitted through plant tissue at the same wavelength.

Radiation reaching the embryo was determined by combining the transmission data through the pod and testa and determining absolute values as above.

ii) Chlorophyll content

Chlorophyll content was measured using the method of Arnon (1949) and expressed on a fresh weight or whole organ (pod, testa, embryo) basis. Two samples were measured for each growth stage of pod, testa and embryo.

iii) Stomatal counts

Stomatal counts were made on isolated pieces of epidermis stripped from adaxial surfaces of leaves and pods. Pieces were floated on distilled water and counts were made under a Zeiss microscope at a magnification of x 10 with 10 fields of view per leaf or pod. The mean was taken of 5 samples per structure.

iv) Pod Thickness

Pod thickness was measured across 4 mm sections cut dorso-ventrally from the centre of the pod using an electronic digital caliper accurate to + 0.03 mm. Five samples were measured for each genotype at a single age, stage IV.

D. IN VITRO STUDIES

i) Culture media

Immature embryos were cultured using the liquid media of Stafford (1978, Table 3.1). All media were filter sterilised to prevent the chemical composition and osmotic pressure from changing. The pH was adjusted (with 0.1M NaOH) to 5.6 to match that of the endosperm (personal observation).

TABLE 3.1

Liquid medium for culturing immature pea embryos (after Stafford, 1978)

Component	$mgdm^{-3}$	Component	$mgdm^{-3}$
macro-nutrients:		amino acids:	
KNO_3	1900	alanine	500
NH_4NO_3	1650	arginine	250
$MgSO_4.7H_2O$	370	asparagine	100
$CaCl_2.2H_2O$	440	aspartic acid	100
KH_2PO_4	170	glutamic acid	500
		glutamine	500
micro-nutrients:		glycine	250
$MnSO_4.4H_2O$	22.3	histidine	100
$ZnSO_4.7H_2O$	8.6	isoleucine	250
H_3BO_3	6.2	leucine	100
$CuSO_4.5H_2O$	0.025	lysine	50
$Na_2MoO_4.2H_2O$	0.25	methionine	500
$CoCl.6H_2O$	0.025	serine	250
KI	0.83	threonine	500
		valine	150
vitamins:			
myo-inositol	100		
thiamin HCl	0.1		
pyridoxin HCl	0.5		
nicotinic acid	0.5		
$FeSO_4.7H_2O$	27.9		
Na_2EDTA	37.5		

Sucrose was added as a nutrient / osmoticum at various levels between 2 % and 20%. In order to determine the effect of sucrose and osmoticum, sucrose was replaced by mannitol, which affects the osmoticum but is essentially inert.

ii) Culture method

Seeds were sterilised in 1% hypochlorite + Tween 80 (a surfactant) for 5 minutes, rinsed 4 times with sterile distilled water before the embryos were dissected out. Six embryos of known ages/ size classes were placed in 9 cm petri dishes containing 20 ml media per dish. Dishes were sealed with nesco film and left in a constant environment room at 15°C, 16h photoperiod, photon flux density of 35 $\mu molm^{-2}s^{-1}$ for 9 days. Three replicate dishes were made for each size class or treatment.

Resulting fresh and dry weights were plotted on growth curves of the *in vivo* data obtained by fitting a quadratic curve through the points, using the "Maximum Likelihood Programme" of Ross (1980).

Results were analysed using the hierarchical analysis of variance with cross-classified effects (Steele and Torrie, 1980). This distinguished between genotypes, between treatments, between dishes within treatments, between replicates within dishes and the genotype x treatment interaction. Significant differences were determined using t-values.

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"'fore autumn when the leaves turn brown,
please take a pen and write it down..."

Through the Looking Glass

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Genetic variation for fruit development of Pisum sativum L., with special reference to the rugosus locus.

Abstract

This study has compared various aspects of pea fruit development, using two green-podded lines near-isogenic except for the r locus and a yellow-podded mutant having the genotype gpgp.

The gp gene is associated with reduced chlorophyll in the pod wall which then appears yellow. The r gene is associated with a wrinkled appearance of mature dried seed and cotyledonary starch grains which are small and fissured.

An ultrastructural survey of tissues from pods, testas and cotyledons showed there to be no effect of the r locus on chloroplast structure. The structure of amyloplasts however, appeared to be affected by the r locus; starch grains in the cotyledons having a rugged outline in the wrinkled type.

Chloroplasts from the inner tissues of the yellow pod were similar to those in leaves, green pods, green-podded testas and cotyledons. Chloroplasts in the yellow pod mesocarp and in the testa of yellow pods had dilated thylakoids, less starch and more lipid than the green-podded types.

Growth analysis of the near-isogenic lines showed there to be no difference between the round (RR) and wrinkled (rr) lines in pod and testa growth, but embryo growth differed. The wrinkled embryo contained more water during development but had a lower final dry weight than the round embryos.

Water potential (an indication of the osmotic regulation) was lower in the wrinkled embryos than in the round embryos, except very early in development when it was higher in the wrinkled embryos.

In vitro culture of embryos showed that optimum growth was obtained in liquid media containing 10% sucrose as a carbon source. Replacing sucrose with mannitol determined that it was the sucrose which was important, not its resulting water potential.

These gp and r gene effects and their relevance to future breeding programmes are discussed with other biochemical studies on similar genotypes.