DOCTOR OF PHILOSOPHY

Aspects of root growth in cotton seedlings

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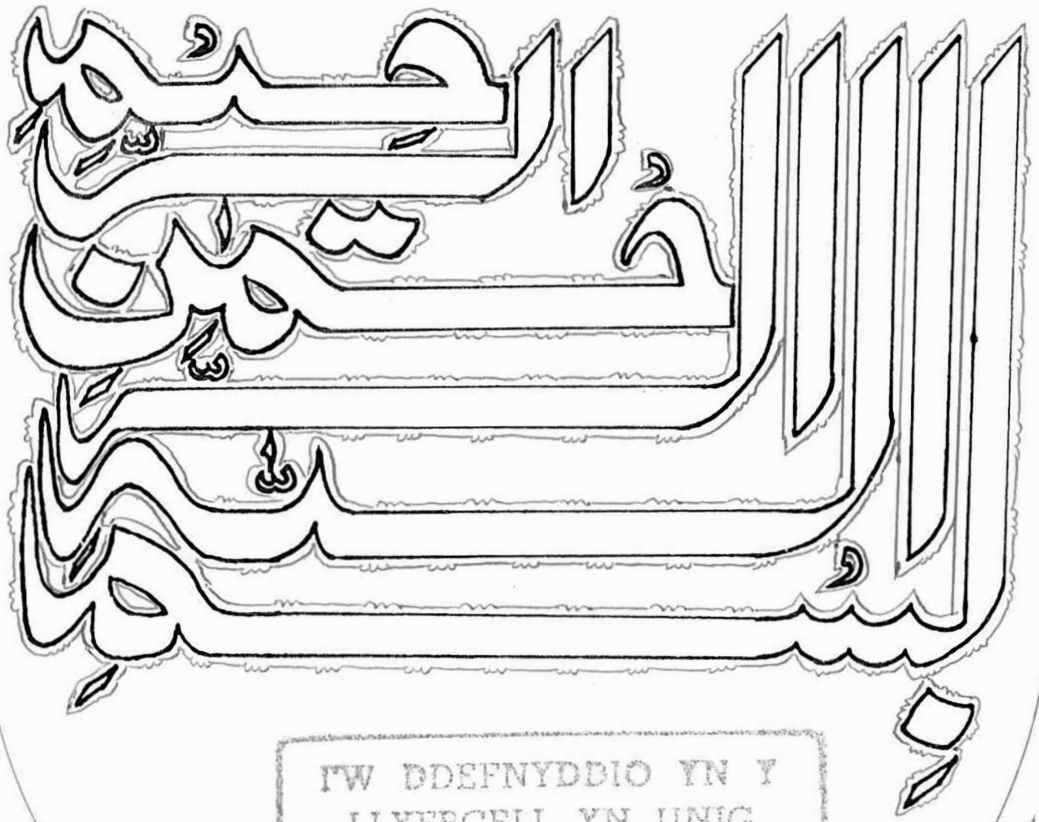
IN THE NAME OF

ALLAH

THE MOST GRACIOUS THE MOST MERCIFUL



THANKING

HIM

WITH FULL HEART AND DEVOTED TONGUE

بسم الله الرَّحمن الرَّحيم

« وَهُوَ الذِيْ أَنْزَلَ مِنَ السَّمَاءِ مَاءً فَأَخْرَجْنَا بِهِ نَبَاتَ كُلِّ شَىءٍ فأَخرجْنا مِنْهُ خَضِراً نُخْرِجُ مِنْهُ حَبًّا مُتَرَاكِباً وَمِنَ النَّخْلِ مِنْ طَلْعِهَا قِنْوَانٌ دانِيةٌ وَجَنَّاتٍ مِنْ أَعْنَابٍ والزَّيْتُونَ وَالرُّمَّانَ مُشْتَبِهاً وَغَيرَ مُتَشَابِهٍ أُنْظُرُوا إِلى ثَمرِهِ إِذَا أَثْمَرَ وَيَنْعِهِ إِنَّ فِي ذَلِكُم لآيتٍ لِقَومٍ يُؤمِنُون »

سورة الأنعام آية 99

" He It Is Who sendeth down water from the sky, and therewith We bring forth buds of every kind; We bring forth the green blade from which We bring forth the thick-clustered grain; and from the date-palm; from the pollen therof, spring pendant bunches; and (We bring forth) gardens of grapes, and the olive and the pomegranate, alike and unlike. Look upon the fruit therof, when they bear fruit, and upon its ripening. Lot herein verily are portants for people who believe. "

The Holy Quran, Chapter 7, verse 99

ASPECTS OF ROOT GROWTH IN COTTON SEEDLINGS

A thesis submitted to the University of Wales

by

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M.Sc. (Hons.) Agri.

In candidature for the degree of

Doctor of Philosophy

Dedicated to

my Parents,

to my Brother Ali Akbar,

to my Wife Bhain Bheern and

to my Sons Sadaruddin

Badaruddin and Azharuddin

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Abstract

Experiments were carried out to study differences in physiological and biochemical properties between slow-growing and fast-growing cotton seedling roots.

The root length, fresh weight and dry weight increased with germination time. The difference between the relative fresh weights of long and short roots was greater than the difference between the corresponding dry weights. In other words, long roots contained more water than the short roots, at least during early germination. Short roots were fatter than long roots and they had more root tip fresh weight. Root elongation occurs in the tip region, while a second area of elongation was observed in the hypocotyls after 48 hour germination. Lateral root initiation occurred away from the root tip over a fairly extended range up to the root-hypocotyl junction.

In the tip region, cell elongation took place mostly between 2.5 mm and 6.0 mm from the tip. Long roots had longer cells as compared with short roots, but the age of the root had no significant effect on cell length. During early germination, long roots had wider cells and greater cell volume as compared with short roots, but this effect disappeared by 84 hours. The number of cell files decreased with increasing root length at all germination times. Younger seedling roots contained more cell files as compared with older ones. These changing parameters of cell length, cell width and file number determined the size and the shape of the roots.

During early germination, (Me-^{3}H) thymidine incorporation was greater in long roots than in short roots, but it was almost the same in the roots of all lengths at later germination times. Incorporation values were higher in younger roots than in older roots. It was highest in the tip region of the root. Total DNA levels were greater in short roots than in long roots and more in younger than in older roots. It was concluded from these data that cell division does not play an important part in root growth in cotton seedlings.

Soluble peroxidase activity decreased as germination time increased. The insoluble (cell wall) peroxidase activity increased as germination time increased. On a per root basis, soluble peroxidase activity did not vary much with decreasing root length, but cell wall peroxidase activity increased. On a per gram fresh weight basis, both soluble and cell wall activities decreased progressively with decreasing root lengths. The distribution of both soluble and cell wall peroxidase activities in both long and short roots showed high activities in the first segment and a second area of high activity in segments further away from the tip.

Root elongation was slightly stimulated by 10^{-5} to 10^{-7} M GA_3. ABA also slightly stimulated root growth up to 24 hours, but by 48 hours the effect was reversed. High IAA concentrations (10^{-3} and 10^{-4} M) inhibited growth. Low IAA concentrations (10^{-6} to 10^{-10} M) promoted growth. The high concentration caused a large increase in the tip fresh weight.

With the high IAA concentrations, soluble peroxidase activity on a per root basis decreased up to 24 hours, but then increased again up to 48 hours. On a per gram fresh weight basis, the activity decreased with time all the way to 48 hours. Insoluble peroxidase activity, on a per root as well as on a per gram fresh weight basis, decreased with time all the way to 48 hours. At low concentrations, IAA had no effect on soluble peroxidase activity, but the insoluble (cell wall) peroxidase activity was increased. It was concluded from the peroxidase studies results that enzyme had no significat role in root elongation.

LIST OF ABBREVIATIONS

Me-^{3}H	methyl-radiolabelled hydrogen
DAPI	4',6'-diamidino-2-phenylindole
HCl	hydrochloric acid
NaCl	sodium chloride
EDTA	ethylene diamine tetra acetic acid
TCA	trichloro acetic acid
DNA	deoxy ribonucleic acid
cDNA	complementary (copy) DNA
H_2O_2	hydrogen peroxide
IAA	indole-3-acetic acid
GA_3	gibberellic acid
ABA	abscisic acid
Fig.	figure
Δ	change of
A	absorbance
min	minute
min^{-1}	per minute
h	hour
hr^{-1}	per hour
cm	centimetre
cm^3	cubic centimetre
mm	millimetre
μm	micrometre
nm	nanometre

ml	millilitre
μl	microlitre
M	molar
mM	millimolar
μM	micromolar
g	gram
mg	milligram
μg	microgram
ng	nano gram
Ci	curie
mCi	millicurie
μCi	microcurie
dpm	disintegration per minute
cpm	counts per minute
kPa	kilopascal
MPa	megapascal
dSm^{-1}	deci Siemens per metre
wt.	weight
$^{\circ}C$	degrees celsius
%	percent
$\pm$	plus or minus
sd	standard deviation
se	standard error
ANOVA	analysis of variance
S1	segment number
Q1	quantile number

p	probability
>	greater than
<	less than
rmp	revolution per minute
i.e.	that is
cf.	compare with
Ca.	about
ha	hectare

CONTENTS

CHAPTER ONE

Chapter One

General Introduction

This chapter gives a general description of the cotton plant, its production and importance in daily use. The chapter also explains the germination process and the various changes which takes place during germination. A general account of the physiology of root growth, cell division and cell elongation are also explained. The cell wall metabolising enzymes, especially peroxidase are then discussed.

1.1 General description of the cotton crop

Cotton is one of the most important crops grown commercially in about 60 developing countries between 47°N and 35°S (Bielorai *et al.*, 1983). These countries include Pakistan, India, China, Egypt, Mexico and Peru. The United States of America is also a major producer. According the United Nations Food and Agriculture Organisation's statistics, the world's planted area in cotton is about 33 x 10^6 ha, about 33% of the worldwide cultivated arable land (FAO, 1978). It has been reported that cotton has evolved in several different locations, although its exact origin is unknown. The earliest written records indicate that cotton was known in Mexico as early as 5000 B.C., Peru in 2500 B.C. and in the Indian sub-continent at least by 3000 B.C.

Cotton belongs to the genus *Gossypium* of the Family *Malvaceae*. The modern cultivated cottons fall into two different groups consisting of two diploid (2n=26) "Old World" species (*G. arboreum* L. and *G. herbaceum* L.) and two tetraploid or amphidiploid (2n=52) "New World" species (*G. barbadense* L. and *G. hirsutum* L.). In addition to the modern cultivated species, the genus also includes 46 wild and lint-

less species, that are widely scattered geographically and which can grow at high temperature (Fryxell *et al.*, 1992; Hutchinson, 1962; Berger, 1969; Arnon, 1972). These wild species may be of considerable interest to plant breeders. Some of them have already been used in crosses to improve the quality of cultivated cottons.

The diploid species (*G. herbaceum* and *G. arboreum*) are commonly referred to as asiatic cottons and they include the short staple type of cultivated cottons. They have a long history of cultivation in the old world and cotton fragments of about 5000 years old have been found in the Indus valley of Pakistan (Gulati and Turner, 1928). During the last 100 years, however, the new world tetraploid species have replaced the diploids in most of the world, except for small areas of the Indian sub-continent (Phillips, 1976). The "New World" species *G. hirsutum* has become the most popular species and it supplies over 85% of the world's raw cotton for factory use. The remaining 15% of production is covered by the remaining three cultivated species, *G. barbadense, G. arboreum* and *G. herbaceum*.

Cotton is a warm-season crop growing in subtropical and tropical countries and requiring a temperature range of 20-30°C from germination to harvest (Bhatti, 1975; Abdel Majid and Osman, 1977). It can be grown as an annual summer crop in more temperate zones (Christiansen, 1963; Buxtan and Sprenger, 1976; Munro, 1987). It is not grown in regions in which the mean annual temperature is below 16°C (Chapman and Carter, 1976). It is a sun-loving plant and produces best if the weather is relatively cloudless during the active growth period. As a day-neutral plant it responds mainly to the total received illumination or accumulated radiant energy. Therefore, the length of the daily illumination period has little effect on growth and fruiting (Tharp,

1960). The suitability of the climate for cotton cultivation also depends upon elevation and wind speed, while topography of land, nature of the soil, and availability of irrigation water are other important factors affecting the crop's adaptability.

Commercially grown cotton is a long-season annual crop requiring about 180-200 frost-free days. It grows to a height of 2-5 feet (60-120 cm) and develops a deep tap root and a widely branching secondary root system. It requires adequate moisture (Arnon, 1972) and continuous warm days with relatively warm nights for optimum growth and development (Gipson and Joham, 1968). The interaction between day and night temperatures appears to the most influential of the environmental parameters, since day/night variations have a significant impact on the growth pattern. Low night temperatures (11 $^{\circ}$C) slow the development of the plant so that the plants are less productive than the plants produced at high night temperatures (21 $^{\circ}$C to 27 $^{\circ}$C). Variation in the night temperatures can also influence boll formation and lint properties (Gipson and Ray, 1970), seed development and chemical composition of seed (Gipson, 1970). High day-time temperatures of 37 $^{\circ}$C can delay the flowering of upland cotton, because plants become tall and bushy, resulting in poor crop yield (Bhatt, 1977).

Cotton requires high light intensity during most of the growing season for efficient growth and good economic yields. Sunshine and rainfall are of great importance, particularly in the early growth period and at full bloom. Lack of sunshine, for example, may prevent boll ripening (Berger, 1969). According to Chapman and Carter (1976), plenty of sunshine is vital for cotton and areas with more than 50% cloudiness are not suitable for the crop.

Cultivated cotton requires moderate to fairly large amounts of water. The optimum rainfall for economic yield is about 60 inches (150 cm) distributed throughout the season. Irrigation requirements can vary from 24 to 72 inches (60-180 cm). The minimum is about 20 inches (50 cm) of rainfall per year. Heavy rains can injure the young seedlings, however, as well as the fully grown plant. Moderate rainfall is desirable during the period of vegetative growth, preferably during the night so as to allow for maximum sunshine during the day. A drier period is desirable during later growth to allow the bolls to ripen and to be picked. Excess moisture, whether from irrigation or rainfall, delays maturation and interrupt harvesting. Therefore, at harvesting time, relatively dry and cool conditions are preferable. Apart from drying the soil, moderate winds do little harm to cotton plants. Strong winds seriously affect delicate young growth, however, and at maturity they can blow away lint from the open bolls.

The crop can be produced on a variety of soils. Deep, friable soils with good humus content and favourable moisture-holding capacity are preferred. Generally, those that are high in organic matter produce higher cotton yield. Loamy soils (clay and sand mixtures) and alluvial soils are ideal for cotton. Very sandy soils can produce poor yields, however. Cotton can be grown over a wide range of soil pH, but the optimum lies between 5.0 and 8.0 (Arnon, 1972; Chapman and Carter, 1976). In Pakistan, the cotton crop is mainly grown in the Indus valley in Sindh and Punjab Provinces, which have a continental type of climate characterized by extreme variations of temperature, both seasonally and daily. The summer growing season starts from the month of April and ends in September. In summer the temperature often exceeds 47 $^{\circ}$C and the diurnal variations may be as much as 20 $^{\circ}$C. The soils of Indus valley are mainly alluvial. The

average annual rainfall is about 7 inches (180 mm), coming mainly during July and August. The type of soil and especially the availability of irrigation water are therefore the main factors which determine the choice of cotton as crop.

Basically, Pakistan is an agricultural society and cotton plays an important role in the country's economy. The country obtains about one-third of its agricultural export earnings and one-tenth of its total export earning from cotton. The low yield per acre has been a major problem however. In addition to several socio-economic constraints, some environmental factors also are responsible for this low productivity. High temperatures and limited water availability are especially critical in this respect. Lack of proper seed handling and storage facilities also lead to poor germination and crop establishment, which ultimately results in a reduced yield of lint.

Cotton production in Pakistan is dominated by the tertaploid "New World" species *G. hirsutum*. The cultivar of *Gossypium hirsutum* used in the present study was MNH-93 and its parental back-ground is C-158 x MS-39 x MEX12 derived originally from United States material imported into Pakistan.

1.2 Cotton products and their importance

The cotton plant produces a wide range of products for daily use. Cotton lint and cotton seed oil are the most useful products and they can be used in many ways. Cotton lint is a main source of raw material for the textile industry. It is stronger than the fibres of other crops (eg flex, hemp etc) and it has qualities of primary importance in many textile products. Several workers have reported that cotton seed production and processing also ranks second among the five major oilseed crops, which are soybean,

cotton, sunflower, peanut and rape (Arnon, 1972; Van Wallwijkn van Doorn, 1982).

The process of lint production by the cotton plant passes through several phases. Within the boll, the epidermis of the seed coat produces two kinds of hairs. One is long, thick and white or creamy in colour and it is called lint or fibre. The other is very short and it is attached more strongly to seed coat; it is called fuzz or linters. The cotton lint, after ginning is graded according to fibre length, strength and fineness. Continuous exposure in the field to weathering and the action of microorganisms can cause white cotton to loose its brightness and become darker. Both forms of fibre (the lint and the fuzz) arise as single cell outgrowths from the epidermis. The cells producing the lint begin to elongate at about the same time as the flower opens (ie before seed development begins), while the growth of the fuzz begins 5 or 6 days after flowering. The lint and fuzz hairs develop as thin tubular structures. At their active growing stage, these hairs (which are parts of the living cell) contain protoplasm and organelles including a nucleus (Berger, 1969). The primary lengthening process continues for about 13 to 25 days, and the lint eventually achieves a length of 25 to 35 mm depending upon the cultivar and the environment (Tharp, 1960; Quisenberry and Kohl, 1975). When the lint reaches its maximum length, the lint cell wall starts to thicken by the deposition of consecutive layers of cellulose in a spiral fashion on their inner surface. This secondary thickening of the cell wall continues until lint maturity at about 35 to 60 days post-anthesis. Mature cotton lint consists of 87 to 90% cellulose, 5 to 8% water, and 4 to 6% of other constituents. By this point it is dead and its cytoplasmic contents are mostly lost. Gipson (1982) reported that the rate and duration of boll and lint development are highly influenced by temperature. Decreased night temperatures, in particular, slow down the rate by reducing the amount of cellulose synthesised, resulting in under-developed lint

with characteristically poor physical and chemical properties (Gipson and Ray, 1970).

As stated above, cotton lint has qualities of primary importance in many textile products. Lint strength, length, smoothness and maturity are the main properties that determine the quality of cotton. The longer lint is finer and more expensive, where as short staple is relatively less valuable. Long staple is mainly used in making shirts and other fine quality goods. Short staple is used for making carpets, rugs, blankets, ropes and other rough materials.

The length of modern cotton lints are usually classified as follows:

Short staple : less than 25 mm.

Medium staple : 25 to 28 mm.

Long staple : 29 to 34 mm.

American-Egyptian cotton has an extra-long staple, measuring 35 mm and above (Berger, 1969).

After removing the lint, the cotton seed is further processes to yield about 16% crude oil, 9% linters, 20% hull and 45% meal (Chapman and Carter, 1976). The oil is the most valuable among these products, accounting for about 50% of the value of the crop. It is used mainly for human consumption. Cotton seed oil stands second after soybean oil in the world production and consumption of edible oils (Cherry and Leffler, 1984). The cottonseed cake or meal is rich in protein and is mainly used as livestock feed supplement. The linters are a source of cellulose used for making rayons, explosives, film, shatter proof glass, plastic etc. (Berger, 1969). The hull is less valuable and it is mainly used as roughage in livestock feed.

1.3 Structure of the cotton seed

The structure of the cotton seed is shown in Figure 1.1. It is simple in structure and about 10 x 6 mm in size. It consists of an embryo covered by a seed coat or testa. The embryo consists of two large but very thin cotyledons, an epicotyl (or plumule), hypocotyl and a radicle. Structurally, the cotyledons are analogous to leaves. They are attached at the upper end of the short, thick stem-like axis of the embryo called the hypocotyl. Most of the storage materials (mainly oil and protein) are present in these cotyledons. The plumule is located just above the point at which the cotyledons are attached to the hypocotyl. The primary root (radicle) of the plant develops from the lower end of the hypocotyl. The embryo is surrounded by a thin endosperm, nuclear fragments, and the hard seed coat consisting of five or more cell layers (Hearn, 1976; Duffus and Slaughter, 1980; Christiansen and Moore, 1959). The colour of the seed coat is dark brown or black.

Several workers have studied the ultrastructure of the cotyledons and reported that the cells have many clear-cut structural components including nuclei, spherosomes (fat storage bodies), protein bodies (protein storage bodies), globoids (phytin particles within the protein bodies), and cellular protoplasm in which the nucleus, spherosomes, and protein bodies are located. Gossypol glands containing toxic polyphenols and gossypol can be seen in the cell walls of the seed coat (Yatsu, 1965; Engleman, 1966; Dieckert and Dieckert, 1972, 1976). These cellular structures, especially those of the cotyledons are strongly reflected in the chemical composition of the seed.

1.4 Chemical composition of the cotton seed

According to their storage materials, the seeds of different species can be divided into

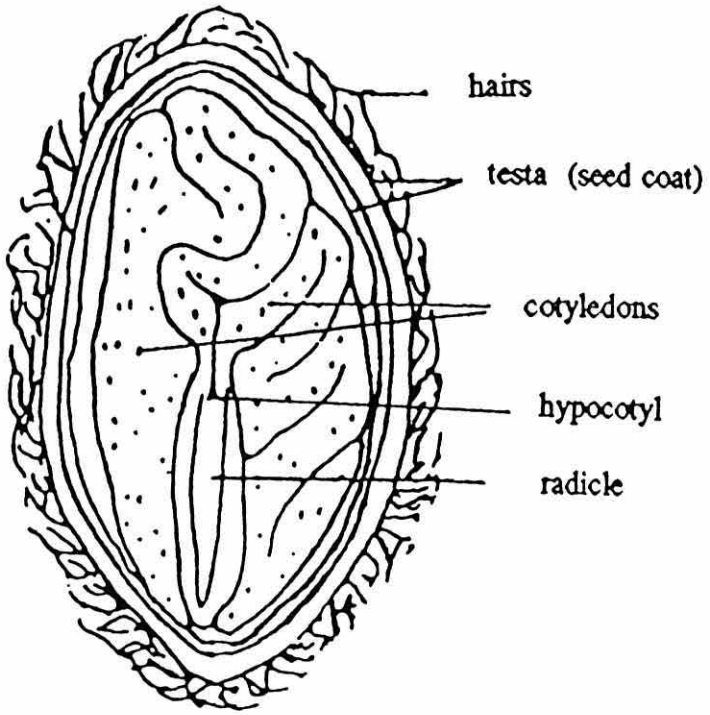


Fig. 1.1 Structure of the cotton seed.

two groups.

1. Those with lipids as their main storage material.
2. Those with carbohydrates as their main storage material.

Cotton belongs to first group. The chemical composition of the seed can vary among cultivars, location and according to environmental factors (Cherry, 1983; Copeland, 1976). In all cases, however, the seed is an excellent source of both lipids and proteins. Its oil content is about 20%. Brohult and Sandegren (1954) determined the total protein content of *G. herbaceum*, and reported that it was approximately 20-22% of the dry seed weight with 90% of the proteins being globulin and the remaining 10% glutelin. Cherry *et al.* (1981); Cherry (1983); Ergle and Guinn (1959) reported that phytin, a major phosphorus-containing compound, is also present in the cotyledons of the cotton seed and it is located within the protein bodies. This phytin is a valuable source of potasssium, calcium and magnesium. Small quantities of phytin are also present in the embryo axis of the seed (Albaum and Umbreit, 1943). The seeds also contain a small amount of other mineral material which tends to accumulate in the hull and other structural tissues. The typical structural and chemical composition of cotton seeds (Cherry *et al.*, 1981; Cherry, 1983) is given in Table 1.1.

1.5 The germination process

A seed remains in a state of rest or dormancy after formation and development on the parent plant. In this state, it is characteristically low in moisture and has a low metabolic activity. It remains in this condition until the right conditions are provided. Under suitable conditions of water availability, oxygen tension and temperature, the seed absorbs water, increases its metabolic activity and then germinates (Abdul Baki, 1969;

Table 1.1 Structural and chemical composition of cotton seeds *G. hirsutum*.

Component	Content (%)
Structural composition:	
Hull	41.6
Kernel	45.4
Lint	14.0
Chemical composition:	
Oil	19.0
Protein	22.3
Free fatty acids	0.2
Free gossypol	1.0
Total gossypol	1.1
Phosphorus	0.9

The hull, kernel, and lint values are percentages of the whole seed. The oil, free fatty acid, protein, free gossypol, total gossypol and phosphorus values are percentage of delinted seed.

Mayer and Mayber, 1975). The entire metabolic apparatus of the dry seed becomes operative in a sequential fashion. The term germination can be defined as those activities which begin with water uptake and which terminate with the emergence of the radicle or hypocotyl (Bewley and Black, 1978; Roberts, 1988; Feldman, 1984).

In the case of cotton, the radical or primary root emerges first from the seed and elongates downward (Figure 1.2). Cotton seed has an epigeal germination system in which the cotyledons emerge above the ground. The root is a tap root system, which may reach a length of 20-25 cm before the cotyledons begin to emerge above the ground. Lateral roots begin to develop after emergence and unfolding of the cotyledons (Arnon, 1972). In the latter process the hypocotyl extends upward pulling the attached cotyledons towards the soil surface. The seedling emerges from the soil 5-12 days after sowing (Horowitz, 1962; Bielorai *et al.*, 1983). After opening of the newly-emerged cotyledons, they quickly become green and begin photosynthesis. Plumule growth proceeds and later the exhausted cotyledons wither and fall to the ground in a few days time.

Water is essential for germination. It is needed for enzyme activation, thus permitting the breakdown, translocation and utilization of reserve food material. The water is absorbed through natural openings in the seed coat and diffuses into the seed tissues. Laffler and Williams (1983) have reported that during the first few hours, the water content of delinted seeds increased sigmoidally. After a rapid increase in the rate of uptake, further absorption occurs but at a reduced rate (Krieg and Bartee, 1975). When the water first enters the seed, it reaches the interior of the seed coat. There follows a two-fold increase in size of the seed coat, which becomes turgid and the chalazal pore

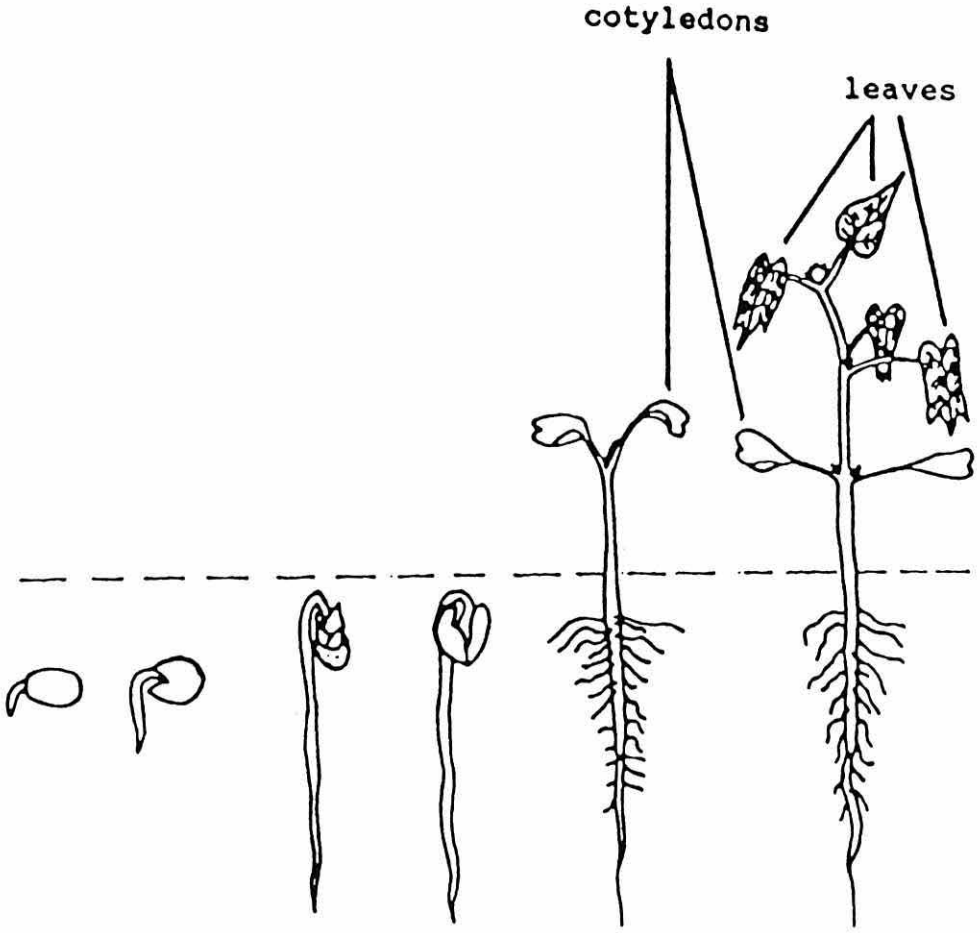


Fig. 1.2 Epigeal germination in cotton.

opens. The seed coat then becomes permeable to oxygen and carbon dioxide which are necessary for respiration. The further uptake of large amounts of water causes expansion and the rupture of seed coat. Cotton seed are normally fully hydrated in approximately 4 to 5 hours at 30°C at a soil moisture level of 55% (Benedict, 1984).

The hydration of the seed initiates metabolic activity by activating enzymes, polysome formation, transcription and translation, which are required for growth and cell division. As mentioned above, the hydration process activates various enzymes which are already available in the seed and which then support metabolism and ATP production (Christiansen and Moore, 1961). Proteinases have been observed in many seeds and their activity increases rapidly during germination (Ryan, 1973). The early phase of germinative metabolism in cotton is dominated by metabolization of the lipid reserves. Lipase enzymes are reported to be present in the cotton seed and are activated during germination (Yuldashev and Rakhimov, 1970). Lipase acts on glycerides to produce free fatty acids, although only low amounts of free fatty acids are reported during germination (Christiansen and Moore, 1961; St. Angelo and Altschul, 1964). The fatty acids are converted into carbohydrate via the glyoxylate pathway. Reserve carbohydrates are broken down by various enzymes (eg. starch by amylase). These carbohydrates serve as substrates for respiration and energy production. Proteinases (endopeptidases, carboxy-peptidases and aminopeptidases) are responsible for breaking the peptide bonds of the storage proteins and releasing small peptides and amino acids (Ryan, 1973). Phytin is hydrolysed by phytase which is also present in the quiescent seed. Rakhimov *et al.* (1986) reported that, under dry conditions, cotton seeds possess low phytase activity, but it increases gradually during germination, reaching a peak on the fifth day when its activity is nearly seven times higher than the original value. Newly activated

enzymes also catalyse the chemical reactions needed for the synthesis of new materials which support the growth of the root-shoot axis. Although the changes in the activities of these various enzymes have been studied in many seeds the same general trend is observed in most species (Mayer and Mayber, 1975). Many other enzymes required for germination (eg. enzymes of intermediary metabolism such as glycolysis, the tri-carboxylic acid cycle and oxidative phosphorylation) are already synthesized during embryogenesis (Trelease, 1984). As proteins break down during seed germination, there is a concomitant rise in amino acids in the cotyledons followed by protein synthesis in the growing parts of embryo (Copeland, 1976). The dry seeds also store pre-existing mRNA. The early protein synthesis following imbibition is fully dependent on these pre-existing messengers. Later events, however, associated with cell division and differentiation, require protein synthesis using newly synthesised mRNA. In cotton seeds, some preformed mRNAs are immature and require polyadenylation during early imbibition before being translated (Mayer and Mayber, 1975; Copeland, 1976). It has been reported that, in cotton seed cotyledons, glyoxysomes increased 7-fold in volume within 36 hours after imbibition (Kunce *et al.*, 1984) presumably reflecting membrane synthetic activities in the imbibed seeds.

During the first few days of germination, seedlings undergo a net loss in dry weight. The loss occurs due to the high respiration rate achieved and by the exudation and leakage of solutes through the seed coat. Ingle *et al.* (1964) found that in germinating maize seedlings, for example, dry weight decreased from 220 to 180 mg over a 5 day period. A similar loss can be expected in cotton seeds during germination.

Several important environmental factors which affect the germination process and

seedling growth are as follows:

Temperature: Each crop has its own temperature requirements for optimum germination. Ong and Baker (1985) reported that changes in temperature can affect germination and seedling growth. Seed germination is a complex process involving many individual reactions, each affected by temperature (Reddy *et al.*, 1992). The overall effect on germination can be expressed in terms of the minimum, optimum and maximum temperatures. Christiansen and Moore (1961) reported that the mobilization of cotton seed reserves are adversely affected by low (10-15°C) temperatures. Arndt (1945) reported that each stage of cotton seed germination has its own optimum temperature, however, so that the temperature response may change through the germination period. The overall optimum temperature range for cotton seed germination is between 20 and 30°C (Krieg and Carrol, 1978; Abdel Majid and Osman, 1977; Sethar, 1993).

Moisture: All seeds require sufficient moisture for imbibition and germination. Different seeds have different water requirements for their germination however. For example, in seeds such as beetroot (*Beta vulgaris*) and spinach, an excess of water can reduce the permeability of the seed coat to oxygen and inhibit germination (Gulliver and Heydecker, 1973). Low or high moisture levels thus increase the susceptibility of seeds to germination failure by causing either too slow or too rapid imbibition. The amount of water absorbed by the cotton seed is also influenced by the species of cotton. Low soil water potentials reduce the germination rate of cotton (Jordan, 1982) and once germinated, hypocotyl elongation is reduced more than radical extension (Wanjura and Buxton, 1972). At optimum temperatures, cotton seedlings emerge in about 5 days at -

0.03 MPa, 7 days at -0.3 MPa and no emergence was evident after 13 days at -1.0 MPa water potential (Wanjura and Buxton, 1972).

Gases: The availability of oxygen is necessary for oxidative metabolic processes. Germination in most species may therefore be retarded if the oxygen concentration is reduced substantially below that of air (21%). The influence of carbon dioxide on seed germination is usually the opposite to that of oxygen and most species fail to germinate if the carbon dioxide partial pressure is increased beyond the atmospheric level (0.03%). Some cotton seedlings may tolerate a slightly increased concentration of the gas, however (Patterson *et al.*, 1988). A decrease in concentration of carbon dioxide usually does not effect germination, but it does effect ethylene synthesis. Otani and Ae (1993) reported that ethylene and carbon dioxide concentrations of soils varied considerably depending on the presence or absence of rhizosphere. Ethylene was much higher in non-cropped areas (ie. with out rhizosphere) than in the rhizosphere region of a field which maize or soybean were grown. On the other hand, carbon dioxide concentrations were higher in non-rhizosphere soils. Colinas *et al.*, (1994) reported that seedling root growth was stimulated by the soil volatile organic compounds, especially, ethylene. Nitrogen concentration in the soil environment has little or no influence.

Light: While moisture, oxygen and favourable temperatures are essential for germination of all seeds, certain species seeds also require specific light conditions (Munro, 1987). Cotton is not one of these species.

Osmotic pressure: High osmotic pressure in the germination medium makes imbibition more difficult and retards germination (Rogers *et al.*, 1957). The ability of seeds to

germinate under high osmotic pressure depends upon species as well as on variety, and all seeds are affected. The germination of cotton is only moderately salt tolerant. Its growth is reduced by salinity greater than 6 dSm^{-1} (Hamdy, 1992).

Soil pH: Seed germination can proceed over a wide range of hydrogen ion concentrations. The germination of most cultivated species occurs readily between pH values of 4.0 and 7.6 (Justice and Reece, 1954). Cotton, however, can be germinated and grown successfully at between pH 5.0 and 8.0, because it is not unduly sensitive to soil pH (Arnon, 1972).

Presoaking: Presoaking of seeds in water is a way to speed up germination in several species (Heydecker and Coolbear, 1977), especially seeds like cotton with hard coats. Imbibition and metabolic processes begin during the presoaking, resulting in the early availability of energy and metabolic precursors to be utilized for biosynthesis upon germination.

Seed damage: Damage during harvesting, processing and handling is a major problem in many seed industries. Injury symptoms such as damage to the seed coat, internal physical damage (visible only after germination), susceptibility to microorganisms, and hidden injuries of a physiological nature all reduce germination vigour, subsequent plant growth rate, and ultimately reduce the yield. Crop establishment is thus significantly influenced by the quality of seeds that are planted. Good seed quality is therefore one of the first pre-requisites of cotton production (Krieg and Bartee, 1975; Leffler, 1981; Christiansen and Rowland, 1981).

Germination stimulants: Some seeds which look healthy may perform poorly and may be chemically treated in various ways to improve their germination. The seed treatments are generally designed to overcome adverse affects of biotic and abiotic environmental factors. For this purpose, protectants, growth regulators and nutrients are often applied by incorporating them into coatings or pelleting materials. In addition, physiological treatments such as moist chilling, wetting (Heydecker and Coolbear, 1977), drying or hardening and pregerminating or chilling (Bradford, 1986), and osmo-conditioning or priming (Heydecker *et al.*, 1973) are used to improve germination performance. It has been reported that cotton seed germination can be accelerated by the pretreatment of seeds with gibberellins, nicotinic acid, hydrogen peroxide, hetero-auxin, 2,4,5-trichlorophenyl acetic acid or succinic acid (Tsinikov, 1971; Babaev, 1972). Seed treatment with these chemical substances can also increase the germination rate and the eventual yield and quality of cotton lint. To avoid reduced germination rates due to fuzz on the cotton seed, chemical delinting is desirable to improve the absorption of water by the seed.

1.6 Growth of the cotton plant

The cotton plant has a very prominent erect main stem which is monopodial (vegetative) in growth and which carries both leaves and branches. The first true leaf reaches the size of the cotyledons (about 14 cm^2) in about 10-12 days after emergence. The plant has spiral phyllotaxy (arrangement of leaves on the main axis and branches) with each leaf oriented at about 135° from the last leaf (Mauney, 1984). The stem can have either counter-clockwise (dextrose) or a clockwise (sinistrose) phyllotaxy, with half the plants showing clockwise and half showing anti-clockwise phyllotaxy. The leaves vary in size, shape, texture and hairiness and have numerous stomata on both sides. Leaf size and

shape can even vary within an individual plant. The variability is much greater between cultivars and between species, however (Berger, 1969).

Branch meristems develop in the axil of each leaf. The type of branch can be either monopodial (vegetative) or sympodial (fruiting). In the early growth of the plant, the two types of branch development are indistinguishable. Environmental conditions (eg plant population), and to some extent the variety, decide the number of vegetative and flowering branches formed. For example, abortion of the terminal bud or lodging of the plant increases the number of vegetative branches. Only a few vegetative branches develop when the plants are grown under crowded conditions, while several branches arise when plants are widely spaced and grown under conditions of high nitrogen and soil moisture (Tharp, 1960).

The lower branches on the main stem are always vegetative, while the upper ones tend to be fruiting. The first fruiting branch is normally produced at the 6th, 7th or 8th node on the main stem. Growth of fruiting branches is more rapid than that of the vegetative branches and the first fruiting branches are less vigorous than subsequent ones. Each fruiting branch of average length produces 6 to 8 flower buds.

Flowering. Flower induction and the development of the flower and boll are the most crucial and decisive stages in the plant's life, which determines the final yield of lint and seed (Berger, 1969). As far as possible, optimum conditions for these processes must be provided at this time.

The main events taking place from budding to the maturity of the boll are summarised

as follows. The flower bud appears first as a small, green, pyramidal structure known as a square. This consists of a whorl of three triangular shaped green bracts which protect and completely enclose the tender growing flower parts. Inside the bracts, a cup-shaped calyx encloses the basal end of five conspicuous petals which collectively form the corolla. The colour of the corolla varies between species and varieties and it varies from white or ivory to yellow or red. Inside the corolla is the stamenal column, containing numerous stamens, each with a two-lobed anther. In the centre of the flower, there is a superior ovary composed of 3 to 5 united carpels, each containing several ovules (Tharp, 1960; Berger, 1969). The ovary is surrounded by a style ending in a lobed stigma which protrudes from the tip of the stamenal column.

The first flower bud square can usually be recognised at about 35 to 45 days after emergence of the seedling. About 25 days are required for it to develop into an open flower. The flowers open at dawn and wither before the evening of the same day. The interval between flowers on the same fruiting branch is about 6 days. Shortly after the flower opens, the stigma becomes receptive. The anthers split open and discharge free pollen grains, which adhere to the sticky surface of the stigma and pollination takes place. Cotton is mainly self-pollinating and only about 10-15% of cross pollination takes place through insects or wind (Arnon, 1972). Growth of the pollen tubes takes 12 to 30 h after which fertilisation is accomplished (Stewart, 1980). The cell division of the zygote (fertilised ovary) takes place 4 to 5 days after anthesis or pollination, and then enlargement of the young capsule (boll) begins. In upland *G. hirsutum* varieties, the ovary or young boll (fruit) reaches its full size in about 3 weeks and takes a further 3 to 7 weeks to mature. The total time from flowering to the opening of the boll thus ranges between 6 to 10 weeks, depending to some extent upon the variety but mainly

upon temperature, sunlight, rainfall and soil fertility (Berger, 1969).

Environmental conditions during germination and root formation in crop plants have important effects on the subsequent yield. This period from sowing to seedling establishment is a crucial phase in the plant's life cycle. In cotton, uniformity and percentage seedling emergence have a major impact on final yield, including the quality of the cotton lint.

1.7 Seed testing

Laboratory seed testing is performed in order to obtain a measure of seed quality for cultivation purposes. In field conditions, however, the germination percentage of a seed lot is often considerably lower than the germination percentage of the same seed lot in the laboratory. This is largely due to pre-emergence mortality caused by soil-borne organisms and to unfavourable environmental conditions in the field. Therefore, testing a seed lot for its potential germination capability is not necessarily a good guide to its performance in the field. Consequently, in evaluating seed quality, two terms are of great importance - viability and vigour. Viability can be defined as the ability of a seed lot to germinate under ideal conditions; that is, in the laboratory. Vigour can be defined as the ability of the seed lot to germinate under stress. The stress may be abiotic and/or biotic in nature.

Seeds can retain their ability to germinate, provided they are in a state of desiccation. The period of viability (longevity) of a seed is variable and depends on the storage conditions and on the type of seed. In general, viability is retained best under conditions in which the metabolic activity of seeds is greatly reduced, ie. low temperature, low

oxygen tension and low humidity (Mayer and Mayber, 1975).

Seed viability and vigour are not only determined by storage conditions. A variety of environmental factors to which the parent plant is exposed during seed formation and ripening can also affect the subsequent viability or vigour of the seeds (Heydecker, 1972). Such factors, include water supply, temperature, humidity, mineral nutrition and light. Genetic control of seed viability is also of primary importance.

Numerous tests exist for determining the viability and vigour of seed lots in the laboratory, by which reasonably reliable prediction of their field performance can be made. A seed lot is a term used in seed testing circles and in the seed trade. It is a uniform consignment of seeds from which representative tests can be made on a random sample (Roberts, 1972).

Some of the testing procedures currently being used are as follows:

Germination test: This simple test is widely used to determine seed viability. It has become accepted that the percentage seed germination under optimum conditions in the laboratory and seed viability can be considered as one and same. The test has, however, some limitations. In particular, it is merely an estimation of viability and it does not provide sufficient information about germination vigour. Also, it sometimes takes several days to get the final results.

The Association of Seed Testing Analysts (ASTA) has recommended a set of standardised conditions for germinating seeds. Their official rules specify the optimum

germination conditions for each crop species. In the case of cotton, the official germination test is the same as the germination procedure used in the present study (see Sections 2.5 and 2.6). It takes 3 to 4 days to produce a result.

Biochemical tests: The best known of these methods is the tetrazolium test. It is widely recognised as a fairly accurate means of estimating seed viability in several species. The method was developed in Germany by Lakon (1945), who had been trying to distinguish between individual live and dead seeds. The test utilises the activity of dehydrogenase enzymes to estimate the respiration rate and thereby seed viability. A colourless tetrazolium salt is reduced to a red formazan product. Seed viability can be expressed according to the topographical staining pattern of the embryo and the intensity of coloration. The staining pattern reveals the live and dead areas of the embryo, which indicate if seeds have the capacity to produce normal seedlings. A result in this test can be obtained in a few hours. It is also a valuable research technique, since it distinguishes between viable and dead tissues within a single embryo on the basis of their relative respiration rates in the hydrated state.

A Swedish seed analyst (Gadd, 1950) developed a number of biochemical tests which can be summarised as follows. One method is based on the differential coloration of live versus dead tissues when they are exposed to certain dyes, for example indigo carmine. Dead tissues absorb the dye and take on a blue colour, but the dye is incapable of penetrating live cells which remain unstained. Other methods are based on enzyme activities in extracts from imbibed seeds. Hydrolytic enzymes capable of splitting high molecular storage compounds (protein, starch and fats) into water-soluble products have been used for this purpose. Another group of enzymes that has been used in this way

are the oxidases and hydrogenases. Since these enzymes are directly involved in respiration processes, their activities are closely related to seed viability.

Fat degradative reactions may occur in stored seeds as deterioration progresses, especially under conditions of high moisture levels, high temperatures and microorganism infestation. The process results in the formation of free fatty acids, suggesting that the level of free fatty acids might be usable as an index of viability. Unfortunately, this test has never attained the status of a recognised viability test.

Conductivity test: Conductivity tests are based on the premise that, as seed deterioration progresses, the cell membranes become progressively disorganised and more permeable, allowing the cell contents (especially electrolytes) to escape into the surrounding water. This increases the electrical conductivity of the water. The test is a simple method of determining viability by merely subjecting the seed elutes to an electrical current. The most reliable electrical conductivity method has been proposed by Fick and Hibbard (1925) who found that, after soaking a seed sample in water for a few hours under controlled temperature conditions, the conductivity of the solution reflected the viability of the seed sample.

Excised embryo test: This test provides a unique way of assessing the potential viability of dormant seeds. Flemoin (1948) observed that, when the embryos of many dormant seeds are carefully removed from the seed and placed on a moist paper, they germinated readily. One disadvantage of this test is the high level of skill and time needed to prepare the embryos for the test.

Vigour tests: These tests are performed to determine the vigour or strength of germination. Typically, a seed lot is put to germinate in the laboratory under a single stress, for example low temperature, high osmotic pressure provided by a solution of polyethylene glycol, or physical impedance provided by a seed bed containing brick gravel (Heydecker, 1972). After germination for a specific period of time, the percentage germination is determined. Shoot or root length, cotyledons weight or seedling weight are other parameters that have been used. Some of the physiological and biochemical tests mentioned above (eg leachate test, tetrazolium test) have also been modified to provide an estimate of germination vigour. The greatest value of a vigour test lies with the possibility of developing it to the point where it can give an accurate prediction of seedling emergence rates in the field.

1.8 The general physiology of root growth

Root growth is a consequence of the functioning of an apical meristem and the initiation of new meristems in the pericycle leading to lateral root emergence and growth. At the centre of the apical meristem is a quiescent region, a group of meristematic cells from which all other cells in the root are derived (Figure 1.3). Their rate of cell division is much slower than that of the cells in the meristem around them (Clowes, 1956b). Cell division towards the apex of the root gives rise to the root cap. The root cap cells are large parenchyma cells which are pushed through the soil as the root grows. They are therefore constantly being worn away and replaced. They also act as a gravity sensor in the process of geotropy (Barlow and Rathfelder, 1985). The root cap secretes mucigel over its outer surface which lubricates the root as it slides through the soil. This mucigel harbours microorganisms and probably influences the establishment of the *mycorrhiza* and of root nodules in leguminous plants (Barlow, 1975; Foster, 1982).

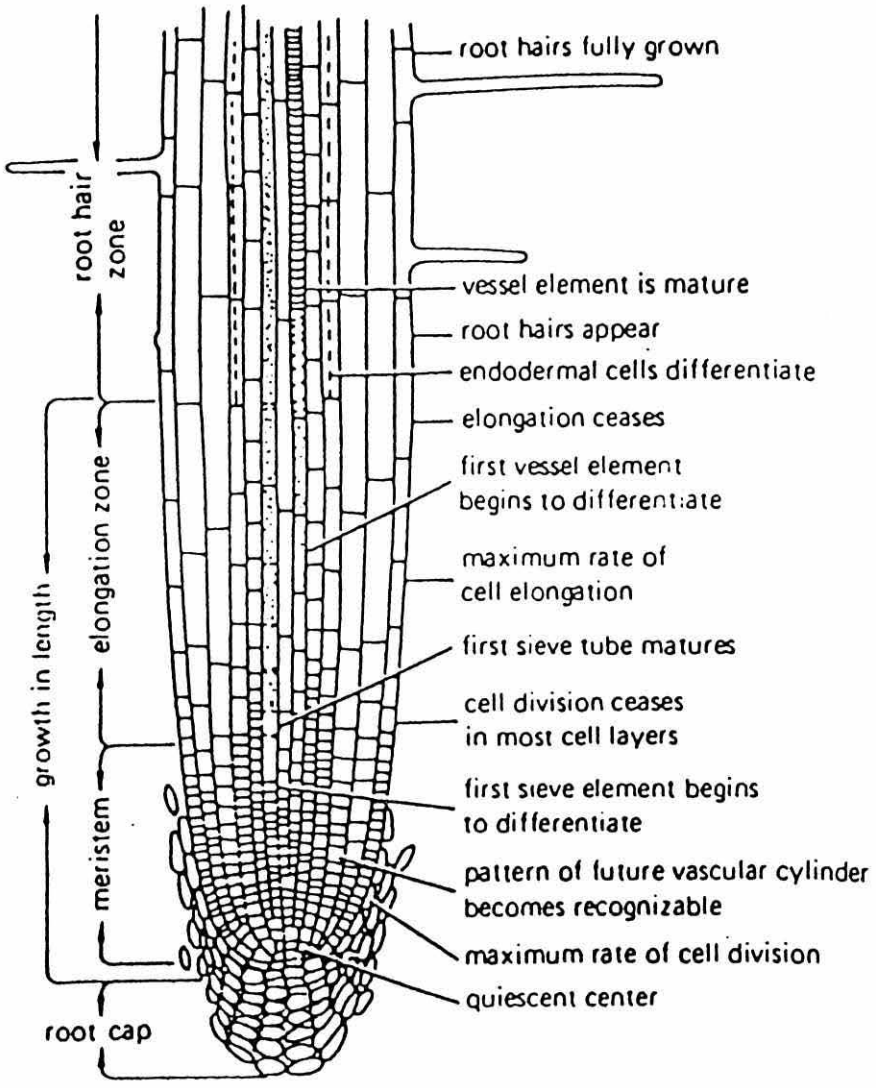


Fig. 1.3 Simplified diagram of the growing zone of a root in longitudinal section. (from Salisbury and Ross, 1992)

Behind the quiescent region, rows of cells can be seen forming within the meristematic region. In cotton, cell division in this region extends 1 to 2 mm back from the root tip and overlaps slightly with a zone of cell elongation. This zone of elongating cells extends to a point about 6 mm behind the root tip. Their increasing length forces the root tip down through the soil.

Further back in the zone of elongation, xylem vessels start to differentiate from just inside the cortical tissue inwards (see Figure 1.3). The first vessels to be formed are protoxylem vessels. They become metaxylem which develops and matures in a zone of differentiation after elongation has stopped. The xylem often expands to fill the centre of the root, in which case no pith develops. Mature xylem (metaxylem) vessels can not stretch or grow because they are dead, rigid and fully lignified tubes.

Phloem resembles xylem in possessing tubular structure (Figure 1.3). It is located between the pericycle and the xylem in the vascular cylinder (stele). The first phloem formed is called protophloem. Like protoxylem, it is produced in the elongation zone of the growing roots. As the tissue around it grows and elongates, it becomes stretched and much of it eventually collapses and becomes non-functional. Meanwhile more phloem continues to be produced. The phloem that matures after elongation has stopped is known as metaphloem. Metaphloem is made up of sieve elements, companion cells, parenchyma, fibres and sclereids. The sieve elements are arranged end to end to form sieve tubes in which each element is separated by a sieve plate.

After cell elongation has stopped, further processes are completed. These include the development of root hairs from the epidermis and the formation of lateral roots. The

former are the major site of water and nutrient uptake from the soil. The latter originate from the pericycle and they grow out through the cortex and epidermis (Salisbury and Ross, 1992). Lateral root formation is probably genetically controlled, although it is possible to recognize marked differences in the extent of lateral root formation between species and between strains within species.

1.9 Shoot-root interactions and sink-source relations

In the growing plant, the roots and the shoots are dependent upon to each other. Shoots trap solar radiation and, through photosynthesis, they produce the metabolites on which all growth depends. Roots anchor the plant in the soil and absorb water and mineral nutrients from the soil (Scott and Russell, 1977). Interaction between the two parts can be seen at many levels. For example, the shoot might increase the root's absorption of mineral salts by rapidly using them in growth processes. The shoot supplies carbohydrates to the root, where they are needed to produce the ATP that drives mineral salt absorption (Gastal and Saugier, 1989). The shoots also supply the roots with hormone(s) that affect growth and absorption processes. Similarly, roots synthesise hormones which pass to the shoots of the plant. Thus, the respiration rate of roots over long time periods are highly correlated with the rate of photosynthesis. Root respiration has been correlated with the rate of sugar translocation to the roots. Also, the rates of absorption of nitrate and ammonium ions are correlated with photosynthetic rates (Ingestad and Agren, 1988; Cooper and Clarkson, 1989).

Water and mineral nutrients are taken up from the soil by the roots and transported to the shoots. Both materials (water and minerals) move from higher to lower water potential along a gradient. The initial uptake of water and minerals by roots is a

combination of passive uptake (diffusion) and active uptake. Active uptake is selective and dependent upon respiration and energy metabolism, whereas passive uptake tends to be non-selective and it is not dependent on respiration.

Most water and minerals enter the plant via the root hairs. They travel across the root cortex to the xylem by diffusive or active processes. Because of the highly permeable nature of plant cell walls, each cell of the root cortex is bathed in a solution similar in composition to that of the soil solution as a result of passive movement. Thus, there is a large cellular surface area for mineral uptake by the roots. This movement of water and minerals from root hairs to the xylem occurs by two pathways (symplastic and apoplastic). The symplastic pathway involves transport through the cytosol of the cells on the way to the xylem. The apoplastic pathway involves movement through the cell wall network as far as the Casparian strip, then movement through the symplasm up to the xylem. Once in the xylem, they are redistributed throughout the plant in the transpiration stream, in which they move by mass flow. Water from the plant is lost by evaporation from the surface of the leaf mesophyll cells. It finally diffuses out through the stomata by transpiration (Salisbury and Ross, 1992). It is estimated that less than 1% of the water absorbed by the roots is retained by the plant, the other 99% being lost by transpiration.

Plant growth can be considered in terms of the relationship between two sources and a sink, whereby photosynthesizing leaves and absorbing roots provide the sources and growing tissues provide the sink. The coordination between root and shoot growth in cotton, as in other species, must be compatible with the maintenance of such constant source-sink relationships during the period of most rapid vegetative growth (Pearsall,

1927).

The influence of the source-sink relationship in determining growth can vary depending on the extent to which organic substances are translocated from the leaves to other tissue (Neales and Incoll, 1968). In the leaf source region, loading occurs into the sieve element by active transport via the companion cells and via the numerous plasmodesmata that link the companion cells with the sieve elements. In the sink region, unloading occurs from the sieve tubes by pressure flow (Ho, 1988). Generally, the nutrients are unloaded into the apoplast (Lemoine *et al.*, 1988). Oparka (1986), however, has reported phloem unloading from sieve tube directly into cortical cells of the potato tuber, that is by a symplastic process.

A decrease in the supply of mineral nutrients result in a decreased shoot-root ratio (Wilson, 1989). Any theory of how the shoot-root ratio is regulated must, therefore, be able to explain the effects of inorganic nutrient supply. A first attempt at an explanation goes as follows. When nitrogen is scarce, for example, sugars will accumulate in the root, but they will not stimulate root growth as nitrogen limits it; the usual stimulatory effects on respiratory enzymes will not occur (Farrar and Williams, 1990). In the leaf, sugars will then accumulate and repress the genes that regulate photosynthesis and stimulate those of carbohydrate storage. If the shoot is repressed more than the root, the shoot-root ratio will decrease; this may also depend upon effects on gene expression affecting nitrogen status itself (Redinbaugh and Campbell, 1991) or phosphorus status (Fife *et al.*, 1990).

1.10 Cell division

Cell division is cyclical; a cell grows, divides into two, and then the two daughter cells in their turn grow and divide. The activities of cell division in roots takes place in the meristematic region. The division of the meristematic cells can occur longitudinally or laterally (Gunning *et al.*, 1978). Thus, in the plant meristem, cells progress through a series of events termed the cell cycle. The stages within the cell cycle were first defined in a model proposed in 1953 by Howard and Pelc. They divided the cycle into four stages: G_1, S, G_2 and M (Figure 1.4). G_1, S and G_2 together make up the interphase, while M is the mitotic (division) phase of the cycle. Specific biochemical reactions occur during each of these stages, and specific RNAs and proteins including enzymes, are needed to progress from one stage of the cycle to the next (Rost, 1977).

After the M phase, which consists of nuclear division (mitosis) and cytoplasmic division (cytokinesis), the daughter cells begin the interphase of new a cycle. Interphase starts with the G_1 phase, in which the biosynthetic activities of the cell, which proceed very slowly during mitosis, resume at a high rate. G_1 is followed by the S phase, which begins when DNA synthesis starts. It ends when the DNA content of the nucleus has doubled and the chromosomes have replicated (each chromosome now consists of two identical "sister chromatids"). The cell then enters the G_2 phase, which is resting phase and which continues until mitosis starts. Completion of the G_2 phase initiates the M phase (mitosis), in which the chromosomes that were replicated during S phase are separated into two groups. During the first phase of mitosis the nuclear envelope breaks apart, and the characteristic shapes of the chromosomes can be observed. Then the chromosomes move to the centre of the cell and they become aligned along a region termed the metaphase plate. This is metaphase. During the next phase,

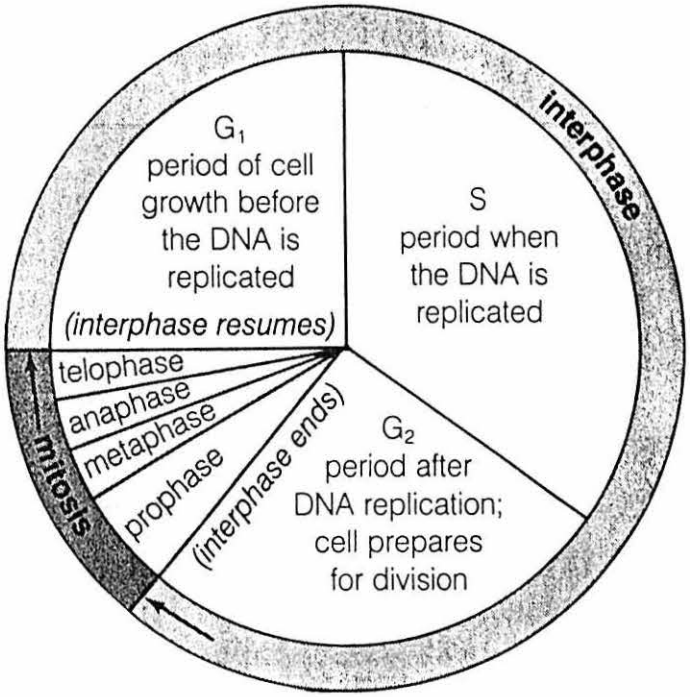


Fig. 1.4 A generalized diagram of the cell cycle. (from Salisbury and Ross, 1992)

anaphase, the duplicate chromosomes are separated into individual but complete sets, while moving from the metaphase plate region to opposite ends (poles) of the cell. Then a new cell wall is synthesized between the daughter sets of chromosomes, and a nuclear envelope reforms around each set of chromosomes. This phase is termed telophase and represents the terminal phase of mitosis. The individual daughter cells are then considered to be in interphase were they again can proceed through G_1, S, and G_2.

Francis (1992) has reported that a group of protein kinases is involved in regulating the transition from both post-synthetic interphase (G2) to mitosis and from pre-synthetic (G1) to DNA synthetic (S) phase. The transition from G_1 to S phase is linked to rapid changes in gene expression. Furthermore, the G_1 phase becomes protracted when plants are stressed by temperature and toxic metals.

An important component of the mitotic mechanism is the spindle apparatus. It is the structural component of the mitotic cell that is responsible for physically moving the chromosomes during the mitosis. The spindle apparatus is not a single structure but consists of hundreds of proteinaceous fibers called microtubules. During prophase microtubules form at the kinetochores of the chromosomes and also as a continuous network of fibers between the poles of the mitotic cell. The microtubules move the chromosomes to the central planes of the cell during metaphase and then to the opposite ends of the cell during anaphase.

Microtubules also are involved in cell wall formation during telophase. In this instance, the microtubules are thought to be involved in the movement of vesicles to the cell plate region where vesicle fusion results in the formation of the new wall. During interphase, microtubules are found preferentially aligned near the plasma membrane (Pickett-Heaps, 1967). They do not move from one location (near the plasma membrane at interphase) to another location (attached to the kinetochores at prophase) during the cell cycle. Instead, they are depolymerized at one location into a pool of protein subunit dimers called tubulin. At the new location, they are reformed by polymerization from this tubulin pool.

The naturally-occurring auxin, indole acetic acid, has been shown to be necessary for the initiation and maintenance of cell division in many plant systems (Das *et al.*, 1956; Torrey, 1961; Partanen, 1963); Furthermore, there is evidence (Braun, 1962; Klein and Vogel, 1956) that autonomy with respect to cell division is a consequence of the ability of the cells to synthesize their own growth factors, including auxin. It is also generally accepted that the root apex synthesizes the auxin necessary for root growth (Aberg, 1957) and hence probably for cell division in the apical meristem. Many processes that are involved in root growth and are dependent on auxin have also been shown to be affected by the alkaloid colchicine. These include the polarity of cell elongation (Levan, 1938; Huskins and Steinitz, 1948), the pattern of xylem formation (Davidson, 1963) and the over-all rate of root growth (Davidson *et al.*, 1965). These observations have led to the suggestion (Davidson and MacLeod, 1966; Davidson *et al.*, 1965) that colchicine exercises some of its effects by changing auxin levels in roots. It has also been suggested (Davidson and MacLeod, 1966) that the high mitotic index observed in root meristems following colchicine treatment is not only the result of metaphase arrest, but also reflects a stimulation of mitotic activity. That such an effect might reflect a colchicine-induced change in some auxin-controlled step in the mitotic cycle has been suggested (Davidson and MacLeod, 1966).

In addition to colchicine, many herbicides that inhibit plant growth are also reported to interfere with the mitotic process.

1.11 Cell enlargement and cell wall metabolism

New cells are generally small in comparison to their final size. To accommodate subsequent cell growth, the walls of such cells, called the primary cell wall, are thin and

only semi-rigid. They may later be thickened to become secondary walls. For these fundamental reasons, the plant cell wall is receiving increasing research attention. There is indeed a growing interest in the biological roles of some of its normal components (carbohydrates and proteins) and in the physiological functions of some specific polymers including lignin, cutin and suberin. In addition, the cell walls are important in relation to different utilization of plant materials. The possibility of manipulating cell wall composition for a better adaptation of plant products is now realistic through plant genetic engineering (Boudet *et al.*, 1995).

Plant cell walls consist of cellulose microfibrils, hemicelluloses, pectic compounds, lignin, suberin, proteins and water (Cassab and Varner, 1988; Carpita and Gibeaut, 1993). In dicotyledonous plants, such as cotton, the primary cell wall is approximately 30% cellulose, 30% hemicellulose, 35% pectin, and 5% protein (Fry, 1988). Additionally, cell walls contain several structural proteins which strengthen the wall (Cassab and Varner, 1988). So far, the best characterized structural protein is extensin (Cooper *et al.*, 1987; Tierney and Varner, 1987; Wilson and Fry, 1986; Biggs and Fry, 1990; Miller and Fry, 1992). Extensin is one member of a class of hydroxyproline-rich glycoproteins present in a wide variety of plants and algae (Catt *et al.*, 1976; Lamport, 1965). It has been proposed that the extensins are the major protein component of the primary cell wall and that they play a vital role in cell wall architecture (Lamport, 1980). The polymers of growing plant cell walls contain important phenolic groups, such as the tyrosine residues of extensin (Fry, 1982), and ferulic acid and p-coumaric acid esterified to galactopyranose and arabinopyranose residues of the hemicellulose polysaccharides (Fry, 1983).

Current models of the wall show three interwoven polymeric networks; a network of cellulose microfibrils linked together by a matrix of hemicelluloses, a gelled network of pectins ionically linked by calcium bridges, and a network of structural proteins (extensin) covalently cross-linked to one another and perhaps to other elements in the wall matrix (Figure 1.5) (Talbott and Ray, 1992; Carpita and Gibeaut, 1993; Albersheim *et al.*, 1973; Northcote, 1972). In both monocotyledonous and dicotyledonous plants, hydrogen bonding is the major force binding the hemicellulosic constituents (xyloglucans in dicots, arabinoxylans in monocots) to the cellulose microfibrils. This, together with covalent attachment of the hemicellulose to the pectin and extensin fractions, forms a tightly cross-linked network. At least one of the networks must bear the mechanical stresses in the wall (Cosgrove, 1985). Little is known, however, about the distribution of the stresses between the various wall components in growing cells. Nevertheless, biochemical and biophysical analysis of the cell wall indicates that the matrix is the most significant component for governing the growth properties of cells. Recent work on cultured cells with genetically modified walls has shown, however, that the matrix and other components can be modified to a remarkable degree and the wall still maintains its structural integrity (Shedietzky *et al.*, 1992). Evidently, plant cells can adapt to a wide range of structural changes in their cell walls and still survive and grow slowly, at least in culture. The aspects of matrix structure and metabolism that control cell growth must therefore be of a more subtle nature than the simple proportions of the components.

Lockhart (1965); Ray *et al.*, (1972) were among the first to recognize that cell enlargement begins with a reduction, or relaxation of wall stress. Cosgrove (1993) later summarized the view that wall expansion in growing cells arise from wall stress

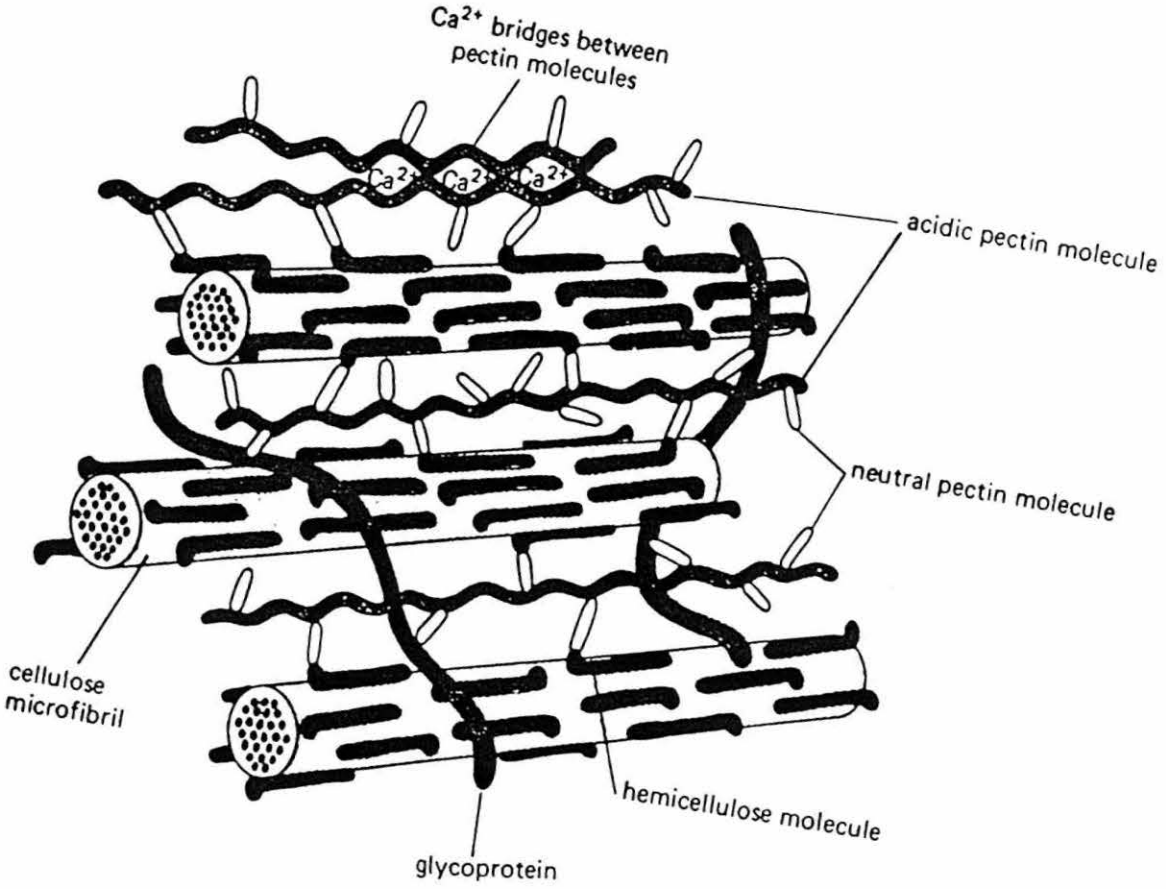


Fig. 1.5 Proposed structure of the primary cell wall. (from Alberts et al., 1989)

relaxation, which in turn lowers the cell water potential and thereby draws water into the cell to physically extend the wall. Cell elongation is thus driven by turgor pressure. The strength of the wall nevertheless constitutes a physical resistance to the cell enlargement and it must influence changes in shape which are a requirement for cell growth and differentiation. In elongating cells, for example, the cellulose microfibrils are found wrapped transversely around the longitudinal axis of the cell. This orientation of the microfibrils prevents the growing cell from becoming spherical, forcing it to enlarge into a cylindrical shape. The non-cellulose matrix of the primary wall (the hemicellulose and pectic substances) also contributes resistance to the forces generated by turgor. These polysaccharides are the 'glue' that holds the cellulose microfibrils together. Changes in their interactions with the cellulose microfibrils must occur during cell enlargement. The orientation of the cellulose microfibrils thus determines the ultimate cell shape, whereas the dynamic interaction of the cellulose and the non-cellulose matrix dictates the rate of cell expansion (Carpita, 1985). Once elongation is completed, the primary wall is locked into shape by the hydroxyproline-rich glycoprotein extensin.

When plant cells enlarge, chemical changes must take place in the cell wall to accommodate the expansion. The bonds which hold the various components together, (ionic, hydrogen and covalent bonds), need to be broken and then reformed. Such cell wall metabolism could occur through a variety of chemical modifications of the wall components (Labavitch and Ray, 1974). It has been proposed that cell wall extension involves the breakdown of hydrogen bonds at the xyloglucan-cellulose interface and that this is promoted by auxin-induced wall acidification (Taiz, 1984). This idea has been supported by other researchers who have shown that indoleacetic acid can stimulate

growth by increasing stress-relaxation in the wall (Cosgrove, 1985). The effect is usually accompanied by a mechanical weakening of the wall (Cleland, 1984) and enhanced xyloglucan turnover (Bret-Harte *et al.*, 1991). Cytokinins and gibberellins can also increase cell wall extensibility by lowering stress-yield thresholds (Cleland, 1981). Thus, cell wall metabolism appears to be an important component in plant growth, because of its role in determining extensibility for cell enlargement.

It is clear from above discussion that the plant cell wall is a metabolically active compartment of the cell and that it must contain numerous enzymes (Lamport, 1970). From several sources of evidence, it is suggested that specific enzymes are involved in loosening the wall by changing the structure of the polysaccharides (Cassab and Varner, 1988). These cell wall enzymes are capable of degrading and resynthesizing the cross links between the polymers, but their precise role in cell wall extension remain uncertain. Xyloglucan endotransglycosylase (XET) is an ideal candidate for wall loosening, which can cut xyloglucan molecules spanning adjacent microfibrils and rejoin the free ends (Smith and Fry, 1991). Increase in XET activity has been found to precede wall loosening along the apical 5 mm of maize roots (Pritchard and Tomos, 1993). Pectinesterase (Goldberg, 1984) and ascorbate oxidase (Lamport, 1965) activities also seem to be related to growth and numerous glycanases associated with the cell walls have been implicated (Cassab and Varner, 1988). Galactosidases (Labrador and Nicolas, 1985), arabinosidases (Dopico *et al.*, 1989), xylosidases (Masuda *et al.*, 1985), glucosidases and glucanases (Wong and MacLachlan, 1980; Hayashi *et al.*, 1984) have been found associated with cell walls in growing cells of dicotyledonous plants. Studies carried out with dicotyledonous plants have shown that galactose and arabinose are the main sugars released by cell wall autolysis during cell elongation (Labrador and Nicolas,

1985). Glucose, xylose and mannose were also released in small amounts (Revilla *et al.*, 1986). A protein extracted from *Cicer arietinum* cell walls showed endo-glycanase activity against water-soluble hemicellulosic polysaccharides (Acebes and Zarra, 1993). This endo-glycanase activity was able to shift the molecular mass distribution of the arabinogalactan fraction of the wall to a lower average value, as well as releasing small oligosaccharides consisting exclusively of arabinose. Exo-glucanase activities, such as glucosidase and galactosidase were also present. In wheat roots, esterases, which may cleave some wall cross-links, were mainly in the outer epidermal cell walls of the growing zone (Smith and O'Brien, 1979). The epidermis and outer cortical cells of maize roots had higher cell wall ß-glucosidase activity than the stele (Ashford and McCully, 1970). It has been subsequently spectulated that ß-glucosidases could modify the side chains of wall polysaccharides, resulting in changes in wall properties (Fry, 1988).

Peroxidase is other important cell wall enzyme which is believed to play a major role in cell wall extension (Masuda and Pilet, 1983). Its activity associated with the cell walls of dicotyledonous plants has been reported (Fry, 1982). The peroxidase enzymes are hemeproteins which can utilize H_2O_2 to oxidise a wide range of hydrogen donors, for example phenolics, aromatic amines, heterocyclics such as indole-3-acetic acid and inorganic ions such as iodine (Shannon, 1968).

Peroxidase exists in several isozyme forms, a few of which are present in the cell walls. Those isozymes function first by forming hydrogen peroxide (H_2O_2) from NADH and oxygen. Next they remove a hydrogen atom from each of two aromatic phenols and combine these two hydrogen atoms with one H_2O_2 to release two H_2O molecules as by-

products. The remaining oxidised phenol is now a free radical, in which several kinds of electronic shifts allow migration of the unpaired electron to various parts of the molecule. These free radicals combine together spontaneously to form polymers. Not only do plants contain several peroxidase isozymes, but their pattern of expression is tissue specific. They are developmentally regulated and highly responsive with respect to exogenous stimuli (Gasper *et al.*, 1982). Their broad substrate specificity may be indicative of a diversity of functional roles for the various iso-forms of the enzyme.

Peroxidases can catalyze phenolic crosslinks between macromolecules such as lignin (Gross, 1977), protein (Labella *et al.*, 1968), hemicellulose (Whitmore, 1976), and ferulic acid (Fry, 1983). They are therefore believed to be involved in cell wall lignification and the crosslinking of extensin monomers and feruloylated polysaccharides (Lamport, 1986). Peroxidase also catalyses the formation of covalent cross links between cell wall components such as the dityrosine link between tyrosine sub-units of extensin (Fry, 1987). Similar diferulate cross links are formed as intramolecular bonds between pectin molecules (Wallace and Fry, 1993). Peroxidase may also crosslink proteins by oxidative deamination of lysine (Stahmann and Spencer, 1977). Its activity might, therefore, be expected to correlate with wall tightening. Peroxidases have also been implicated in the induction of cell wall biosynthesis as a part of the defensive response to pathogen attack (Sequeira, 1983). The enzymes are induced by wounding and they have been implicated in the repair of the damaged walls (Birecka and Miller, 1974). They are believed to do this by forming a watertight barrier over a wound by depositing polymeric aliphatic and aromatic substances (Espelie *et al.*, 1986). Thus, cell wall enzymes appear to be very important components of the wall where they help determine cell structure during development.

1.12 Strategic basis for the present study

It is quite obvious from previous studies that, even under ideal conditions of germination, cotton shows a very high variability in the rate of root growth at the seedling stage. Because of this problem, the present study was under taken to study some basic but important physiological and biochemical parameters in the growth and development of cotton seedlings. It has been reported that root growth depends on two basic processes, cell division and cell elongation. The purpose of this study, therefore, was to study differences between slow and fast growing roots in cotton seedlings. In particular, differences in the rate of cell division and in the rate and extent of cell elongation were determined.

Because of lack of a general research on the physiology of the cotton crop, especially on root growth, the present research work was carried out to answer the following questions:

1. Where does root elongation take place ?
2. Where does initiation of lateral roots takes place ?
3. Is there any difference in cell dimension between slow and fast growing roots ?
4. Are there any differences in the uptake and incorporation of (Me-^{3}H) thymidine between slow and fast growing roots ? Any difference should reflect differences in rates of cell division.
5. Is there any difference in DNA content between slow and fast growing roots ?
6. Are there any differences in peroxidase activities between slow and fast growing roots ? Any difference might reflect differences in cell wall metabolism between the roots.
7. Is there any effect of hormones on root growth and peroxidase activities ?

CHAPTER TWO

Chapter Two

Materials and Methods

2.1 Plant material

Cotton (*G. hirsutum* L.),cultivar MNH-93, was supplied by the Pakistan Central Cotton Committee's Research Institute at Multan (Punjab), Pakistan. The seeds used in these experiments were from the 1991 to 1994 harvests. They were stored for up to one year at 4°C in sealed glass or plastic containers.

2.2 Glassware

All glassware was cleaned by soaking overnight in a phosphate-free detergent (Lipsol liquid, LIP Equipment and Servicing Ltd., Shipley, U.K.). After scrubbing with a brush, the glassware was washed with distilled water and placed for one hour in dilute hydrochloric acid (to remove remaining traces of detergent). It was finally washed three times with distilled water and dried in an oven at 110°C.

Glassware contaminated with radioactive material was pre-soaked in Decon detergent (Decon Laboratories Ltd. Hove, U.K.) for 4-5 hours followed by the above cleaning with Lipsol.

2.3 Reagents

Unless otherwise stated, reagents were obtained from BDH Ltd. Poole, Dorset, U.K. Radiolabelled (Me-^{3}H) thymidine was supplied by The Radiochemical Centre, Amersham, U.K. Guaiacol was obtained from Janssen Chimica, Geel, Belgium, and Na dihydrogen orthophosphate from Koch-Light Laboratories Ltd., U.K. Non-radiolabelled

thymidine, N-lauroylsarcosine, $MgCl_2$, Na acetate, IAA, GA_3, ABA, and hydrogen peroxide were all supplied by Sigma Chemical Co., St. Louis, MO. U.S.A.

Buffers were prepared according to standard procedures (Dawson *et al.*, 1969) and their pH was checked before use using a laboratory pH meter (Corning Model M 220, Ciba Corning Diagnostics Ltd. Sudbury, U.K).

2.4 Solvents

All solvents used in these experiments were AnalaR grade. They were obtained from BDH Ltd. Poole, Dorset, U.K.

2.5 Seed treatment

Cotton seeds were delinted by the following procedure. The seeds (20 g) were placed in a 200 cm^3 beaker and approximately 4 cm^3 of concentrated sulphuric acid were added (1 cm^3 per 5 g seeds). The seeds and acid were stirred thoroughly with a glass-rod for 3 minutes and left for 5 minutes. They were then stirred for a further 2 minutes and transferred to a Buchner funnel where they were washed with running tap water for 3 minutes. The seeds were then placed in 200 cm^3 1% Na bicarbonate solution for 10 minutes to neutralise remaining acid and washed thoroughly with distilled water. The delinted seeds were soaked for an hour in 1 litre of distilled water. After this time, the floaters were discarded and the sinkers were placed in a 1.5% Na hypochlorite solution (0.1% available chlorine) for 20 minutes to further sterilize them. Finally, the seeds were washed eight times with sterile distilled water to remove the hypochlorite.

2.6 Germination of seeds

The method adopted to germinate the seeds has been described previously by Sethar (1993). Fifty delinted and sterilized seeds were placed in 10 rows, with 5 seeds in each row, on a sheet of Whatman No.1 filter paper (23 x 57 cm). The filter paper was moistened with 40 cm^3 sterile distilled water. It was then rolled up round a wooden stick (32 x 0.5 cm) and placed in a polythene bag (35 x 23 cm). The top of the bag was folded over and tied lightly to maintain sterile conditions and allow air exchange. Finally, the bag and its contents were stood upright in a 500 cm^3 beaker and incubated in an incubator in the dark at 25°C. After 24 and 48 hours, the bag and its contents were rolled to re-moisten the seeds with water that had collected at the bottom of the bag. For each analysis, 100 seeds were germinated in this way in two filter paper rolls.

2.7 Selection of seedling roots

After various germination periods, the seeds were harvested. Ungerminated seeds were discarded and the remaining seedlings were divided into groups according to their root lengths. The details of the latter are given in the individual results chapters.

The selected seedlings were dissected using a scalpel to remove their cotyledons and shoot epicotyl. This left the root and hypocotyl for use as the experimental material. The root plus hypocotyl will be generally referred to as the root through most of this thesis.

2.8 Determination of root length, fresh and dry weight

The length of each root was measured using a ruler. For fresh weight determination, the selected batches of root were weighed on a five-place analytical balance (Mettler Model

AE 240, Mettler Instruments Ltd. High Wycombe, U.K). The roots were then dried for 48 hours in an oven at 70°C and re-weighed to determined their dry weight. The percent moisture content was calculated from the fresh weight and dry weight values.

2.9 Measurement of root diameter and segment fresh weight

The roots plus hypocotyl were dissected with a scalpel into 1 cm segments and weighed on a five-place analytical balance to give the segment fresh weight. Each segment was then put onto a slide for viewing under a light microscope (Vickers Laboratories Ltd. Ilkley, U.K.). The root diameter was measured by using a calibrated graticule fitted into the eye piece of microscope. The diameter was recorded at 1.0, 5.0, 9.0, 19.0, 39.0, 59.0, and 79.0 mm from the tip of the root.

2.10 Identification of the region of root elongation

The roots of duplicate sets of 36, 48, 60, 72, 84, and 96 hour seedlings were marked with lines at 2 mm intervals from the tip to the base using a fine felt pen containing water-proof ink. One set of roots was taken straightaway for photography (control). After taking the photographs, these roots were discarded. The other set of seedlings with marked roots were reincubated for 12 hours at 25°C. After that period, the roots were harvested and photographed. Comparison of the photographs from the two sets of roots, allowed us to identify the regions of the root where expansion was taking place.

2.11 Lateral root initiation

Seeds were germinated as described in Section 2.6. The roots were harvested at 96 hours germination period. The five longest roots were selected for further growth. They were grown individually in conical flasks containing half-strength Long Ashton nutrient

solution with air bubbled into the medium from a fish tank pump. The flasks and their contents were maintained for 10 days at 25°C in a plant growth room with a light intensity of 100 μmol m^{-2} s^{-1} in a 16 hour photoperiod with 75% relative humidity. After this time, the roots were take out from the conical flasks and they were photographed to show the lateral roots.

2.12 Histological analysis

Seedling roots were grown for 36, 60 or 84 hours. Upon harvesting, two root groups (long and short) were selected with 10 roots of same length in each group at each germination period (see Section 2.7). The apical 10 mm from each root was excised and placed in 3% glutaraldehyde in 0.1M phosphate buffer, pH 6.8, for 24 hours under vacuum (67 kPa) at room temperature (Vacuum oven manufactured by Townson and Mercer Ltd. Croydon, U.K.). The apices were then dehydrated in a series of ethanol : water mixtures (25%, 50%, 75% and 100% ethanol;12 hours each) at 4°C. The dehydrated roots were placed overnight in a 50/50 mixture of ethanol and historesin infiltration solution (50 cm^3 basic resin + 0.5 g activator) (Leica UK Ltd. Milton Keynes, U.K.) on a rotator. This was then changed to pure infiltration solution and left on the rotator for a further 2 days. The infiltrated roots were finally embedded in historesin. Serial longitudinal sections were cut (4μm thick) on a Richert-Jung 2050 microtome using disposable metal knives. The sections were floated out on to water in a water bath at 40°C, collected on glass slides and dried on a hot plate at 40-50°C. The sections were stained for 1-2 minutes with 0.05% toluidine blue in benzoate buffer, pH 4.4, (Feder and O'Brien, 1968) and rinsed in distilled water until the plastic was free from stain. The slides were dried and mounted with neutral Gurr mounting medium (BDH Ltd. Poole, Dorset, U.K.). The slides with their sections were examined under

a light microscope (Vickers Laboratories Ltd. Ilkley, U.K.). The cell size (cell length and diameter) was measured by using a calibrated graticule fitted in the eye-piece of microscope. The cell size was recorded at 6.0, 7.5 and 9.0 mm from the root tip for all the germination periods and root lengths mentioned above. At each distance from the tip, the number of cortical cell files were counted and the cell size measurements were made on the two "middle" cell files with 10-15 cells per file on each side of stele. The final value for the cell size at each point was then taken as an average of all these values. Finally, the cell volumes were calculated from the mean cell length and cell diameter values by the following equation:

$$\pi r^2 l$$

Where: π = 3.14 (constant)

r = cell radius

l = cell length

2.13 Uptake and incorporation of (Me-^{3}H) thymidine

The method used to measure the incorporation of (Me-^{3}H) thymidine has been described previously by Baiza *et al.* (1989). Ten whole roots were washed for 1 minute in 200 cm^3 0.5% Na hypochlorite followed by four washes with sterile distilled water. The roots were then placed in upright positions in a vial (11 x 74 mm) containing 5 cm^3 50 mM Tris-HCl buffer, pH 7.6, 50 mM KCl, 10 mM $MgCl_2$, 2% sucrose, 10 $\mu g/cm^3$ chloramphenicol, and 1 μCi (Me-^{3}H) thymidine (specific activity = 65.8 mCi/mg). Water-saturated air was bubbled into the vials from a fish tank pump and the roots were incubated for 4 hours at 25°C in a reciprocating water bath (Grant instruments Ltd. Barrington, U.K.) operating at 175 oscillation per minute. At the end of this radiolabelling period, the medium was removed from the roots by vacuum filtration

through a sintered-glass funnel. The roots were then washed three times with distilled water, then with 10 cm^3 of 1% Na citrate followed by 10 cm^3 of 80% ethanol (both solutions containing 200 $\mu g/cm^3$ non-radioactive thymidine).

The washed roots were dissected with a scalpel to produce 1 cm segments. The segments from the same part of the roots were bulked together and each group of segment was homogenized for 1 minute in 1.5 cm^3 of 80% ethanol containing 200 $\mu g/cm^3$ non-radioactive thymidine using a Polytron top-drive homogeniser (Philip Harris Scientific. Lynn lane, Shenstone, Lichfield, U.K.) at speed mark 3. The homogenate was centrifuged for 10 minutes at 3000 rpm in a MSE-Microcentaur centrifuge (Fisons Scientific Equipment. Loughborough, U.K.) and the supernatant was discarded. The pellet was resuspended in 0.5 cm^3 1M NaOH and heated for 3 minutes in a water bath at 90°C. After cooling, 2 cm^3 of 10% TCA were added and the resulting suspension was put in ice for 2 hours. It was then filtered through a 2.5 cm Whatman GF/A glass microfibre filter placed in a sintered-glass filter funnel. The filter with the retained particulate material was washed with 5 cm^3 of 5% TCA followed by 10 cm^3 of 95% ethanol. The filter paper and its contents were then dried and placed in a 22 cm^3 glass scintillation vial with 10 cm^3 of scintillation fluid. The scintillant used was Scintillant Cocktail T (BDH Ltd. Poole, Dorset, U.K.). Uptake of radioactivity into the tissue was determined from the sum of the radioactivity in aliquots taken after the centrifugation (supernatant), filtration (filtrate) and in the particulate material collected in the filter. The incorporation of radioactivity into the DNA was determined as the radioactivity contained in the particulate material on the glass micro fibre filter. The radioactivity was determined by scintillation spectrometry using a Beckman Model LS 7500 Spectrometer (Beckman-IRIC Ltd. High Wycombe, U.K.). Raw counting data were converted from

counts per minute (cpm) to disintegration per minute (dpm) by the spectrometer using the Crompton "end effect".

2.14 Determination of DNA

The method used for the determination of total DNA was that described by Wakiuchi *et al.* (1990). In this method, 10 excised roots were placed in a mortar and homogenised with a pestle at room temperature in 5 cm^3 of extraction medium containing 10 mM Tris-HCl, pH 7.0, 2M NaCl, 10 mM EDTA, and 1% (w/v) N-lauroylsarcosine. The homogenate was transferred to a 40 cm^3 centrifuge tube and centrifuged for 10 minutes at 14000 rpm in a Beckman JA2-21 centrifuge (Beckman-IRIC Ltd. High Wycombe, U.K.). The supernatant was removed and the precipitate was resuspended in 2 cm^3 of extraction medium and centrifuged again as described above. The supernatants were combined to form the DNA extract and this was diluted with one and a half volumes of the extraction medium. Chloroform was then added to the diluted extract in the ratio 3:2. The whole was vigorously mixed and then centrifuged at 4000 rpm for 10 minutes to separate the phases. The aqueous upper phase was used for the fluorometric determination of DNA.

The DNA analysis was carried out according to the following procedure. A solution of calf thymus DNA (50 $\mu g/cm^3$) was prepared for use as internal standard and a stock solution of DAPI (1 mg/100 cm^3 in water) was diluted to 100 ng/cm^3 with buffer solution (10 mM Tris-HCl, pH,7.0, 0.1M NaCl, and 10 mM EDTA) to be used as the fluorogenic reagent. For the actual assay, 3 cm^3 of the diluted DAPI solution was placed in a 1 cm x 1 cm cuvette and its fluorescence determined. The fluorescence was measured with a Luminescence Spectrometer (Perkin-Elmer Model LS-5, Perkin-Elmer

Ltd. Beconsfield, U.K.) at excitation and emission wavelengths of 343 nm and 450 nm respectively. Four aliquots (20 μl each) of the plant extract followed by four aliquots (20 μl each) of the internal standard were then added successively. After each addition, the contents of the cuvette were mixed and the fluorescence was determined. A separate calibration curve was prepared using a standard solution of Calf thymus DNA containing 0-8 μg DNA. This calibration 'curve' was linear (see Figure 2.1).

The method of Wakiuchi *et al.* was checked for a number of possible sources of error as follows:

Influence of root water content. The question was raised whether root water content may influence the DNA values because of the very low volume of extraction medium used. The tissue water could contribute significantly to the final volume of extract. Therefore, the values for root water content were calculated from root fresh and dry weight data (Section 2.8). The results for DNA were recalculated to correct for this.

Effect of homogenization time. To check for complete homogenization of the tissue, one set of roots was homogenized for a short time (2 min) and another set for a long time (5 min). When the results were compared, both values were almost the same. It was concluded that the shorter time was sufficient to guarantee complete homogenisation.

Degradative action of DNAase. The question was raised about the presence of DNAase, which might degrade DNA especially in older roots. The advice of Dr. A.H. Shirsat was sought and he advised that there is little possibility that DNAase would interfere with the extraction procedure used.

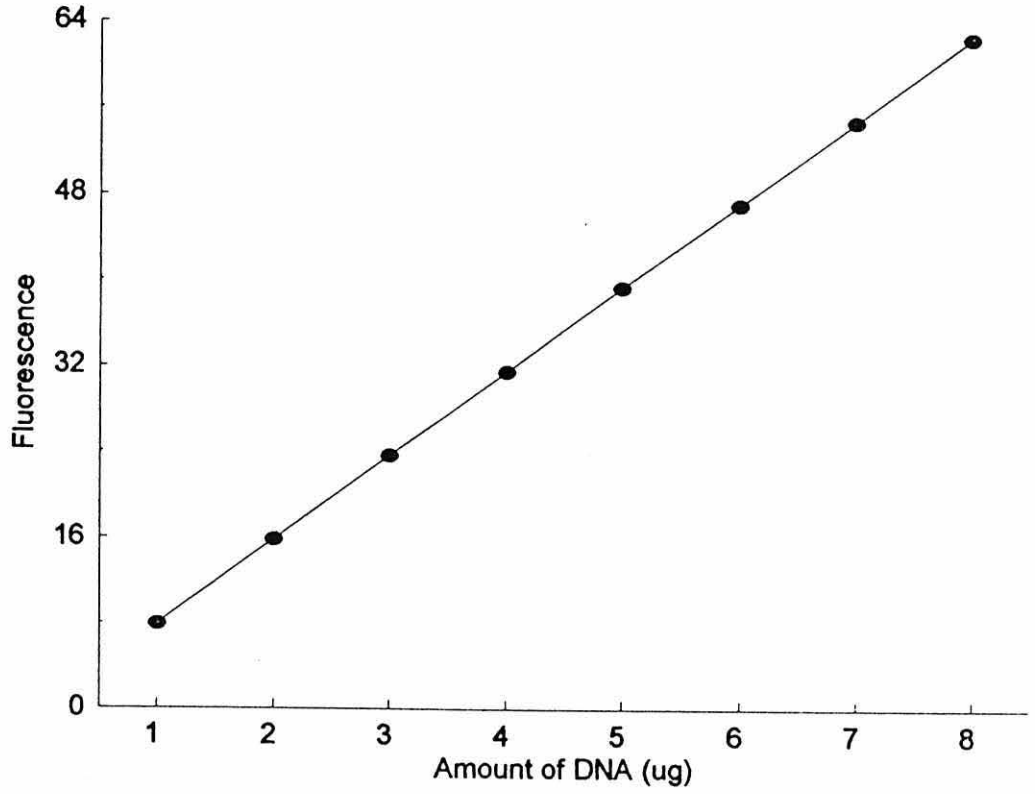


Fig. 2.1 Calibration curve for the determination of total DNA.

Efficiency of the chloroform partitioning. The question was raised whether lipid might interfere with the fluorescence assay for DNA. The interference could arise due to incomplete extraction of tissue lipid by the chloroform used as part of the extraction procedure. This check was done using two sets of roots. For one, slow shaking with chloroform was used and for the other a more vigorous shaking was employed. The latter should safely remove all lipid. When the results for the two samples were compared, no difference was found. It was concluded that the standard Wakiuchi procedure was adequate in this respect.

Presence of fluorescent impurities. This was checked by introducing a control without the DAPI reagent. A small amount of fluorescence impurity was found. It accounted for about 6-7% of the DNA values at early growth stages (24 hours), but it increased progressively with increasing germination period. In older roots (84 hours) it accounted for 40-50% of the obtained DNA values. All experiments therefore included determinations of this source of error.

Presence of quenching impurities. The possible presence of these impurities were checked by measuring the fluorescence of a standard DNA and tissue extracts individually and in combination. Quenching was calculated to cause between 8.5 and 10.5% decrease in the experimental DNA values. This was the same quantatively at all germination times. All raw data were corrected accordingly.

Although the method of Wakiuchi *et al.* was shown to be subject to inaccuracies particularly due to the presence of interfering fluorescent substances in older roots, the resulting errors did not change the overall patterns of DNA through the germination

period under study. Nevertheless, the corrections described above were carried out on all the raw data obtained from the method.

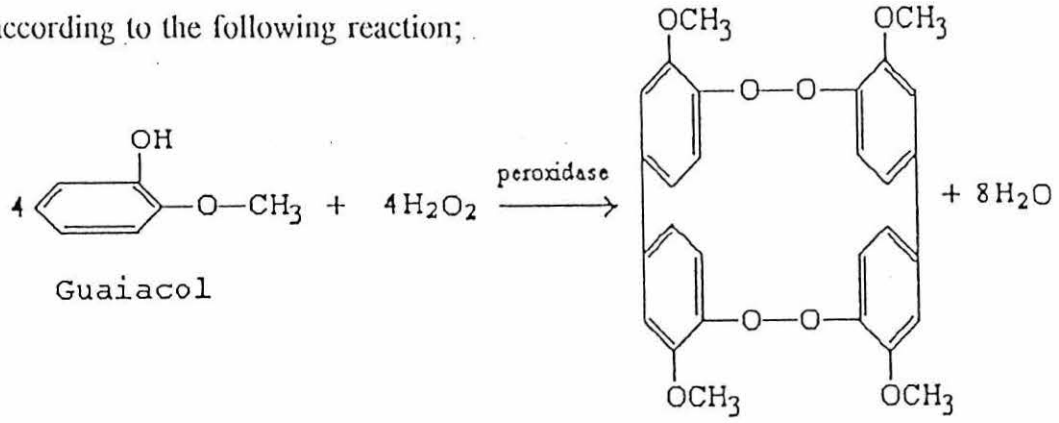
2.15 Determination of Peroxidase activity

Extracts were prepared by grinding the root tissue thoroughly in a cold pestle and mortar with 1 cm^3 of cold (4 °C) 20 mM Na phosphate buffer, pH 7.0. An additional 2 cm^3 of buffer was then added and the extract was poured into 1.5 cm^3 Eppendorf tubes. A further 3 cm^3 of buffer was used to wash the mortar and this was added to the Eppendorf tubes. The resulting mixture was centrifuged for 10 minutes at 13000 rpm (MSE-Micro Centaur centrifuge. Fisons Scientific Equipment. Loughborough, U.K.). This centrifugation was done at room temperature (25 °C). The supernatants (the extract) were bulked and used for the enzyme assay. This extract contained the soluble peroxidase(s). The residue (pellet) from the extraction was re-extracted at 4 °C for 24 hours in 20 mM Na phosphate buffer, pH 7.0, containing 1M NaCl. The supernatant from this extraction contained insoluble (cell wall) peroxidase(s). The extracts were maintained at 0 °C and they were assayed on same day.

The determination of peroxidase activity was based on the method of Chance and Maehly (1964). Activity was measured using a spectrophotometer set at 470 nm (Jenway, Model 6100, Jenway Ltd. Felsted, U.K.) coupled to a potentiometric chart recorder (Kipp and Zonen, Model BD 111/112, Delft, Holland). The stock reagents for the assay were 150 mM guaiacol, 20 mM Na phosphate buffer, pH 7.0, and 40 mM hydrogen peroxide. These were prepared weekly and kept at 0 °C when not in use. All assays were done after the reagents had been allowed to reach room temperature (25 °C). The assay mixture contained 2.5 cm^3 of 20 mM Na phosphate buffer, pH 7.0, 0.1 cm^3

of 0.3 mM hydrogen peroxide, 0.2 cm^3 of 35 mM guaiacol and 0.2 cm^3 of enzyme extract, making a final volume of 3 cm^3 in a 10 mm spectrophotometer cuvette. The enzyme extract was added last to start the reaction and the change in absorbance was followed for 5 minutes. The rate of absorbance change per minute was obtained by determining the slope of the graph plotted by the recorder at the beginning of the assay (initial reaction velocity). A typical example of a reaction curve is shown in Figure 2.2.

This method involves the oxidation of the phenol guaiacol by hydrogen peroxide according to the following reaction;



Guaiacol

Tetraguaiacol

The reaction is catalysed by peroxidase. The product, tetraguaiacol, has a red-brown colour which begins to fade a few minutes after its formation. The formation of the coloured product can therefore be easily followed spectrophoto-metrically if measurements are made quickly (Bertrand, 1904).

2.16 Effects of hormones on root growth and peroxidase activity

Gibberellic acid. Five different concentrations of GA_3 (10^{-4} to 10^{-8} M) were used to study their effects on root growth. Six batches of 100 seeds were germinated as described in Section 2.6. The roots were harvested after 36 hour germination period and the 10 longest roots from each batch of seeds were selected. One batch was employed

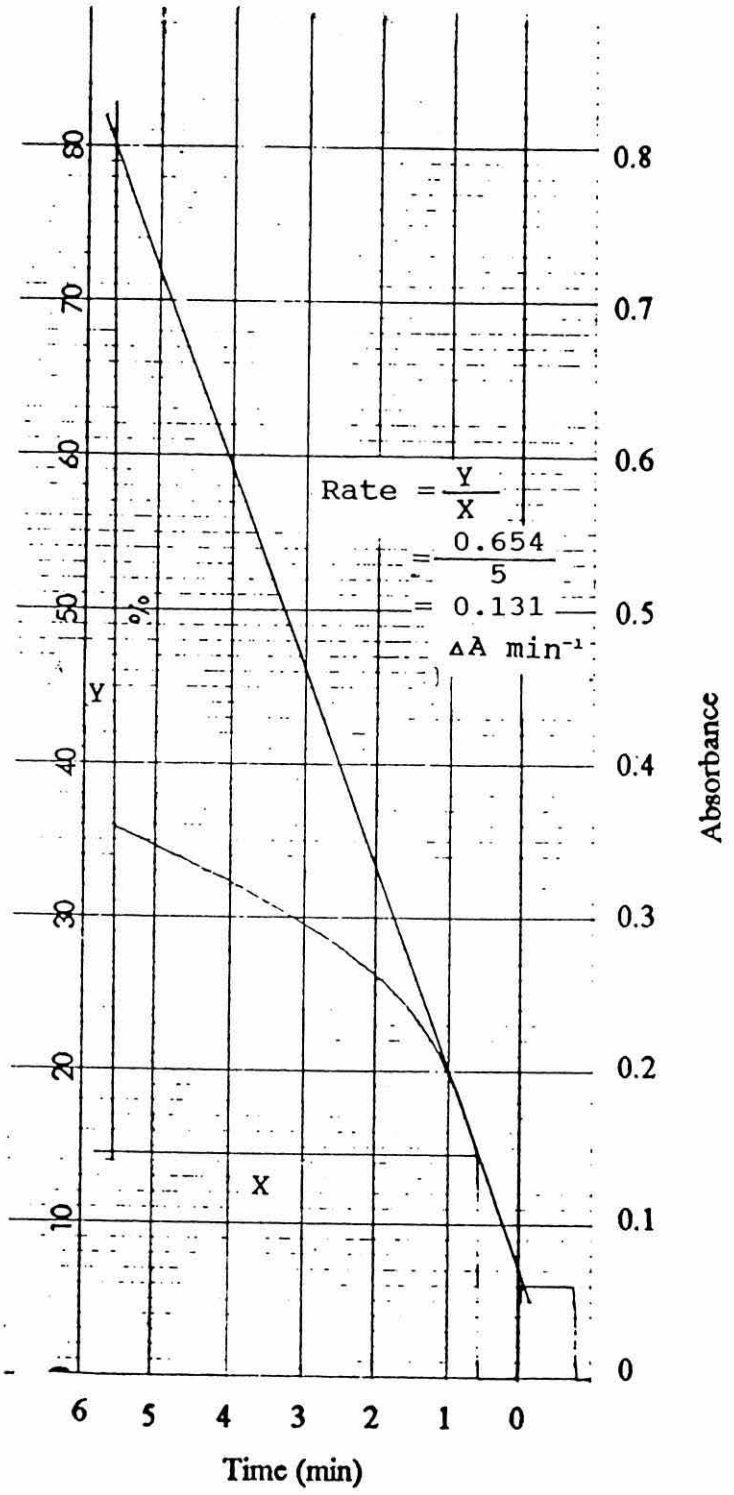


Fig. 2.2 Typical progress curve for the peroxidase determination.

as the control using distilled water and the remaining 5 batches were treated with different concentrations of GA_3. All the seedlings were reincubated for a further 36 hours in paper (see Section 2.6) soaked with distilled water (control) or one of the GA_3 solutions. After this time, all batches were reharvested and the root lengths of each batch were measured as described in Section 2.8. This experiment was repeated 3 times.

Gibberallic acid (10^{-6} M) was used to study the effect of hormone on root growth at different germination periods. Ten batches of 100 seeds were germinated as described in Section 2.6. The seedling roots were harvested after 36 hours germination and the 10 longest roots from each batch of seeds were selected as before. The root length of 2 batches were measured (0 h control) as described in Section 2.8. From the remaining 8 batches, 4 were used as no-hormone controls (reincubated with distilled water) and 4 were reincubated with GA_3. They were reincubated for 6, 12, 24 and 36 hours. At the end of each period, two batches (control and hormone treated) were reharvested. The root lengths of each batch were measured as described in Section 2.8. This experiment was also repeated 3 times.

Abscissic acid. Three different concentrations of ABA (10^{-5} 10^{-6} and 10^{-7} M) were used to study their effects on root growth at various germination periods. For each ABA concentration, 10 batches of 100 seeds were germinated as described in Section 2.6. The seedling roots were harvested after 24 hour germination and the 10 longest roots from each batch of seeds were selected. The root length of 2 batches were measured (0 h control) as described in Section 2.8. From the remaining 8 batches, 4 were used as no-hormone controls (reincubated with distilled water) and 4 were reincubated with ABA. They were reincubated for 12, 24, 36 and 48 hours. At the end of each period, two

batches (control and hormone treated) were reharvested. The root lengths of each batch were measured as described in Section 2.8. This experiment was repeated 3 times for each concentration.

Indole acetic acid. Five different concentrations of IAA (10^{-3}, 10^{-4}, 10^{-6}, 10^{-8} and 10^{-10} M) were used to study their effects on root growth. For each IAA concentration, 10 batches of 100 seeds were germinated as described in Section 2.6. The roots were harvested after 24 hour germination and the 10 longest roots from each batch of seeds were selected. The root length of 2 batches were measured straightaway (0 h control). From the remaining 8 batches, 4 were used as control using distilled water and other 4 were treated with IAA as treatment. They were reincubated for 12, 24, 36 and 48 hours. At the end of each period, two batches (control and treatment) were reharvested. The root length of each batch was measured as described in Section 2.8. This experiment was repeated 3 times for each concentration.

Two concentration IAA (10^{-3} and 10^{-8} M) were selected to see their effects on peroxidase activity. These experiments were the same as those described above up to the point where root lengths were measured. After that, 2 cm root tips were dissected and bulked together. They were weighed straightaway after dissection (fresh weight) and then used for peroxidase activity. The peroxidase activity was determined as described in Section 2.15.

2.17 Statistical analysis

All means, standard deviation and standard error values were calculated using a scientific pocket calculator. Student's T-test was carried out using the Systat/Sygraph

software (Systat Inc. Evanston, IL. USA) mounted on a personal computer. Analysis of variance (ANOVA), based on the general linear model (GLM), was carried out using the Minitab (Version 10.2) statistical package (Minitab Inc., USA) by accessing the University network. In some instances (noted in the text) data were logged in order to minimise positive relationships between mean values and their variance. For all statistical analysis, a probability value of 0.05 or less was considered to be significant and any value above that was considered to be insignificant. All graphs were plotted using the personal computer with the Quattro Pro (Version 6.0) software package (Borland International Inc. USA).

CHAPTER THREE

Chapter Three

On the General Parameters of Root Growth and Development

3.1 Introduction

During the last 20 years the subject of root development has received a great deal of attention, there being a number of reasons for the increased interest. For morphogenic studies, roots can be excised and grown in simple sterile nutrient culture where they grow at near normal rates with normal morphogenesis. The organization of the root apical meristem can be readily explored experimentally; it is less complex than the shoot apex, appendages being formed some distance behind the apex itself. The root is therefore an ideal experimental subject for the detailed study of growth pattern.

The plant root system is important for anchorage and for exploration of the soil for water and mineral nutrients (Ivanov, 1989). Its importance is reflected in the fact that about 20% of the carbon fixed by a plant passes into the root system (Goss, 1991). The root is often a main organ for the storage of nutrients including carbohydrates. In all plants, the roots are a critically important source of several growth regulating substances including hormones (Crozier and Hillman, 1984). They are, for example, a major source of the cytokinin necessary for shoot development and the proper function of apical meristems. Many gibberellins are also synthesized in roots, although others are produced in leaves and transported to the root to be converted to the active form.

Roots are able to grow compensatorily. That is, if part of the root system is restricted the rest will grow faster, or if one root enters a region that is especially favourable it will greatly increase its growth rate while the rest slow down (Brouwer *et al.*, 1981;

Russell, 1977; Reynolds, 1975). Root buds, leading to lateral root development, can apparently form almost anywhere and in almost any density, often being induced more by external factors than by internal ones (Vartanian, 1981). Roots form not only lateral roots as part of the root branching system, but in some plants they from adventitious shoot buds and thereby serve as a means of asexual propagation (Peterson, 1975). Roots act as props or stilts for corn and other grasses. They can be a means of root aeration in plants growing in stagnant water (Chapman, 1939).

Roots are probably the simplest organs in which growth processes can be studied. Root tips contain a meristematic region giving rise to a distal growth zone and a proximal one. The distal growth zone produces the root cap, while the proximal zone produces the root proper. The cells in the proximal growth zone are arranged in longitudinal files. It is convenient to further divide this proximal growth zone into a distal part, where growth occurs by cell division and cell elongation, and a proximal part, where growth occurs by cell elongation without cell division (Green, 1976).

Rapid root growth occurs as the result of high rates of cell division and elongation, although the actual number of meristematic and elongating cells is less than in shoots. Proteins and nucleic acids are synthesized in the growing part of the root at much greater rates than in growing shoots and leaf however. Towards growth cessation, proteins and nucleic acids are degraded. The active metabolism of purines accompanying the synthesis and decomposition of RNA could be the basis for more active synthesis of cytokinins in roots than in shoots (Ivanov, 1989).

The apical region of the root secretes a complex gel-like substance called mucigel (Jenny

and Grossenbacher, 1963). Part of it is secreted by the root cap (Juniper and Pask, 1973; Paull and Jones, 1976) and part by the root hairs (Greaves and Derbyshire, 1972; Leppard, 1974; Werker and Kislev, 1978). The mucigel is the actual contact interface between growing roots and the soil, providing physical protection to the delicate root structures as they grow through the soil and between soil particles. Therefore, the cell wall does not interact directly with soil or the soil solution (Foster *et al.*, 1983; Greenland, 1979; Russell, 1977). Microbes are always found within the mucigel (Foster and Rovira, 1976). It has been suggested that it serves as culture medium for the bacteria, which somehow aid the growth of the root by secreting hormones and/or nutrients (Bowen and Rovira, 1976; Dart and Mercer, 1964; Rovira, 1979).

All germinating seeds have similar patterns of root growth. Under optimum conditions, growth proceeds rapidly with increasing germination time. In the case of cotton germinating at 25°C, root length increases rapidly for at least 72 hours (Sethar, 1993; Sheikh, 1994). Similar results have been reported for root growth in wheat, maize, soybean and millet (Mengal and Barber, 1974; Sivakumar *et al.*, 1977; Gregory, 1978; Stone and Taylor, 1983).

The reason for conducting the experiments on root growth presented in this chapter was to get basic information which would help in dealing with the detailed experiments in later studies. Particular attention was given to the influence of root growth rate on the measurements made. Much of this information for cotton is not available in the scientific literature.

3.2 Methods

3.2.1 Selection of seedling root groups

Three methods of separation of root groups according to root length were used.

Method 3A: Batches of 100 seeds were germinated as described in Section 2.6. After 24, 36, 48, 60, 72, 84 and 96 hours germination, the seedlings were harvested. Ungerminated seeds were discarded and the remainder were arranged according to root length. The 10 longest and 10 shortest roots were retained along with a group of 10 roots of intermediate length. The three classes were designated the 'longest', 'shortest' and 'intermediate' roots.

Method 3B: The seedlings were germinated and arranged as in Method 3A. Six seedlings were selected from the centre of the root length distribution (ie. median roots). Two further sets of 6 seedlings were then selected, one of which shared a longer root length and the other a short root length. The roots were designated the 'long', 'medium' and 'short' roots.

Method 3C: Batches of 100 seeds were germinated as described in Section 2.6. After 96 hours germination, the seedlings were harvested. Ungerminated seeds were discarded and the remainder were arranged according to root lengths. Sets of 6 seedlings with roots of 4, 5, 6, 7, 8 and 9 cm lengths were then selected.

3.2.2 Measurement of length, fresh weight, dry weight and diameter of roots, and root segment fresh weight

The methods used are described in Sections 2.8 and 2.9.

3.2.3 Identification of the region of root elongation and demonstration of lateral root initiation

The procedures used are described in Sections 2.10 and 2.11.

3.3 Results

At the beginning of the study, large variations in root growth rate were noted. Due to this variation, which occurred within a single seed lot, relatively large samples (100 seeds) were used for each analysis. It was also decided to divide the roots into groups according to their length. This procedure reduced the standard deviation values considerably, at least within the selected groups.

3.3.1 Root length

The results of an experiment in which roots were selected into longest, shortest and intermediate subsets (Method 3A; see Section 3.2.1) are presented in Table 3.1. They show that the patterns of root growth were similar for longest, intermediate and shortest roots. Thus, the root lengths in all of the seedling groups increased with increasing germination time. At the 24 hours germination period, the mean lengths for longest, intermediate and shortest roots were 1.52, 1.01 and 0.62 cm respectively. These values increased to 10.0, 7.36 and 5.10 cm at the 96 hours germination period. When the data are presented in graphical form (Figure 3.1A), it can be seen that growth became roughly linear after 36 hours in all three groups of seedlings. It also becomes clear that throughout the period 36-96 hours the longest roots grew faster than the intermediate ones, which in turn grew faster than the shortest roots. Linear regression analysis of the 36-96 hours data gave the following growth rates: 0.109 cm hr^{-1} for the longest roots, 0.075 cm hr^{-1} for the intermediate ones and 0.054 cm hr^{-1} for the shortest roots. Further

Table 3.1 Root length at different germination times.

Germin[a]	Longest roots			Intermediate roots			Shortest roots		
period(h)	mean	±sd	±se	mean	±sd	±se	mean	±sd	±se
24	1.52	0.18	0.03	1.01	0.05	0.01	0.62	0.15	0.02
36	3.33	0.36	0.06	2.66	0.35	0.06	2.07	0.61	0.10
48	4.15	0.31	0.05	3.16	0.24	0.04	2.05	0.22	0.03
60	5.56	0.66	0.10	4.35	0.58	0.09	3.48	0.52	0.08
72	6.63	0.60	0.09	4.80	0.36	0.06	3.57	0.39	0.06
84	7.88	0.66	0.10	5.70	0.69	0.11	4.21	0.78	0.12
96	10.00	0.90	0.14	7.36	0.41	0.06	5.10	0.31	0.05

Each value is the mean length (cm) ±sd and ±se from 4 separate experiments.

statistical analysis by ANOVA (in which the effects of seedling groups and germination time groups on root lengths were investigated) confirmed that root length was highly dependent upon both seedling group (longest, intermediate or shortest roots) and germination time ($P < 0.001$ in each case). The interaction between seedling group and germination time ($P < 0.001$) reflected the difference in the rate of root growth in the different seedling groups.

3.3.2 Root fresh weight

Fresh weight values are presented in Table 3.2 and Figure 3.1B. The data were obtained from the same root samples used for the root length determinations (Section 3.3.1). As might be expected, the fresh weight of the roots was dependent upon both the length of the root and the germination time. At 24 hours germination, the mean fresh weight values for longest, intermediate and shortest groups of roots were 0.124, 0.099 and 0.088 g $(10 \text{ roots})^{-1}$ respectively. The differences between the groups were maintained as germination proceeded. At 96 hours germination, the mean fresh weight values of the longest, intermediate and shortest groups were 1.84, 1.65 and 1.45 g $(10 \text{ roots})^{-1}$ respectively. Obviously, after the first 36 hours the fresh weight of all the groups increased linearly with increasing time of germination. In contrast to the different root growth rates, however, the rates of fresh weight increase were nearly the same for all three groups of seedlings (0.021 g hr^{-1} for longest roots, 0.020 g hr^{-1} for intermediate roots and 0.019 g hr^{-1} for shortest roots as determined by regression analysis). It is concluded from these results that the seedling groups (longest, intermediate, shortest roots) and the age group of the seedling had the greatest effects on the fresh weight. Statistical analysis by ANOVA (in which the effects of seedling group and germination period group on root fresh weights were investigated) confirmed these influences ($P <$

Table 3.2 Root fresh weight at different germination times.

Germin[a]	Longest roots			Intermediate roots			Shortest roots		
period(h)	mean	±sd	±se	mean	±sd	±se	mean	±sd	±se
24	0.124	0.007	0.003	0.099	0.003	0.001	0.088	0.019	0.009
36	0.618	0.059	0.029	0.481	0.069	0.035	0.324	0.106	0.053
48	0.874	0.055	0.028	0.654	0.046	0.023	0.481	0.038	0.018
60	1.124	0.126	0.063	0.983	0.146	0.073	0.805	0.124	0.062
72	1.276	0.075	0.038	1.146	0.067	0.034	0.972	0.104	0.052
84	1.661	0.121	0.061	1.384	0.177	0.088	1.194	0.238	0.119
96	1.840	0.097	0.048	1.654	0.056	0.028	1.451	0.012	0.006

Each value is the mean fresh weight [g(10 roots)$^{-1}$] ±sd and ±se from 4 separate experiments.

0.001 in each case). The marginally significant interaction between seedling group and germination period over the 36-96 hours period (P = 0.05) confirms that there was a slight difference in the fresh weight gain of the different seedling groups during this period.

3.3.3 Root dry weight and percent moisture content

The dry weight pattern for the roots investigated in Sections 3.3.1 and 3.3.2 was qualitatively similar to that of the fresh weights. The values are presented in Table 3.3. The mean dry weight values for the longest, intermediate and shortest groups of roots were 26.3, 24.3 and 23.0 mg (10 roots)$^{-1}$ respectively at 24 hours of germination. These values increased with increasing root age. At 96 hours, the values were 96.8, 90.3 and 83.8 mg (10 roots)$^{-1}$ respectively. Like the fresh weights, the dry weights of all three groups of roots increased linearly with time of germination (Figure 3.1C), after the first 36 hours. The rates of increase were 0.794 μg hr^{-1} for longest roots, 0.769 μg hr^{-1} for intermediate and 0.758 μg hr^{-1} for shortest roots. It was also noted that, during early germination, the relative differences in dry weight with respect to root length were smaller than those in the corresponding fresh weights (cf. Tables 3.2 and 3.3). This effect was especially obvious at 24 hours germination. The observation suggests that the longest, intermediate and shortest roots might have different moisture contents. Indeed, when the moisture values were calculated (Table 3.4), it was found that the longest roots at 24 hours contained 78.7% moisture, whereas the shortest root contained 73.5%. The difference in percent moisture content was thus about 5% between the longest and shortest roots. This difference was rapidly reduced as germination proceeded (Figure 3.1D). At 96 hours germination, the difference between the moisture contents of the longest and shortest roots was less than 1%. At the same time, the actual percentage

Table 3.3 Root dry weight at different germination times.

Germin[a]	Longest roots			Intermediate roots			Shortest roots		
period(h)	mean	±sd	±se	mean	±sd	±se	mean	±sd	±se
24	26.3	1.50	0.75	24.3	0.50	0.25	23.0	4.90	2.45
36	52.3	4.57	2.29	46.0	6.68	3.34	40.8	13.20	6.60
48	61.3	4.11	2.06	52.5	3.69	1.85	41.7	3.40	1.70
60	69.8	7.68	3.84	63.0	9.20	4.60	54.8	8.42	4.21
72	82.0	5.03	2.52	76.0	4.55	2.28	68.0	7.16	3.58
84	93.8	6.75	3.38	82.0	10.55	5.28	72.0	14.31	7.16
96	96.8	4.72	2.36	90.3	1.50	0.75	83.8	0.95	0.48

Each value is the mean dry weight [mg(10 roots)$^{-1}$] ±sd and ±se from 4 separate experiments.

Table 3.4 Root percentage moisture content at different germination times.

Germin[a]	Longest roots			Intermediate roots			Shortest roots		
period(h)	mean	±sd	±se	mean	±sd	±se	mean	±sd	±se
24	78.7	0.10	0.05	75.9	0.33	0.17	73.5	0.06	0.03
36	91.5	0.14	0.07	90.5	0.06	0.03	87.4	0.05	0.03
48	93.0	0.05	0.03	92.0	0.10	0.05	91.4	0.06	0.03
60	93.8	0.05	0.03	93.6	0.06	0.03	93.2	0.05	0.03
72	93.6	0.06	0.03	93.4	0.06	0.03	93.0	0.05	0.03
84	94.4	0.05	0.03	94.1	0.06	0.03	94.0	0.06	0.03
96	94.7	0.08	0.04	94.5	0.14	0.07	94.2	0.05	0.03

Each value is the mean percentage moisture content ±sd and ±se from 4 separate experiments.

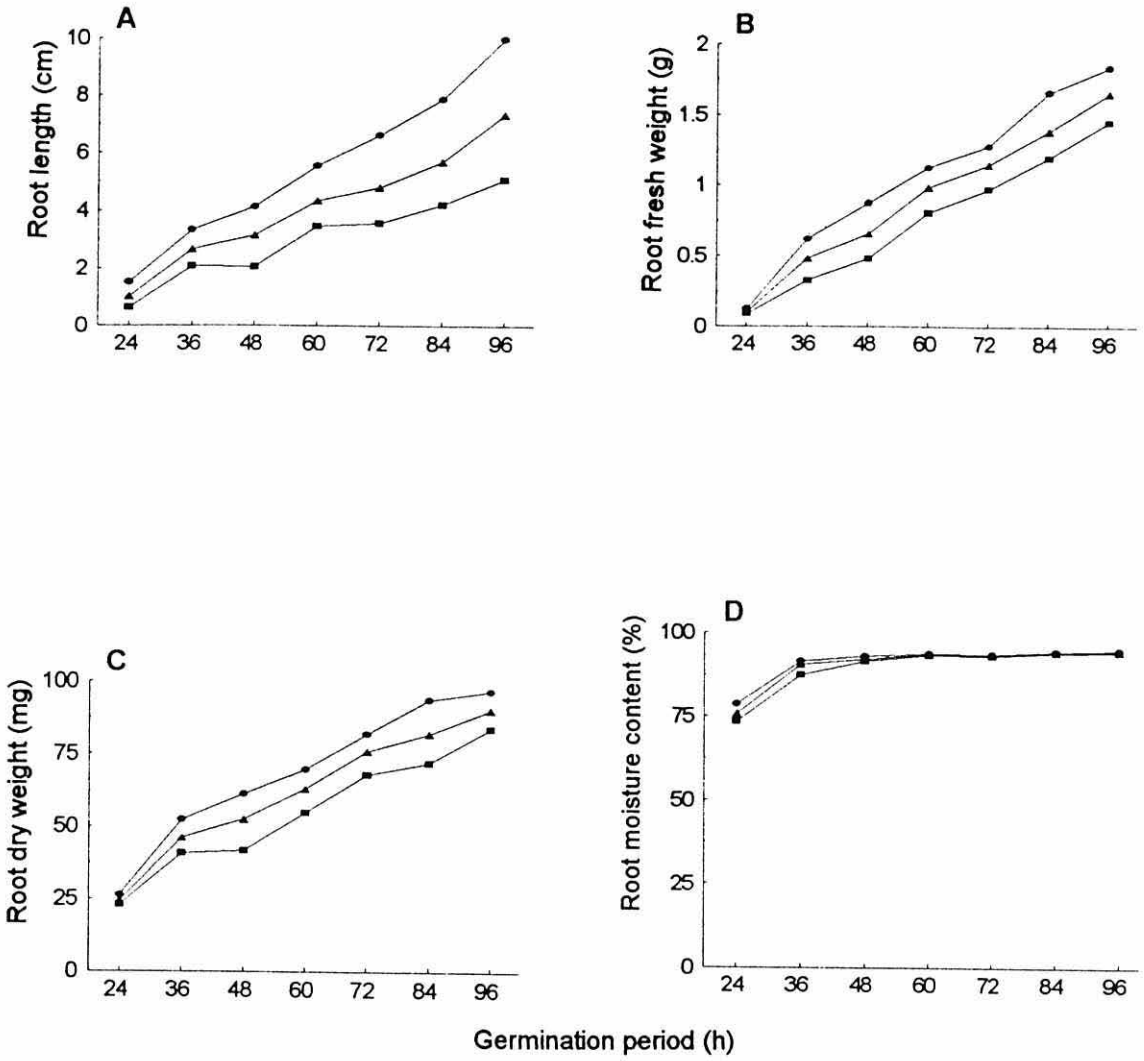


Fig. 3.1. General root parameters at different germination times. A, length; B, fresh weight; C, dry weight; D, moisture content; ●, longest roots; ▲, intermediate roots; ■, shortest roots.

moisture contents increased (94.2 to 94.7% at 96 hours compared with 73.5 to 78.7% at 24 hours).

Statistical analysis of the dry weight data, using ANOVA in which the effects of seedling group and germination period group were investigated, showed that the parameter was highly dependent upon both seedling group and germination time ($P < 0.001$ in each case). The absence of an interaction between seedling group and germination time during the 36-96 hours period ($P = 0.68$) confirm that there was no significant difference in the rate of dry weight gain during this period.

3.3.4 Root diameter

The data on root diameter from an experiment in which roots were selected into long, medium and short subsets (Method 3B; see Section 3.2.1) are presented in Table 3.5. The results are also presented as histograms in Figure 3.2 and again in Figure 3.3. (The two figures contain the same data presented in different formats to emphasise different features). It can be seen in Figure 3.2 that root diameter always increased with increasing distance from the tip. Statistical analysis using ANOVA, in which the effects of observation point (distance from tip), seedling group and germination period were investigated (using data from the 1.0, 5.0 and 9.0 mm observation points to omit missing values), showed that the observation point had the greatest influence on root diameter ($P < 0.001$). It was also noted that root diameter increased suddenly between the next to the last and the last measuring point, especially at later germination times. In long roots at 48 hours, long and medium roots at 60 hours and in all roots at 72 and 84 hours, this increase caused almost a doubling of the diameter. It was noted that the most prominent thickening was associated with the hypocotyl rather than with the root

Table 3.5 Root diameter at different germination times.

Germin[a] period (h)	Root length		Observation point (mm from tip) 1.0	5.0	9.0	19.0	39.0	59.0
36	L	mean	0.59	0.84	1.03	1.70	-	-
		±sd	0.04	0.07	0.07	0.17	-	-
	M	mean	0.61	0.96	1.19	2.20	-	-
		±sd	0.04	0.05	0.08	0.12	-	-
	S	mean	0.73	1.16	1.49	-	-	-
		±sd	0.06	0.09	0.14	-	-	-
48	L	mean	0.55	0.84	0.98	1.30	2.50	-
		±sd	0.05	0.08	0.11	0.12	0.15	-
	M	mean	0.57	0.87	1.05	1.72	-	-
		±sd	0.04	0.06	0.07	0.22	-	-
	S	mean	0.65	1.00	1.29	2.20	-	-
		±sd	0.06	0.08	0.10	0.18	-	-
60	L	mean	0.54	0.75	0.83	1.10	2.20	-
		±sd	0.08	0.08	0.09	0.13	0.11	-
	M	mean	0.62	0.86	0.95	1.10	2.30	-
		±sd	0.08	0.07	0.09	0.09	0.09	-
	S	mean	0.61	0.87	1.00	1.40	-	-
		±sd	0.04	0.08	0.11	0.13	-	-
72	L	mean	0.51	0.76	0.85	1.00	1.60	2.50
		±sd	0.04	0.05	0.09	0.06	0.09	0.11
	M	mean	0.56	0.78	0.88	1.00	1.70	-
		±sd	0.05	0.07	0.09	0.08	0.22	-
	S	mean	0.55	0.82	0.93	1.10	2.20	-
		±sd	0.05	0.07	0.09	0.09	0.08	-
84	L	mean	0.53	0.73	0.77	0.90	1.10	2.20
		±sd	0.03	0.05	0.05	0.01	0.01	0.08
	M	mean	0.51	0.74	0.79	1.00	1.40	2.40
		±sd	0.05	0.08	0.05	0.07	0.24	0.22
	S	mean	0.56	0.78	0.86	1.00	2.10	-
		±sd	0.04	0.04	0.03	0.05	0.29	-

Each values is the mean root diameter (mm) ±sd from 6 roots. L, long roots; M, medium roots; S, short roots.

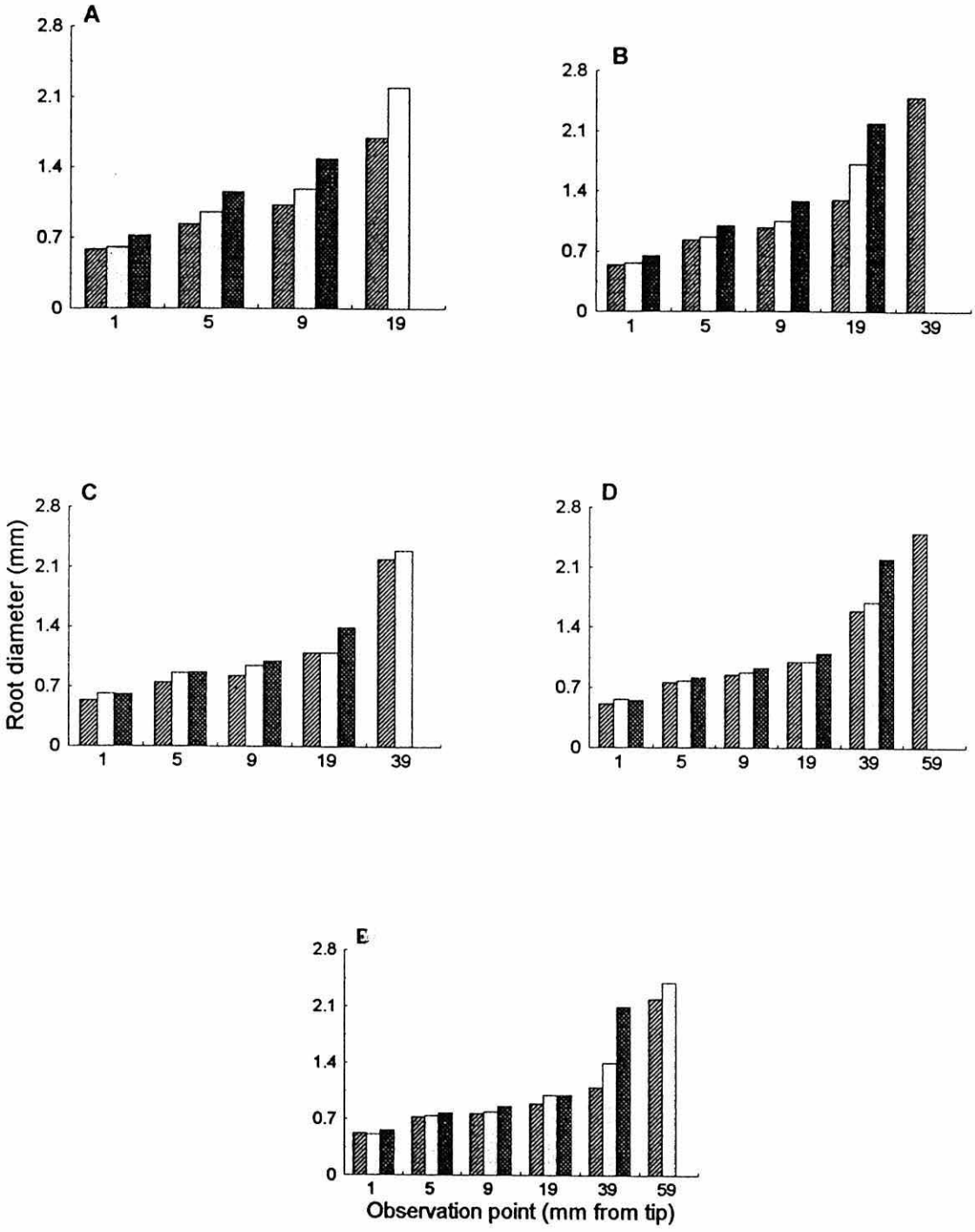


Fig. 3.2. Root diameter at different germination times. A, 36 hours; B, 48 hours; C, 60 hours; D, 72 hours; E, 84 hours; ▨, long roots; ▩, medium roots; ■, short roots.

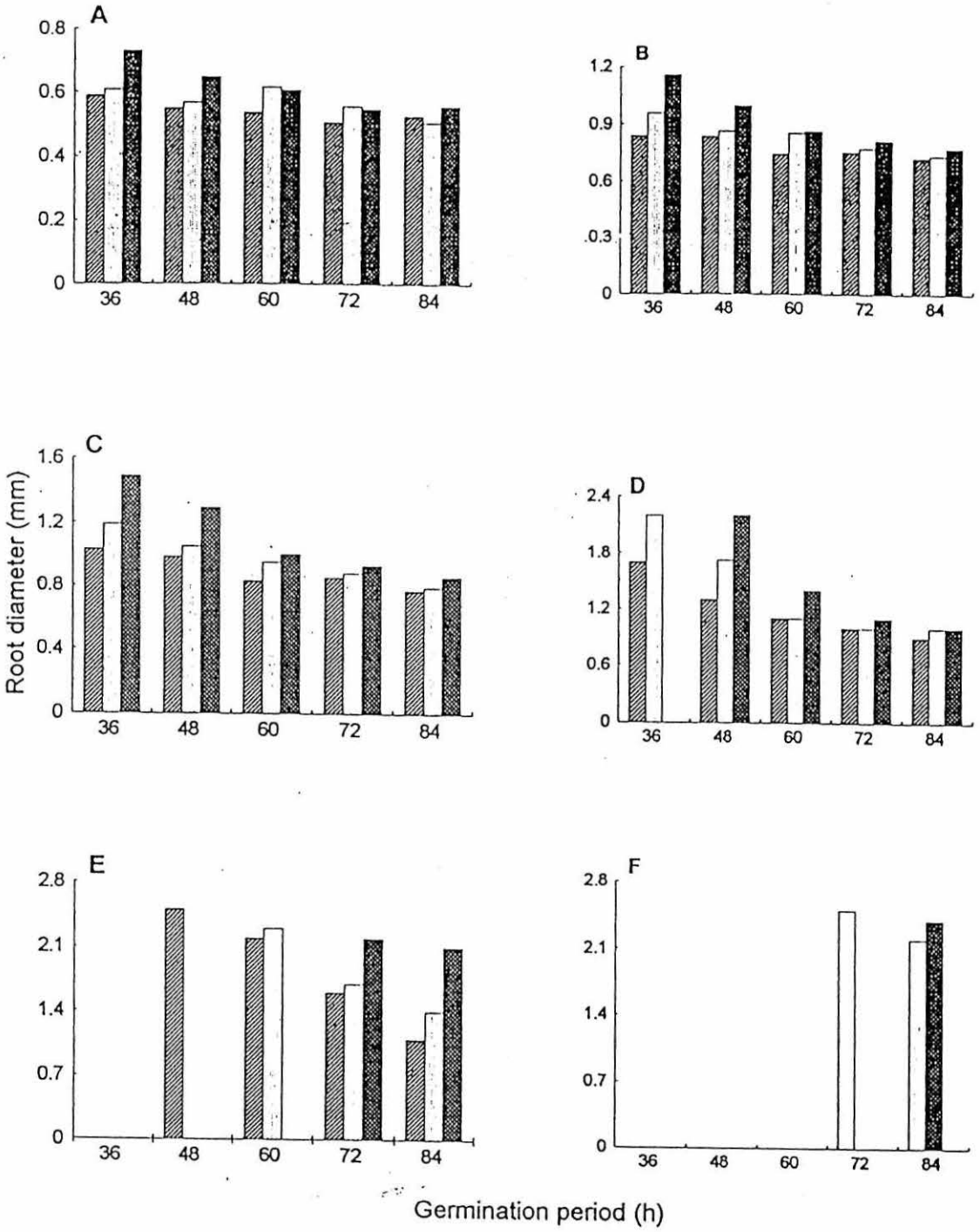


Fig. 3.3. Root diameter at different germination times. A, 1 mm from tip; B, 5 mm; C, 9 mm; D, 19 mm; E, 39 mm; F, 59 mm; ▨, long roots; ▩, medium roots; ▩, short roots.

proper.

Root diameter was less dependent upon seedling group (long, medium or short roots) than on observation point, although short roots generally had greater diameters than long roots especially during early germination. This dependency upon root length was nevertheless significant ($P < 0.001$). Root length had a greater influence over diameter in the basal region of the root than in the tip region. For example, at 48 hours and at 19 mm from the root tip, the long, medium and short roots had diameters of 1.1, 1.4 and 2.1 mm.

Root diameter in the tip region (0-5 mm from the tip) did not change much over the 36-84 hours germination period although a small decrease can be seen (see Figure 3.3A,B). This influence of germination time was statistically significant however ($P < 0.001$). Further back from the tip, root diameter was more strongly affected (reduced) by germination time. This can be seen most clearly at 19 mm from the tip and beyond (Figure 3.3D,E).

The results of a further experiment in which roots were selected as described in Method 3C (see Section 3.2.1) was used to determine root diameters in a wider range of root lengths at 96 hours germination, are presented in Table 3.6 and Figure 3.4. They confirm that root diameter increased with increasing distance from the tip to the base of the root in all cases ($P < 0.001$ using ANOVA). It was also confirmed that there was a relatively small, though significant ($P < 0.001$) negative interaction between root length and root diameter in the tip region (1.0-9.0 mm). Also, it was confirmed that the diameter of the root suddenly increased (doubled) between the next to the last and last

Table 3.6 Root diameter at 96 hours germination.

Root length (cm)		Observation point (mm from tip)						
		1.0	5.0	9.0	19.0	39.0	59.0	79.0
4	mean	0.53	0.79	0.92	1.20	2.20	-	-
	±sd	0.05	0.09	0.07	0.17	0.09	-	-
5	mean	0.57	0.80	0.90	1.00	2.40	-	-
	±sd	0.09	0.06	0.08	0.06	0.22	-	-
6	mean	0.49	0.70	0.78	0.90	1.50	2.20	-
	±sd	0.07	0.07	0.09	0.07	0.20	0.30	-
7	mean	0.50	0.74	0.79	0.90	1.40	2.30	-
	±sd	0.07	0.05	0.06	0.08	0.04	0.21	-
8	mean	0.45	0.67	0.71	0.80	1.00	2.10	2.40
	±sd	0.06	0.06	0.08	0.07	0.05	0.17	0.32
9	mean	0.46	0.71	0.75	0.80	1.00	1.40	2.50
	±sd	0.05	0.07	0.07	0.11	0.09	0.17	0.07

Each value is the mean root diameter (mm) ±sd from 6 roots.

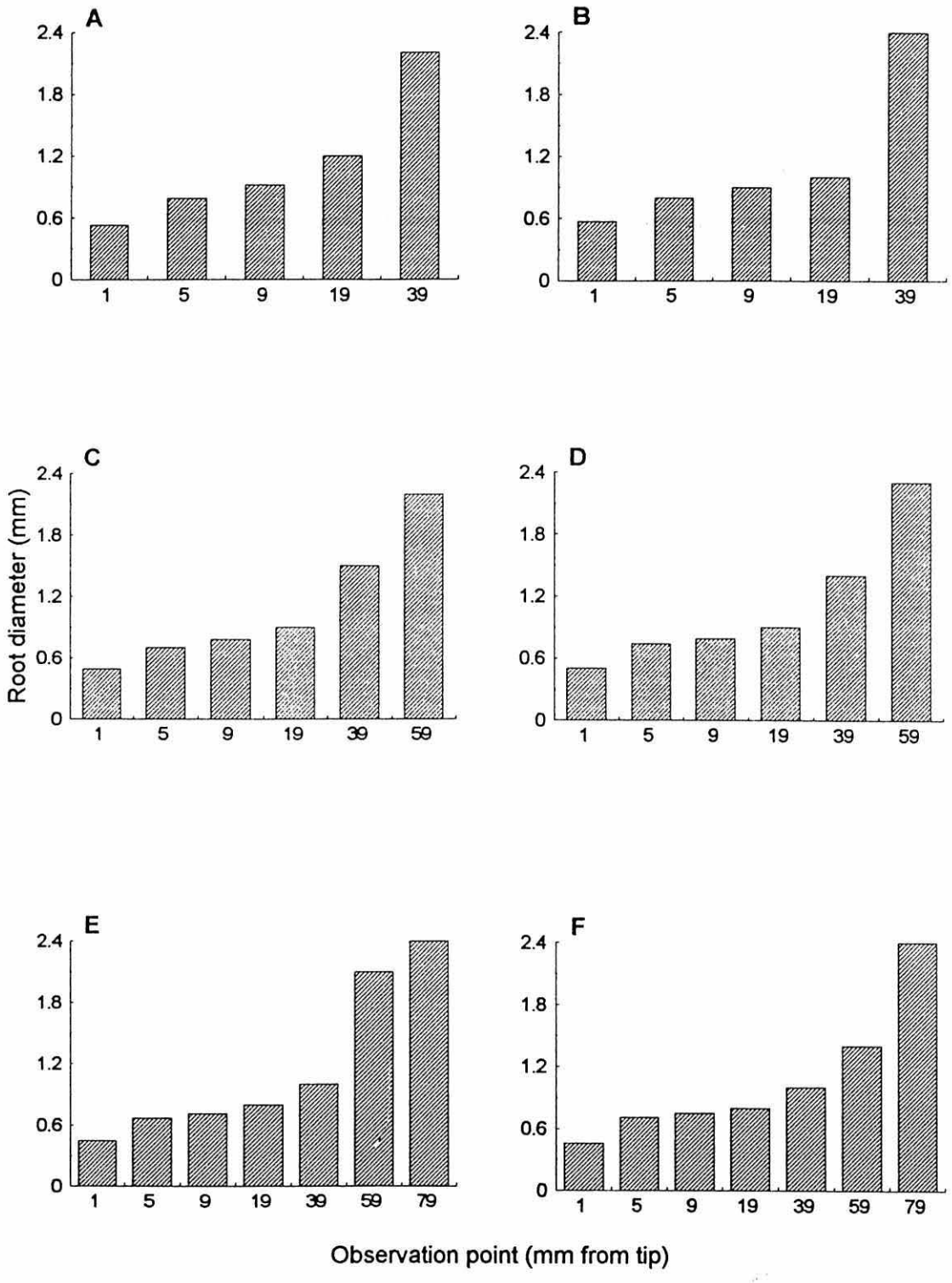


Fig. 3.4. Root diameter at 96 hours germination. A, 4 cm roots; B, 5 cm; C, 6 cm; D, 7 cm; E, 8 cm; F, 9 cm.

measuring points in roots of 4-7 cm in length. The same sudden increase was seen in the last two segments of 8 and 9 cm long roots.

3.3.5 Root segment fresh weight

In this experiment, in which Method 3B was used to select long, medium and short roots, 1 cm segments of the roots were studied. The results are presented in Table 3.7. It shows that the pattern is same as for root diameter. Thus, the segment fresh weight increased progressively ($P < 0.001$ using ANOVA) with increasing segment number from the tip to the base of the root (Figure 3.5). For example, at 36 hours and in long roots, the fresh weight values for the 1st, 2nd and 3rd segments were 6.2, 15.6 and 30.1 mg $(segment)^{-1}$, respectively. It was also found that segment fresh weight decreased with increase in root length ($P < 0.001$), i.e. the fresh weights of the segments of long roots were less than the corresponding segments from short roots. These differences were particularly large during early germination and they became less marked as germination proceeded. Finally, it was noted that the segment fresh weight decreased ($P < 0.001$) with increasing germination time (Figure 3.6).

Segment fresh weight was examined in greater detail in a further experiment in which root from 96 hour seedlings were selected according to Method 3C (Section 3.2.1). The results (see Table 3.8 and Figure 3.7) show more clearly that the segment fresh weights of short roots were much greater than those of long roots ($P = 0.007$). It also confirmed that the segment fresh weight increased with increasing distance from tip to the base of the root ($P = 0.004$). There was one exception to this pattern in the 6-8 cm long roots. Here the fresh weights of the last segment was actually less than that of the next-to-last segment.

Table 3.7 Root segment fresh weight at different germination times.

Germin[a] period (h)	Root length		Segment number (from tip) 1	2	3	4	5	6	7
36	L	mean	6.2	15.6	30.1	-	-	-	-
		±sd	0.2	0.8	0.5	-	-	-	-
	M	mean	8.6	23.9	-	-	-	-	-
		±sd	0.3	1.1	-	-	-	-	-
	S	mean	12.4	-	-	-	-	-	-
		±sd	0.7	-	-	-	-	-	-
48	L	mean	5.6	9.5	18.6	37.5	-	-	-
		±sd	0.5	0.7	0.9	3.1	-	-	-
	M	mean	6.6	14.0	32.1	-	-	-	-
		±sd	0.4	1.0	1.5	-	-	-	-
	S	mean	9.3	26.6	-	-	-	-	-
		±sd	0.5	2.1	-	-	-	-	-
60	L	mean	4.8	6.9	11.3	24.9	37.9	-	-
		±sd	0.3	0.5	1.1	1.4	2.7	-	-
	M	mean	5.4	9.6	19.9	41.5	-	-	-
		±sd	0.6	1.0	1.8	1.1	-	-	-
	S	mean	6.1	11.1	30.9	-	-	-	-
		±sd	0.8	1.1	3.9	-	-	-	-
72	L	mean	4.5	5.9	7.7	13.5	35.3	44.7	-
		±sd	0.3	0.6	0.7	1.3	3.7	1.7	-
	M	mean	4.8	6.6	11.1	30.4	44.2	-	-
		±sd	0.4	0.7	0.7	4.7	3.8	-	-
	S	mean	5.3	9.2	20.3	44.6	-	-	-
		±sd	0.5	0.9	3.3	1.4	-	-	-
84	L	mean	4.3	5.6	6.6	10.1	23.1	46.6	38.4
		±sd	0.6	0.7	0.4	0.7	2.5	4.4	2.9
	M	mean	3.8	5.6	7.3	13.3	33.7	43.6	-
		±sd	0.3	0.3	0.6	1.1	4.2	3.7	-
	S	mean	3.9	6.3	10.9	26.7	42.8	-	-
		±sd	0.4	0.7	1.4	1.6	4.7	-	-

Each value is the mean fresh weight [mg(segment)$^{-1}$] ±sd from 6 roots. L, long roots; M, medium roots; S, short roots.

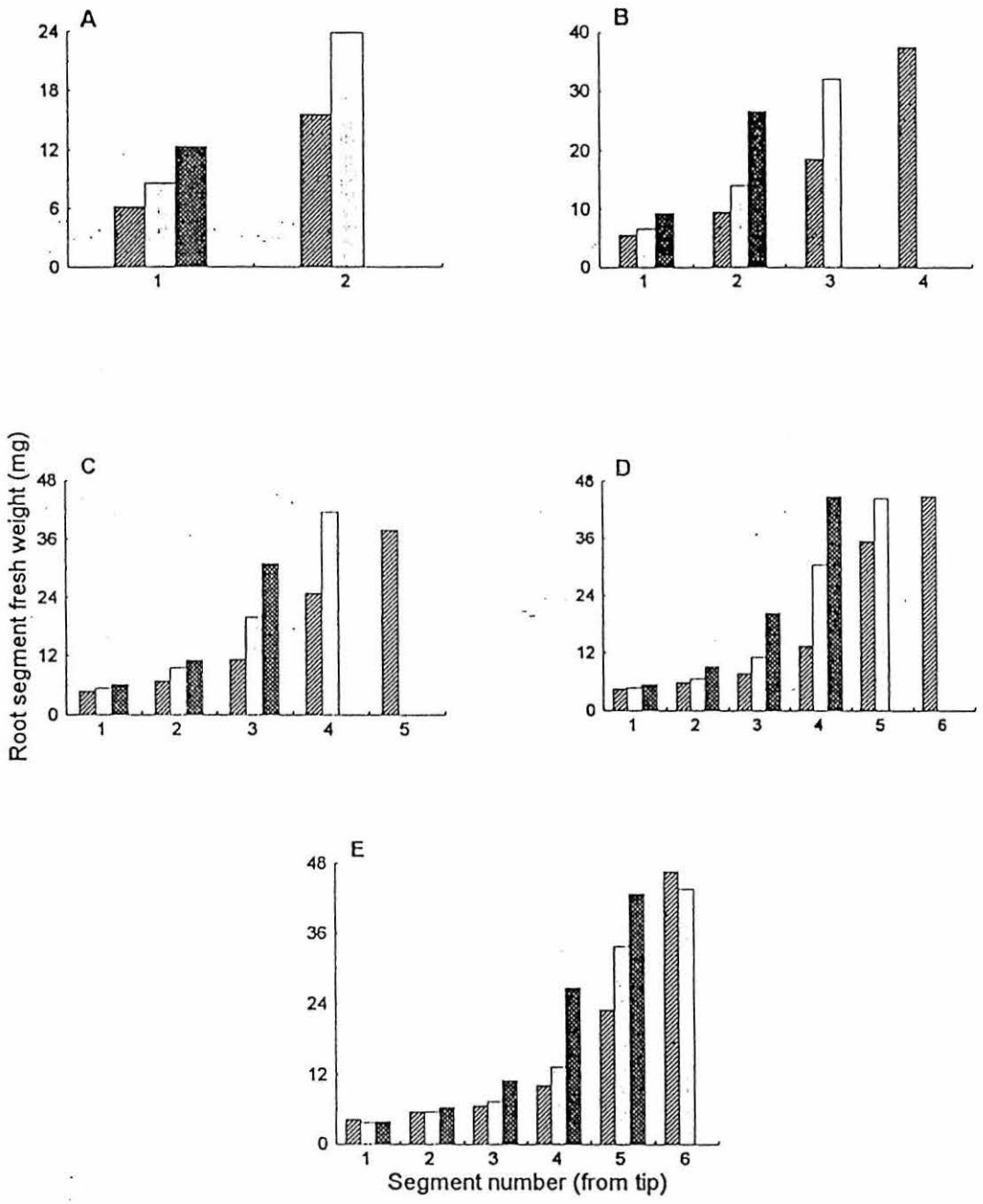


Fig. 3.5. Root segment fresh weight at different germination times. A, 36 hours; B, 48 hours; C, 60 hours; D, 72 hours; E, 84 hours; ▨, long roots; ▩, medium roots; ■, short roots.

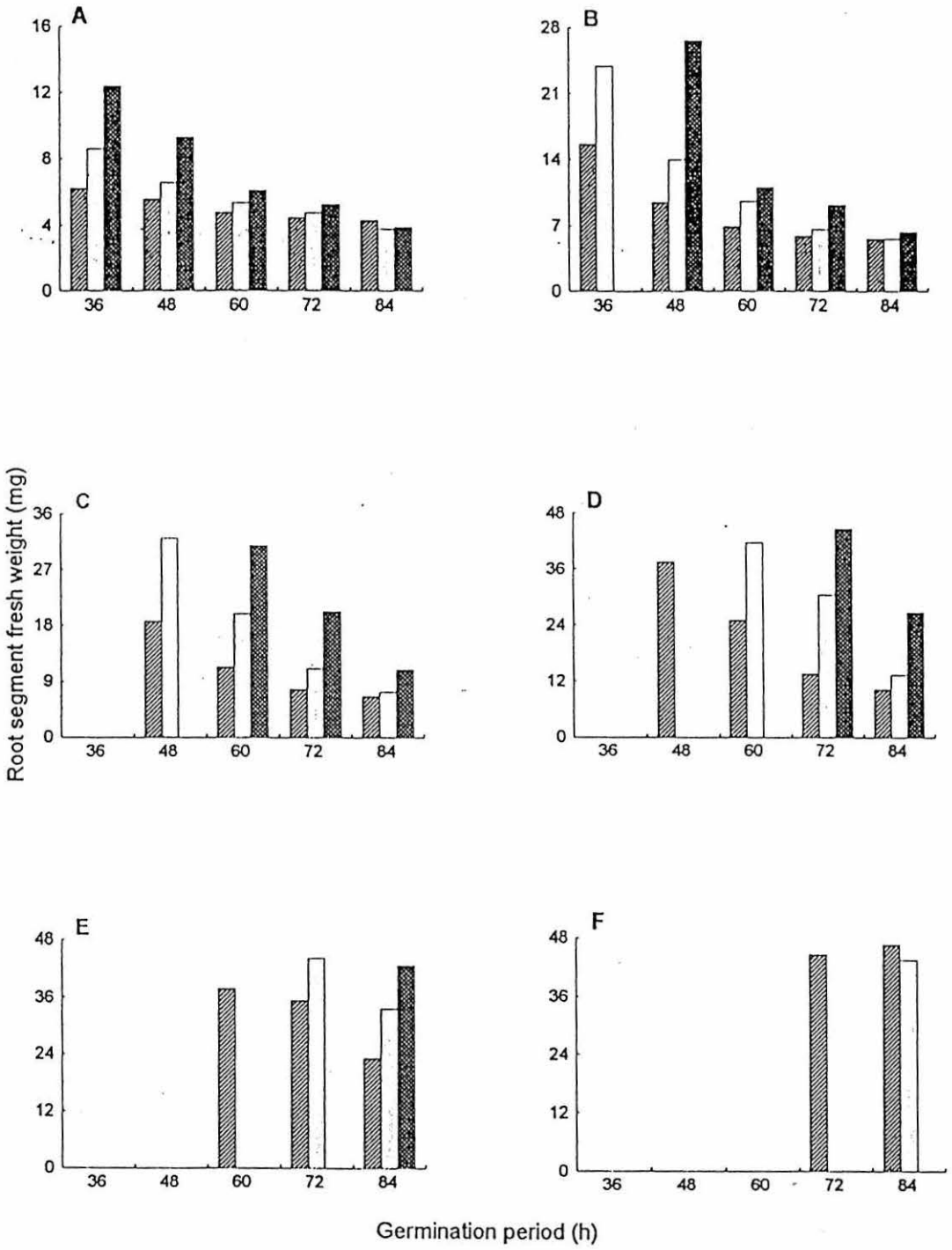


Fig. 3.6. Root segment fresh weight at different germination times. A, segment number 1 from tip; B, segment 2; C, segment 3; D, segment 4; E, segment 5; F, segment 6; ▨, long roots; ▩, medium roots; ▩, short roots.

Table 3.8 Root segment fresh weight at 96 hours germination.

Root length (cm)		Segment number (from tip)								
		1	2	3	4	5	6	7	8	9
4	mean	4.8	8.1	21.4	35.1	-	-	-	-	-
	±sd	0.6	0.9	3.4	2.5	-	-	-	-	-
5	mean	4.9	7.2	15.1	36.1	40.5	-	-	-	-
	±sd	0.6	0.4	2.5	4.0	2.9	-	-	-	-
6	mean	4.4	5.7	9.4	20.9	47.4	38.6	-	-	-
	±sd	0.5	0.6	0.6	2.0	3.3	2.5	-	-	-
7	mean	3.8	5.1	5.6	9.3	25.4	41.9	34.9	-	-
	±sd	0.5	0.7	0.4	0.4	2.4	3.2	2.9	-	-
8	mean	2.3	2.6	3.1	3.9	6.0	11.6	25.9	24.8	-
	±sd	0.2	0.2	0.1	0.3	0.1	0.4	0.4	0.6	-
9	mean	2.4	3.8	3.9	4.3	4.7	5.6	13.6	29.9	33.7
	±sd	0.2	0.2	0.2	0.1	0.4	0.8	1.2	0.8	1.1

Each value is the mean fresh weight [mg(segment)$^{-1}$] ±sd from 6 roots.

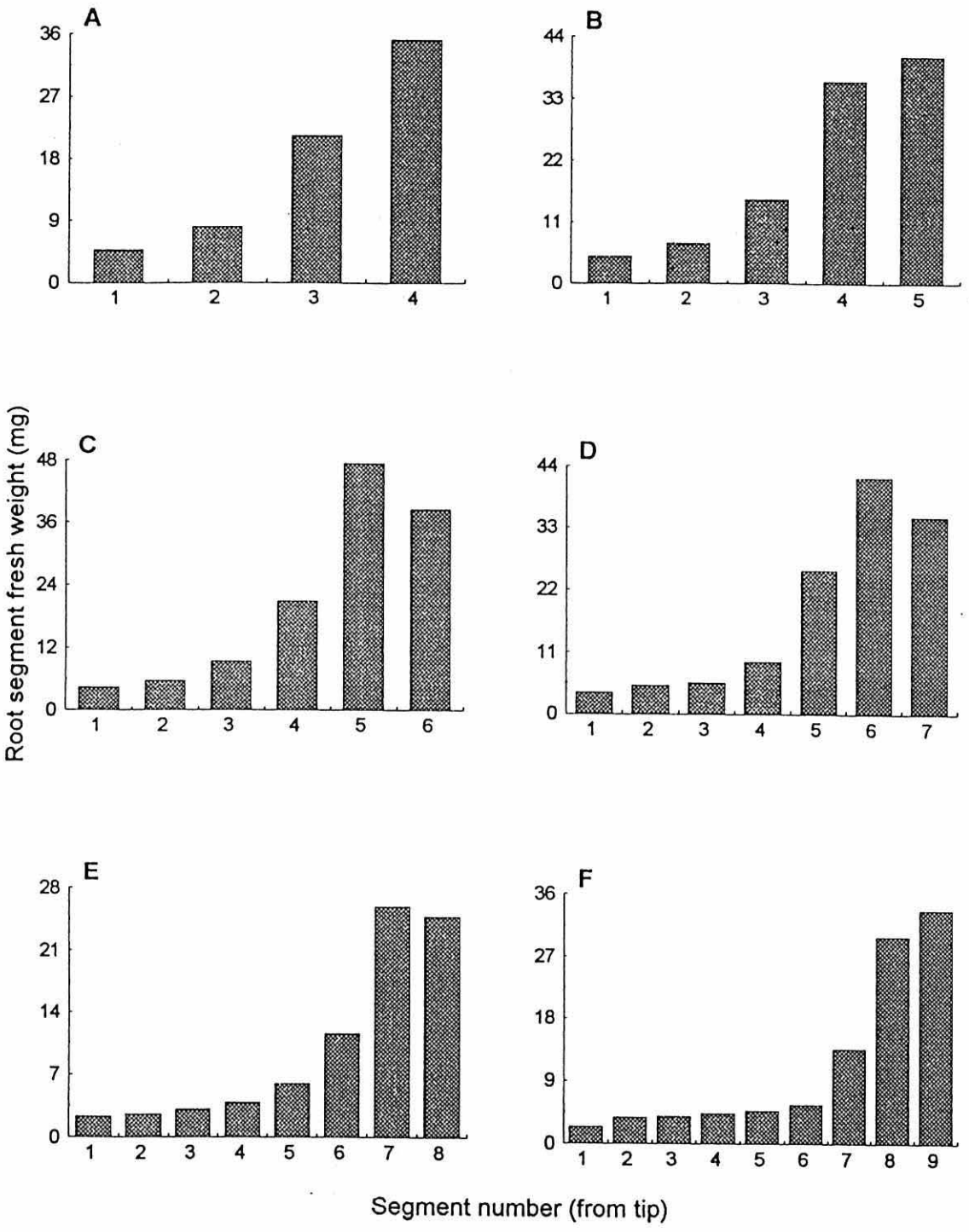


Fig. 3.7. Root segment fresh weight at 96 hours germination. A, 4 cm roots; B, 5 cm; C, 6 cm; D, 7 cm; E, 8 cm; F, 9 cm.

3.3.6 Identification of the elongation region

It can be seen in the photographs of marked roots (Plates 3.1 and 3.2) that root elongation occurred exclusively just behind the tip of the root at all times of germination. As expected, the rate of elongation was greater in the long than in the short roots at all germination periods. It was additionally observed that, after 48 hours, elongation also took place in the hypocotyl region. Again, there was greater elongation in the long roots and less in the short roots.

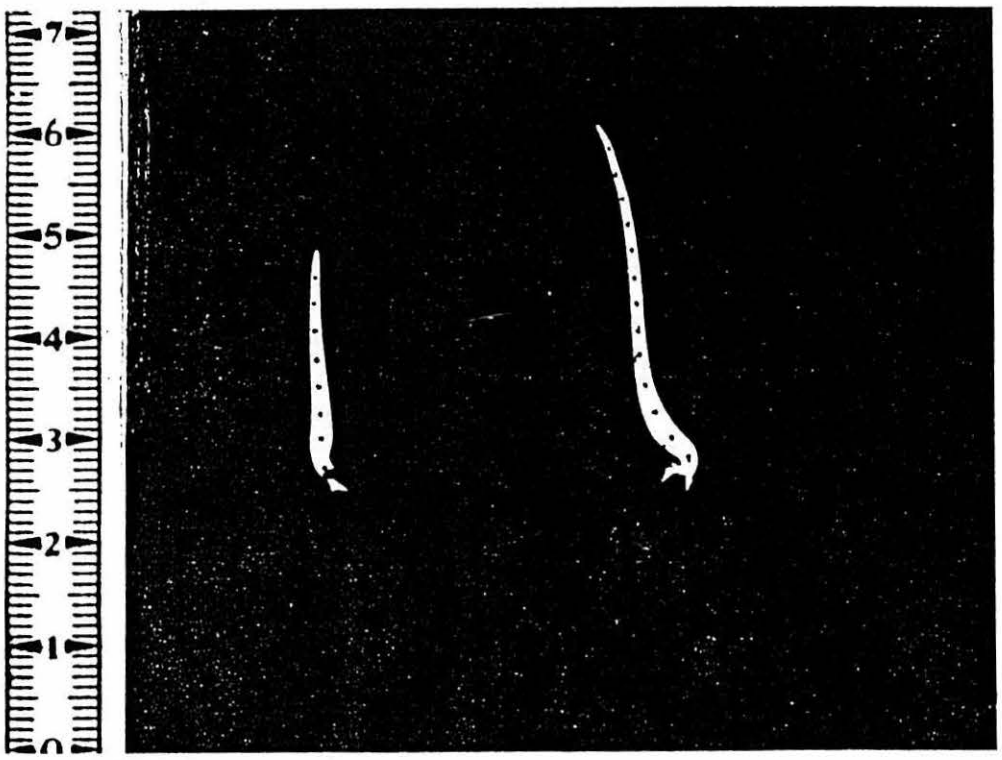
3.3.7 Lateral root initiation

To give enough time for lateral root growth, cotton seedlings were grown for 10 days in nutrient solution. By the end on this period, the seedlings had initiated their lateral roots and it can be seen in Plate 3.3 that the laterals were produced away from the root tip. The main root length at 10 day was about 15 cm and the laterals were evident over a range from 5 cm from the tip to the root - hypocotyl junction.

3.4 Summary and Discussion

It is clear from the results that the length of the cotton root increases with germination time, as expected. Root fresh weight and dry weight also increase with the age of the root. Within these general patterns there are some differences however. In particular, the difference between the relative fresh weights of long and short roots is greater than the difference between the corresponding dry weights. This is especially true at the early germination times, though it becomes progressively less pronounced as germination proceeds. In other words, in roots of the same age, the amount of non-aqueous material (organic matter and salts) differs only moderately according to root length. On the other hand, long roots contain more water than the short roots, at least during early

A



B

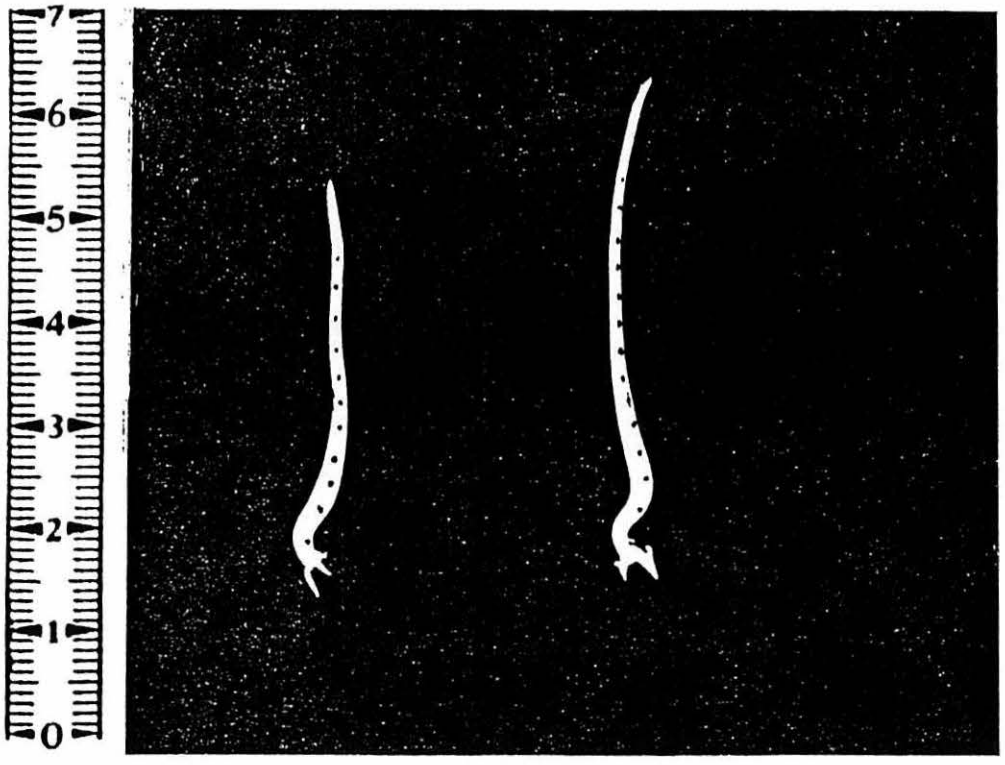
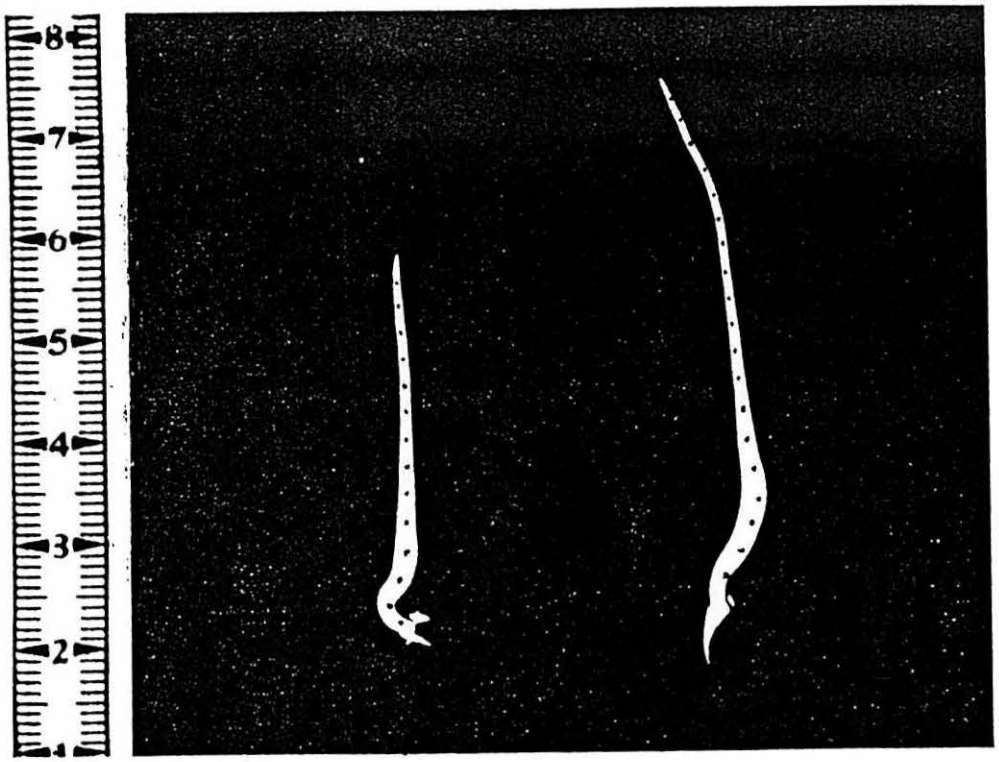


Plate 3.1 Identification of the root elongation region.
A, Roots marked at 48 hour germination (control);
B, Roots marked at 48 hour germination and reincubated for a further 12 hours.
Elongation is restricted to the tip region and the hypocotyl.

A



B

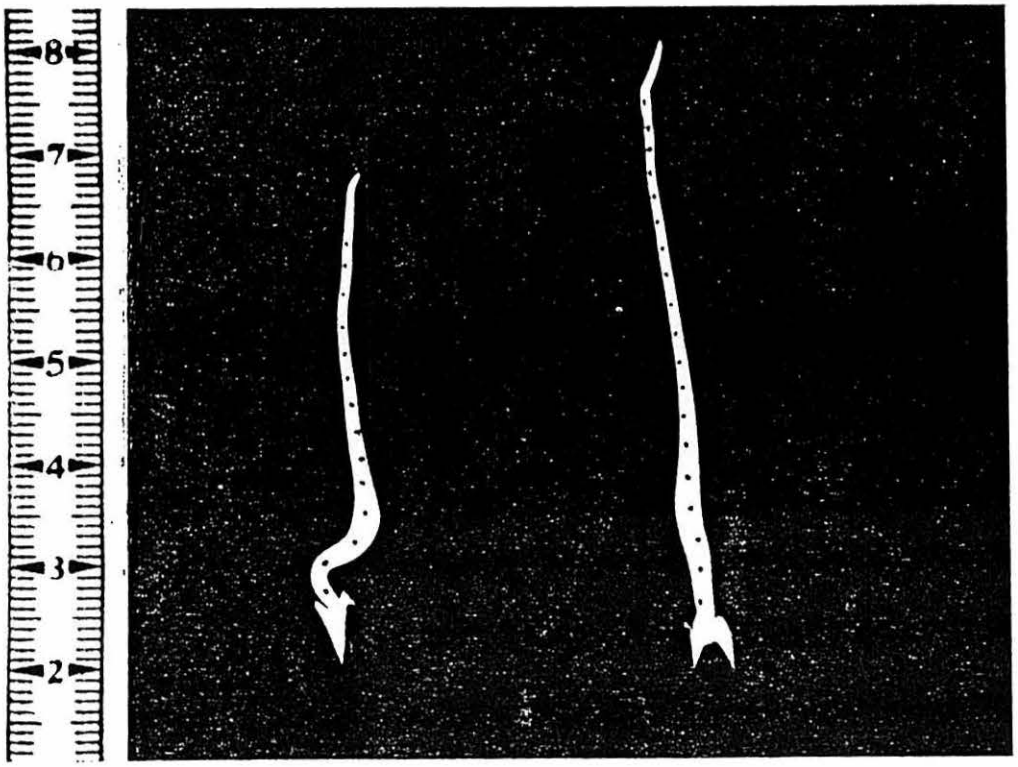


Plate 3.2 Identification of the root elongation region.
A, Roots marked at 72 hour germination (control);
B, Roots marked at 72 hour germination and reincubated for a further 12 hours.
Elongation is restricted to the tip region and the hypocotyl.

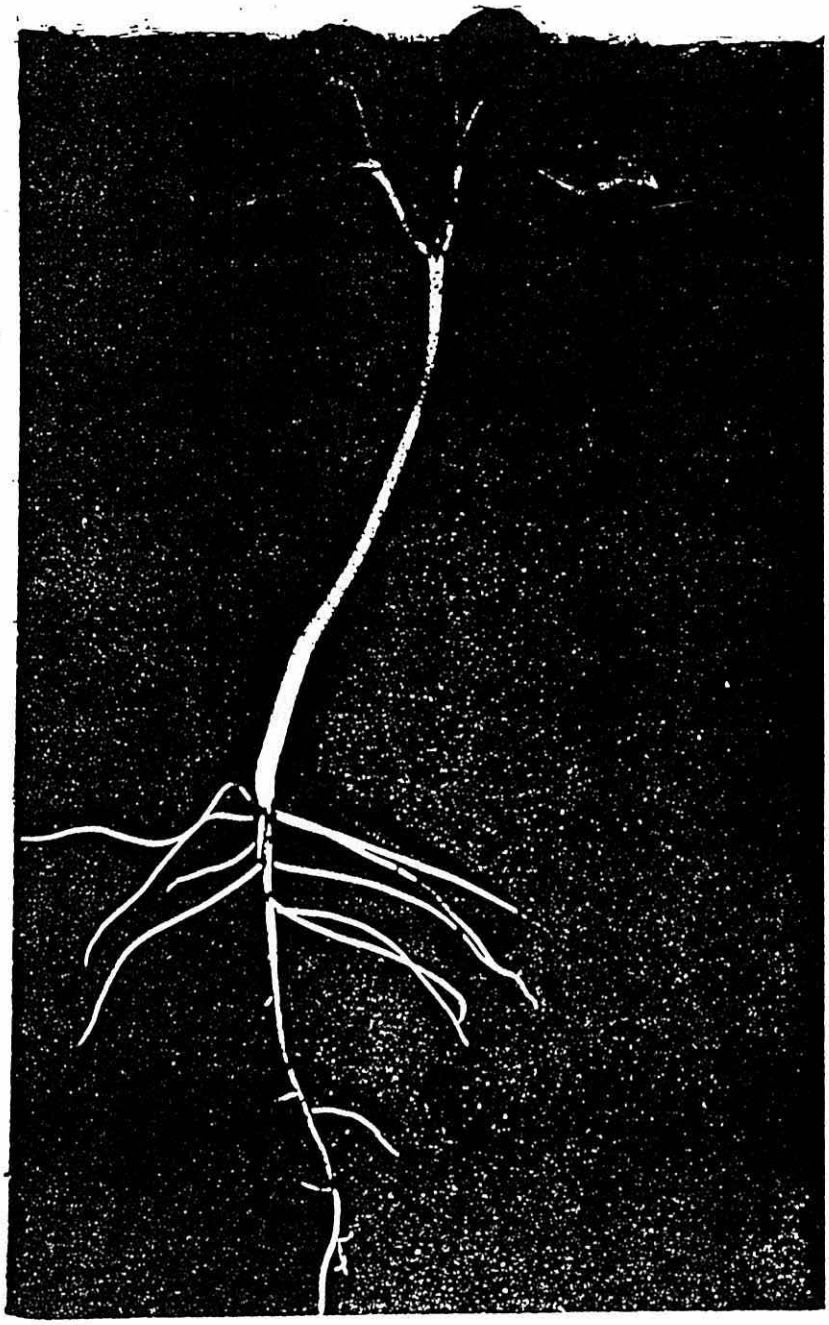


Plate 3.3 Lateral root initiation in a seedling grown for 10 days.

germination. This finding is confirmed by the data for percentage moisture content, which show clear differences during early germination.

The large differences seen between the fresh weights of long and short roots during early germination is presumably a reflection of greater cell elongation in the long than in the short roots (see Chapter 4). These longer cells might be expected to contain more cytoplasm and especially larger vacuoles than the shorter cells, while the amounts of cell wall material and cellular organic material are presumably more similar. The water content of the larger cells will therefore be greater.

The differences between long and short roots is not confined to cell elongation. The differences in cell diameter shown in Chapter 4 is reflected in the observation that short roots are consistently fatter than long roots, particularly beyond the proximal region of the tip. The same kind of difference can also be seen throughout the length of the root during early germination, especially at 36 hours. Clearly, longer roots are also thinner roots. The two effects do not completely compensate each other, however, so that differences in fresh weight are still evident, especially at 36 hours. Because the fresh weight differences are a reflection of differences in both root length and root diameter, it is worth calculating some root volumes. These values should reflect the fresh weight values. This calculation has been made for the tip segment of 36 hour seedlings using the data in Table 3.5. The volume of the tip segment of the short root is about 9.9×10^{-3} cm^3, while the volume of the tip segment from the long root is about $5.3 \times 10^{-3} cm^3$. The volume of the segment from the long root is thus about half that of the short root. As expected, these volumes are a good reflection of the differences between the corresponding fresh weights which are 12.4 mg and 6.2 mg for the short and long roots

respectively (see Table 3.7). Similar calculations can be made for other segments of the roots. The outcome of these complex interactions between length, diameter and moisture content is that rate of root elongation differs between the seedling groups, although their rates of dry weight increase are the same.

The above observations account for the finding (see Figure 3.1) that, whereas the rate of increase in root length in the root groups differs substantially over the 24-96 hour germination period, this is much less true of fresh weight and dry weight. In the latter, the differences are largely confined to the 0-36 hour germination period.

A further conclusion from the present study is that root elongation occurs in the tip region. This is confirmed in later experiments by measuring cell lengths (see Chapter 4), which show that cell elongation takes place between 2.5 to 6.0 mm from the tip. This finding is supported by the earlier work of Erickson and Sax (1956) and Green (1976) on maize, who reported that further away from the root cap the frequency of cell division drops away and cells increase in length. In the present study, a second area of elongation was observed in the hypocotyls after 48 hour germination. This elongation facilitates in the emergence of the cotyledons above the ground during later germination.

Finally, it has been demonstrated that lateral root initiation occurs away from the root tip over a fairly extended range up to the root - hypocotyl junction.

CHAPTER FOUR

Chapter Four

On the Length, Diameter and Volume of Root Cells

4.1 Introduction

This Chapter is concerned with the measurement of root cell dimensions. The study was undertaken in order to analyse the results of experiments on the general parameters of root growth described in Chapter 3. From those experiments, it was noted that the lengths of cotton seedling roots varied widely at all times of germination. These differences in root lengths could be due either to differences in number of cells or differences in cell sizes or a combination of the two. The present study is intended to find out the exact situation with regard to these possibilities.

The apical part (tip) of the root is most important for the root's growth and development. It comprises a root cap, meristem, elongation zone and root hair zone (see Figure 1.3). The root cap is the mass of cells on the surface of root tip, which protects the root as it grows through the soil. Just proximal to the root cap is the root meristem, where cell division takes place. In the proximal part of the meristem, cells are produced which develop into the different cell types of the root and give rise to the epidermis, cortex, endodermis, pericycle and stele or vascular cylinder (xylem and phloem). This differentiation of the different cell types is accompanied by rapid cell elongation. The whole complex process takes place in the proximal meristem and in the elongation zone just behind the meristem.

In the elongation zone and for some distance beyond it, the cortex constitutes the bulk of the root. It lies between the exodermis and the endodermis and it is typically a mass

of parenchyma tissue (Van Fleet, 1942; Kroemer, 1903). The most commonly held view is that the cortex is composed of cells of uniform type, the cytoplasm of each being in contact with its nearest neighbours through plasmodesmata (Arisz, 1956). The arrangement and frequency of these plasmodesmata imply that the cortex as a whole is capable of active cell to cell transport of solutes. There are no reports of experimental investigations of symplasmic transport in the root cortex. There is, however, a fair amount of indirect evidence to suggest that the cortical symplasm does function as a primary nutrient accumulatory system (Arisz, 1956). As well as the uniformity of the cortical cells, their arrangement in the cortex is ordered. Thus, as they become elongated, they come to lie in rows or files which are arranged concentrically and run along the length of the root (see Plate 4.1).

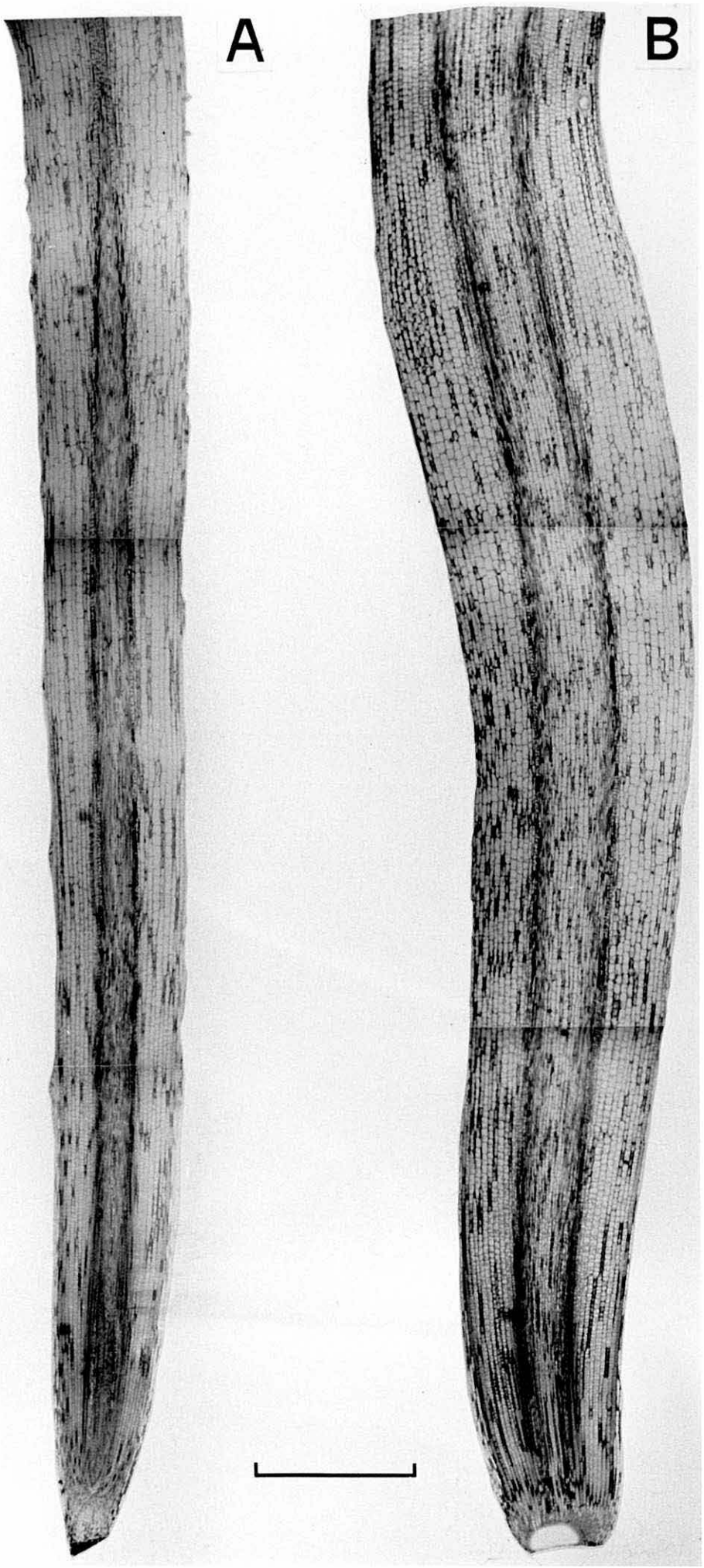
Kurth *et al.* (1986) have suggested that the cortex is the best tissue for the detailed analysis of cellular dimensions in saline-stressed cotton seedling roots, because it consists of a single cell type. In the present study, another reason for studying cortical cells is that they make up the bulk of the root in cotton seedlings and therefore they can be expected to have the greatest influence on root dimensions. Furthermore, there is little recorded information about these parameters for cotton, although detailed studies have been carried out on *Zea mays* L. (Silk *et al.*, 1989; Fraser *et al.*, 1990; Baluska *et al.*, 1990) and *Vicia faba* (MacLeod and McLachlan, 1975). Therefore, it was decided to carry out some histological work on cotton seedling roots.

4.2 Methods

4.2.1 Selection of seedling root groups

Two methods were used for the selection of seedling root groups according to root

Plate 4.1. Longitudinal section of 1 cm tip of cotton seedling roots. A, long root; B, short root. Bar represents 1 mm.


A
B

length (cf. Chapter 3).

Method 4A: Batches of 200 seeds were germinated as described in Section 2.6. After 36, 60 and 84 hours germination, the seedlings were harvested. Ungerminated seeds were discarded and the remainder were arranged according to root length. Two sets of 10 seedlings were then selected, one of which shared a longer root lengths and the other of short root length. The roots were designated 'long' and 'short' roots.

Method 4B: In order to have a wider range of root lengths, a separate experiment was carried out in which 200 seeds were germinated (Section 2.6) for 84 hours. After harvesting, ungerminated seeds were discarded and the remainder were arranged according to root length. Sets of 8 seedlings with roots of 3, 5, 7 and 9 cm were then selected.

4.2.2 Measurements of cell dimensions and number of cell files

The detailed procedures for the measurement of cell dimensions (cell length, diameter, volume) and number of cell files are described in Section 2.12.

In all the experiments, the measurements were carried out on the cortical cells of the root.

4.3 Results

In some preliminary experiments, cell measurements were made at different points through the developmental zones of the cotton root. It was observed from these

experiments that cortical cell elongation takes place mainly in a region between 2.5 and 6.0 mm from the root tip (Plate 4.1). The situation was the same for long and for short roots. It was therefore decided to take more detailed cell measurements (cell length, diameter and volume as well as the number of cell files) in the cortex behind the elongated zone at 6.0, 7.5 and 9.0 mm from the root tip. Measurements at these points should provide accurate data for the dimensions of mature elongated cells, which can be compared with the data for root lengths and diameters reported in Chapter 3.

4.3.1 Cell dimensions at different germination times

The results of an experiment in which seedlings were separated into long and short root groups (Method 4A; see Section 4.2.1) are presented in Table 4.1. They show that the cell lengths in long roots at 36 hours germination were 145.1, 155.2 and 162.5 μm at 6.0, 7.5 and 9.0 mm from the tip respectively. The corresponding lengths for short roots were 121.0, 108.7 and 111.6 μm. At 60 hours germination, the lengths for long roots were 149.9, 160.7 and 160.5 μm respectively at observation points 6.0, 7.5 and 9.0 mm from tip. The corresponding lengths for short roots were 123.7, 131.8 and 133.0 μm. The cell lengths for long roots of 84 hours germination were 154.3, 160.7 and 162.9 μm, again at the same observation points. The lengths for short roots were 116.0, 118.2 and 120.6 μm.

These results, which are presented graphically in Figure 4.1, show that, at 60 hours and 84 hours, cell length increased slightly between 6.0 and 7.5 mm from the tip but thereafter any increase was very small. The picture at 36 hours was less clear cut, but there was again no evidence for any meaningful increase in cell length beyond 7.5 mm from the root tip. It can be seen that there was a considerable difference between the

cell lengths of long and short roots. The seedling groups with long roots had longer cells as compared with the cells of short roots. It was also noted that the age of the root had a small effect on cell length. Statistical analysis of the cell length data, using ANOVA in which the effects of location (distance from tip), seedling group (long and short) and germination time were investigated, showed that the parameter was strongly dependent upon seedling group ($P < 0.001$) and germination time ($P < 0.001$) and the effect of location was also highly significant ($P = 0.003$). The fact that significant interactions were revealed between the effects of root group and germination period ($P = 0.001$) and between root group and location ($P = 0.012$) emphasises the complexity of the changes in cell length.

The results for cell diameter are presented in Table 4.2 and Figure 4.2. These results show that diameter in long roots at 36 hours germination was 42.3, 45.8 and 48.5 μm at 6.0, 7.5 and 9.0 mm respectively from the tip. The corresponding diameters of cells in the short roots were 36.2, 43.1 and 39.7 μm. At 60 hours germination, the cell diameters in long roots were 45.9, 48.0 and 44.9 μm respectively at the same observation points. For short roots, the values were 40.0, 42.6 and 44.9 μm. The cell diameters in long roots at 84 hours were 41.9, 42.6 and 44.4 μm, while the values for short roots were 49.8, 50.4 and 50.5 μm.

The results clearly show that, at early growth stages, i.e. 36 and 60 hours germination, long roots had wider cells as compared with short roots, but at the later growth stage (84 hours) wider cells were seen in the short roots and relatively narrower ones were found in long roots. Further statistical analysis by ANOVA (in which the effects of location (distance from tip), seedling group (long and short) and germination time on

Table 4.1 Cell length at different germination times.

Germin[a] period(h)	Locat[a] (mm)	Long roots mean	±sd	±se	Short roots mean	±sd	±se
36	6.0	145.1	9.12	2.88	121.0	12.67	4.00
	7.5	155.2	6.20	1.96	108.7	6.20	1.96
	9.0	162.5	12.31	3.89	111.6	12.10	3.82
60	6.0	149.9	8.29	2.62	123.7	8.43	2.66
	7.5	160.7	11.71	3.70	131.8	13.09	4.14
	9.0	160.5	9.37	2.96	133.0	11.79	3.73
84	6.0	154.3	6.50	2.06	116.0	9.52	3.01
	7.5	160.7	8.98	2.84	118.2	17.91	5.66
	9.0	162.9	12.25	3.87	120.6	9.28	2.93

Each value is the mean cell length (μm) ±sd and ±se from 10 roots with 40-60 cells measured from 4 cell files in each root. Locat[a] = distance from root tip (mm).

Table 4.2 Cell diameter at different germination times.

Germin[a] period(h)	Locat[a] (mm)	Long roots mean	±sd	±se	Short roots mean	±sd	±se
36	6.0	42.31	3.88	1.22	36.23	6.20	1.96
	7.5	45.76	4.17	1.32	43.13	4.85	1.53
	9.0	48.51	3.46	1.09	39.65	6.92	2.19
60	6.0	45.91	4.93	1.56	40.02	4.52	1.43
	7.5	47.95	2.52	0.79	42.55	3.92	1.24
	9.0	44.90	2.45	0.77	44.91	4.11	1.30
84	6.0	41.85	3.95	1.25	49.75	5.89	1.86
	7.5	42.60	3.59	1.13	50.36	7.82	2.47
	9.0	44.44	2.82	0.89	50.45	7.78	2.46

Each value is the mean cell diameter (μm) ±sd and ±se from 10 roots with 4 cells from 4 cell files measured in each root. Locat[a] = distance from root tip (mm).

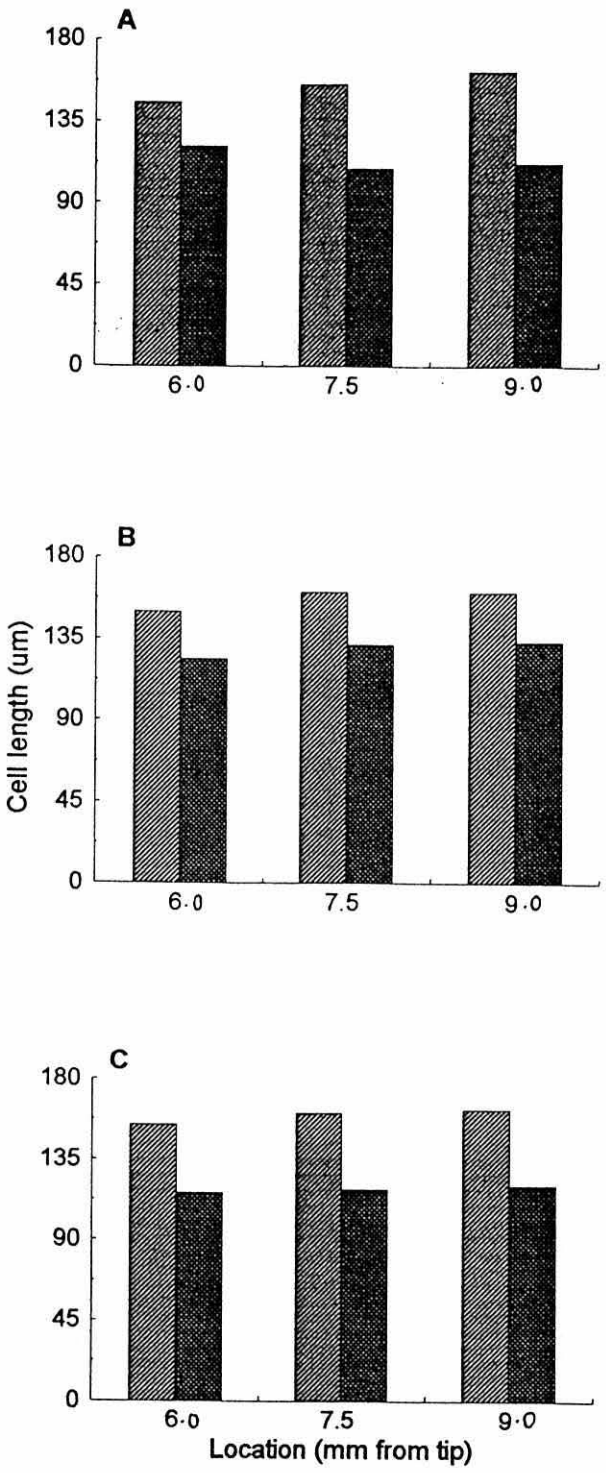


Fig. 4.1. Cell length at different germination times. A, 36 hours; B, 60 hours; C, 84 hours; ▨, long roots; ▩, short roots.

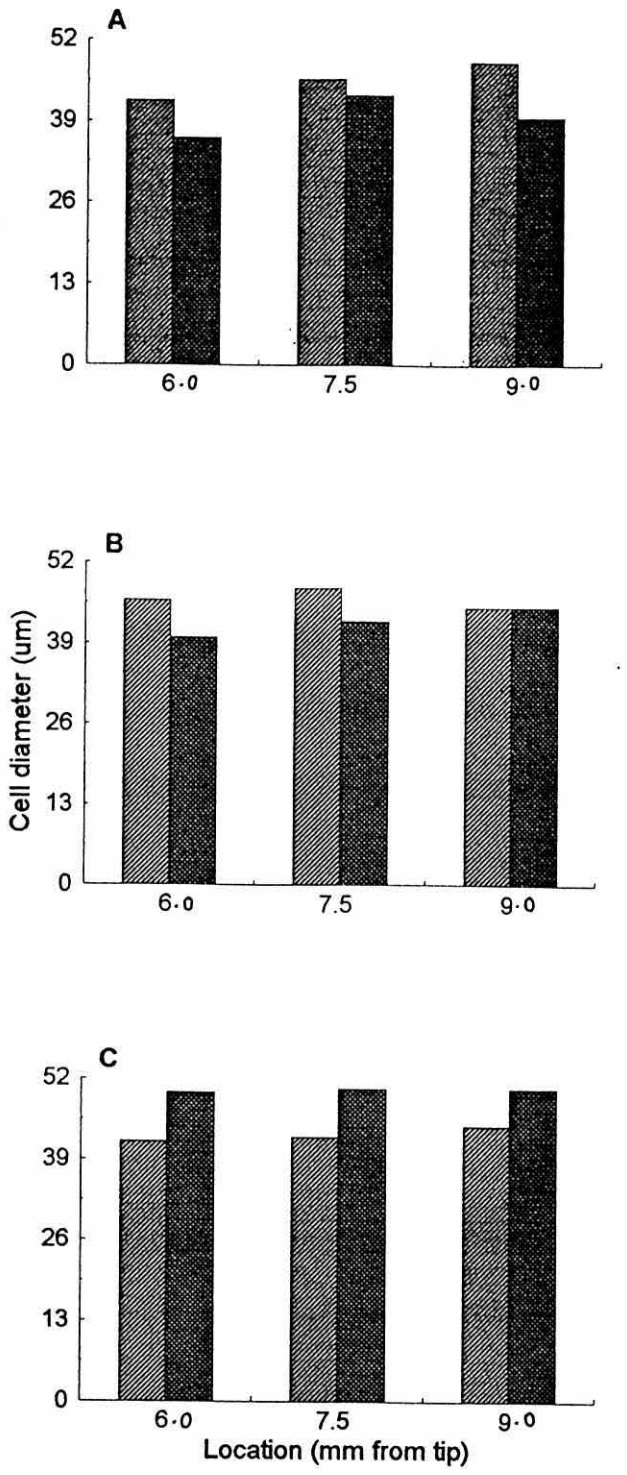


Fig. 4.2. Cell diameter at different germination times. A, 36 hours; B, 60 hours; C, 84 hours; ▨, long roots; ▩, short roots.

cell diameter were investigated) confirmed that cell diameter was strongly dependent upon germination time ($P < 0.001$) and the effect of location was also highly significant ($P = 0.002$). Root group had an insignificant effect ($P = 0.28$) on the parameter. However, the highly significant interaction between root group and germination time ($P < 0.001$) confirmed that the relationship between cell diameter and root length changed with the age of the root as described above.

The cell volumes were calculated using the mean cell length and cell diameter values. The results from these determinations (Table 4.3) show that, as germination time increased, the changes in cell volume were limited. For long roots at 36 hours germination, they were 2.04, 2.56 and 3.0 $x10^5$ μm^3 at 6.0, 7.5 and 9.0 mm from tip respectively. For short roots, the cell volumes were 1.25, 1.59 and 1.38 $x10^5$ μm^3 at the same observation points. By comparison, at 84 hours the cell volumes for long roots were 2.13, 2.29 and 2.52 $x10^5$ μm^3, while the volumes for short roots were 2.26, 2.36 and 2.42 $x10^5$ μm^3.

The above results (see Figure 4.3) show that, at 36 and 60 hours, the elongated cells of long roots had a greater volume as compared with those of short roots, but this effect disappeared by 84 hours. By this time, the cell volumes were almost the same in long and short roots. It can be seen that there was an increase in cell volume with increasing distance from the root tip. Also, cell volumes in the short roots increased with increasing germination time. In contrast, the volumes of fully elongated cells (at 9.0 mm from the tip) in the long roots decreased with increasing germination time. Statistical analysis of these data was not possible, because the volumes were calculated from the means of cell length and cell diameter.

The results for the number of cell files per root are presented in Table 4.4. They show that the number of files in long roots at 36 hours germination were 7.35, 7.35 and 7.60 at 6.0, 7.5 and 9.0 mm from the tip respectively. The number of files for short roots were 9.55, 9.75 and 9.75 respectively at 6.0, 7.5 and 9.0 mm from the tip. At 60 hours germination, the numbers for long roots were 5.90, 6.10 and 6.20, while those for short roots were 7.20, 7.30 and 7.60. At 84 hours, the numbers for long roots were 5.45, 5.50 and 5.65 and for short roots the values were 5.70, 5.80 and 5.85.

These results are also presented graphically in Figure 4.4 where it can be seen that the increase in the number of cell files per root with increase in distance from the tip towards the base of root is barely detectable. The change was, infact, found to be not statistically significant ($P = 0.12$ using ANOVA). On the other hand, the considerable difference between the number of cell files in long and short roots was shown to be highly significant ($P < 0.001$). At 36 hours and 60 hours, long roots had fewer cell files as compared with the short roots. This difference had almost disappeared by 84 hours however. This was reflected in the highly significant interaction between root group and germination time revealed by ANOVA ($P < 0.001$). It was also noted that the age of the root had a significant effect ($P < 0.001$) on the number of cell files. Younger seedling roots contained more cell files compared with older ones.

4.3.2 Cell dimensions at 84 hours germination

The results for a further experiment in which roots were classified as described in Method 4B (see Section 4.2.1) was used to determine cell dimension at 84 hours germination. In this experiment, a wider range of root lengths were examined. The results obtained from the experiment confirm and extend the data reported for 84 hour

Table 4.3 Cell volume at different germination times.

Germinⁿ		Location (mm from tip)		
period(h)		6.0	7.5	9.0
36	LR	2.04	2.56	3.00
	SR	1.25	1.59	1.38
60	LR	2.48	2.91	2.54
	SR	1.55	1.88	2.11
84	LR	2.13	2.29	2.52
	SR	2.26	2.36	2.42

Each value is the mean cell volume ($x10^5$ μm^3) calculated from mean cell length and diameter values in Tables 4.1 and 4.2. LR, long roots; SR, short roots.

Table 4.4 Number of cell files at different germination times.

Germinⁿ	Locatⁿ	Long roots			Short roots		
period(h)	(mm)	mean	±sd	±se	mean	±sd	±se
36	6.0	7.35	0.55	0.17	9.55	0.72	0.23
	7.5	7.35	0.67	0.21	9.75	0.84	0.27
	9.0	7.60	0.44	0.14	9.75	0.93	0.29
60	6.0	5.90	0.50	0.16	7.20	0.80	0.30
	7.5	6.10	0.70	0.20	7.30	0.60	0.20
	9.0	6.20	0.50	0.16	7.60	0.50	0.20
84	6.0	5.45	0.41	0.13	5.70	0.50	0.16
	7.5	5.50	0.44	0.14	5.80	0.50	0.16
	9.0	5.65	0.50	0.16	5.85	0.40	0.13

Each value is the mean number of cell files per root ±sd and ±se from 10 roots. Locatⁿ= distance from root tip (mm).

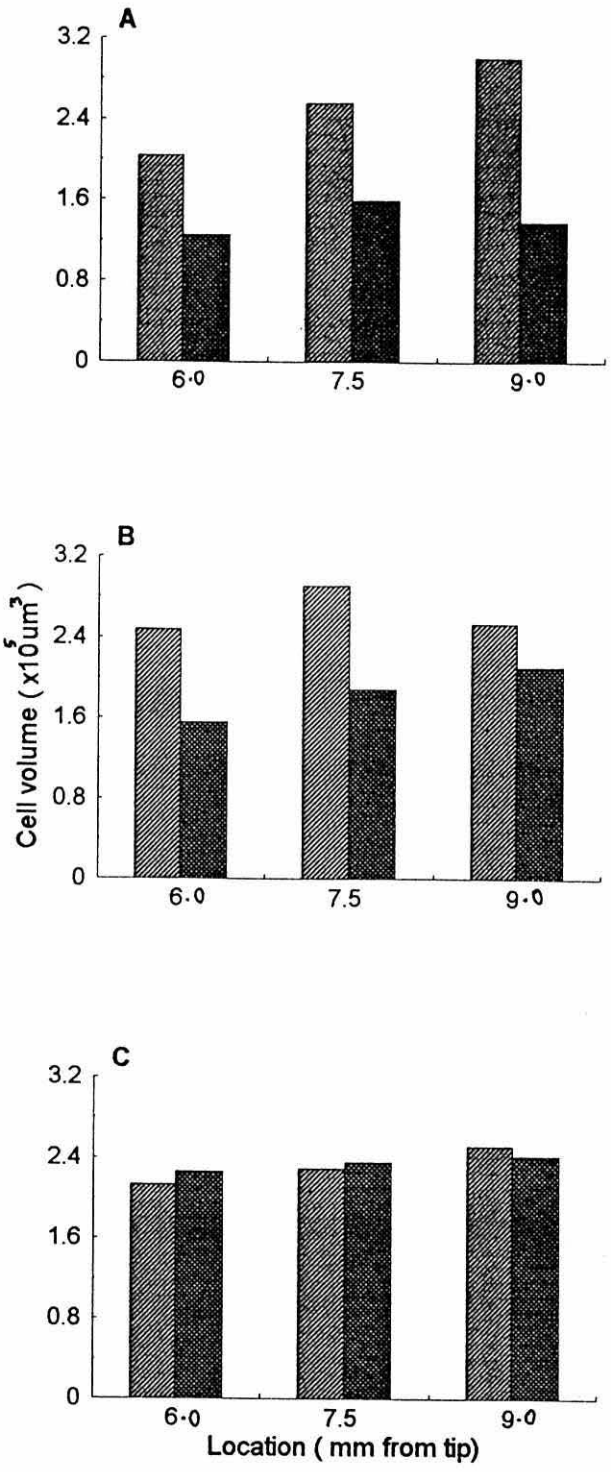


Fig. 4.3. Cell volume at different germination times. A, 36 hours; B, 60 hours; C, 84 hours; ▨, long roots; ▩, short roots.

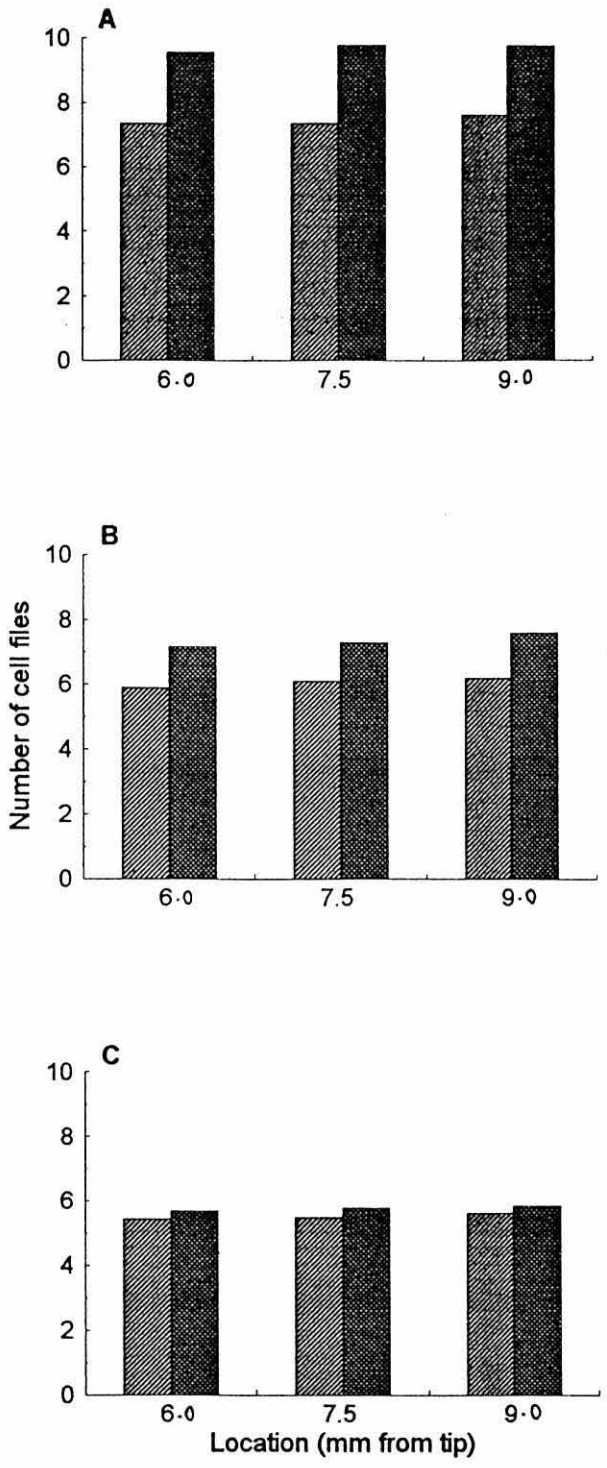


Fig. 4.4. Number of cell files at different germination times. A, 36 hours; B, 60 hours; C, 84 hours; ▨, long roots; ▩, short roots.

seedlings in Tables 4.1 to 4.4.

The results from cell length measurements are presented in Table 4.5 and Figure 4.5A. They show that at 6.0 mm from tip, the cell lengths for 3, 5, 7 and 9 cm roots were 107.7, 123.8, 135.3 and 156.0 μm respectively. The corresponding cell lengths at 7.5 mm from the tip were 113.2, 127.7, 139.8 and 161.2 μm. At 9.0 mm from tip, the values were 118.1, 131, 142.9 and 164.4 μm. These results clearly show that cell length is highly dependent upon root length. Cell length also increased, but to a much smaller extent, from the distal towards the proximal region of the roots. Statistical analysis (ANOVA) of the data confirmed the positive relationship between cell length and both root length ($P < 0.001$) and the distance from the root tip ($P = 0.004$).

The results for cell diameter are presented in Table 4.6. At 6.0 mm from the tip, the cell diameter in 3, 5, 7 and 9 cm roots was 37.6, 40.0, 37.8 and 35.1 μm respectively. At 7.5 mm, the diameters were 39.0, 40.7, 39.7 and 36.3 μm and at 9.0 mm from the tip they were 41.3, 41.6, 40.4 and 37.5 μm. It can be seen from these results (see Figure 4.5B) that cell diameter generally decreased with increasing root length ($P < 0.001$, by ANOVA). Only the shortest (3 cm) roots were an exception to this pattern. Cell diameter increased with increasing distance from the tip ($P = 0.001$). All of these changes in cell diameter were much smaller than the corresponding changes in cell length (cf. Table 4.5).

Table 4.7 and Figure 4.5C contain the results for the cell volumes calculated from the data in Tables 4.5 and 4.6. They show that, at 6.0 mm from the tip, the cell volumes in 3, 5, 7 and 9 cm roots were 1.19, 1.55, 1.52 and 1.51 $x10^5$ μm^3 respectively. The

Table 4.5 Cell length at 84 hours germination.

Locat[a] (mm from tip)		Root length (cm) 3	5	7	9
6.0	mean	107.70	123.80	135.28	156.00
	±sd	6.86	7.19	8.54	13.17
	±se	2.42	2.54	3.02	4.66
7.5	mean	113.20	127.70	139.79	161.20
	±sd	7.56	8.81	8.55	14.18
	±se	2.67	3.11	3.02	5.00
9.0	mean	118.10	131.00	142.95	164.40
	±sd	8.91	8.53	8.23	14.51
	±se	3.15	3.02	2.91	5.13

Each value is the mean cell length (μm) ±sd and ±se from 8 roots with 40-60 cells from 4 cell files measured in each root. All roots were from 84 hour seedlings.

Table 4.6 Cell diameter 84 hours germination.

Locat[a] (mm from tip)		Root length (cm) 3	5	7	9
6.0	mean	37.60	40.00	37.80	35.10
	±sd	2.74	3.03	3.10	1.28
	±se	0.97	1.07	1.10	0.45
7.5	mean	39.00	40.70	39.70	36.30
	±sd	2.42	2.89	3.08	1.48
	±se	0.86	1.02	1.09	0.52
9.0	mean	41.30	41.60	40.40	37.50
	±sd	3.18	3.13	2.93	1.24
	±se	1.14	1.11	1.04	0.44

Each value is the mean cell diameter (μm) ±sd and ±se from 8 roots with 4 cells from 4 cell files measured in each root. All roots were from 84 hour seedlings.

Table 4.7 Cell volume at 84 hours germination.

Locat^n	Root length (cm)			
(mm from tip)	3	5	7	9
6.0	1.19	1.55	1.52	1.51
7.5	1.35	1.66	1.73	1.67
9.0	1.58	1.78	1.83	1.81

Each value is the mean cell volume ($x10^5$ μm^3) calculated from mean cell length and diameter values in Tables 4.5 and 4.6.

Table 4.8 Number of cell files at 84 hours germination.

Locat^n		Root length (cm)			
(mm from tip)		3	5	7	9
6.0	mean	6.81	5.50	5.13	5.00
	±sd	0.24	0.50	0.33	0.50
	±se	0.08	0.18	0.12	0.18
7.5	mean	6.88	5.50	5.19	5.12
	±sd	0.21	0.50	0.24	0.21
	±se	0.07	0.18	0.08	0.07
9.0	mean	6.94	5.63	5.25	5.18
	±sd	0.17	0.48	0.43	0.24
	±se	0.06	0.17	0.15	0.08

Each value is the mean number of cell files per root ±sd and ±se from 8 roots. All roots were from 84 hour seedlings.

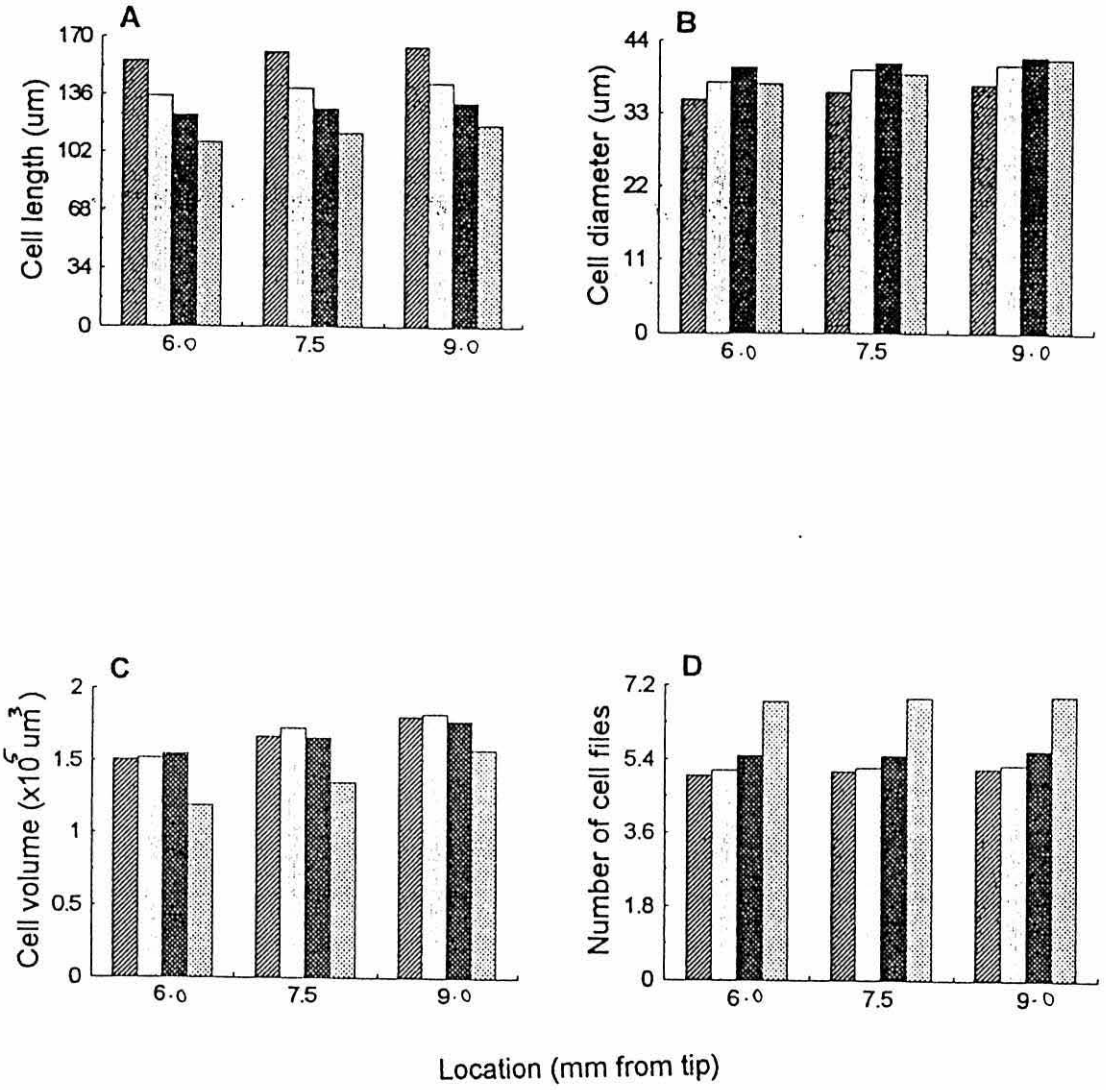


Fig. 4.5. Cell dimensions at 84 hours germination. A, cell length; B, cell diameter; C, cell volume; D, number of cell files; ▨, 3 cm roots; ▩, 5 cm roots ▤, 7 cm roots; ▨, 9 cm roots.

corresponding values at 7.5 mm from the tip were 1.35, 1.66, 1.73 and 1.67 x10^5 μm^3 and at 9.0 mm from the tip they were 1.58, 1.78, 1.83 and 1.81 x10^5 μm^3. It is clear that cell volume increased with increasing distance from the tip. It can also be seen that, except in the very short roots, cell volume was not influenced by root length. Statistical analysis of these data was not carried out.

The results for the number of cell files per root are presented in Table 4.8. They show that at 6.0 mm from tip, the number of cell files for 3, 5, 7 and 9 cm roots were 6.81, 5.50, 5.13 and 5.00 μm respectively. The corresponding numbers at 7.5 mm from the tip were 6.88, 5.50, 5.19 and 5.12. At 9.0 mm from tip, the values were 6.94, 5.63, 5.25 and 5.18. These results (see Figure 4.5D) suggest that the number of cell files increased with increase in distance from the tip towards the base of the root; the increase was very small however and not statistically significant ($P = 0.35$, using ANOVA). It was also confirmed from these results that the number of cell files decreased with increasing root length ($P < 0.001$). In other words short roots had a greater number of cell files and long roots had fewer cell files.

4.4 Summary and Discussion

Pritchard (1994), reported in his review that an increase in cell length is accompanied by a massive increase in the size of the vacuole and an increase in the area of the lateral walls of the cell. There is a small increase in root radius, but this is generally restricted to the apical region of the growing zone. Root growth is therefore the sum of the individual cell expansions occurring along a file of cells. It is the expansion of these individual cells that constitutes root growth.

Our results show that, at 60 and 84 hours, cell length increased slightly between 6.0 and 7.5 mm from the tip but not much thereafter. The picture at 36 hours was less clear cut, but there was again no evidence of a meaningful increase in cell length beyond 7.5 mm from the root tip. This indicates that, at 7.5 and 9.0 mm from root tip, the cells were fully elongated; effectively all the elongation had occurred before the 7.5 mm point. There was a considerable difference between the cell lengths of long and short roots. At any given germination time, long roots had longer cells as compared with the cells of short roots. This observation was further confirmed by the results of cell dimensions in the wider range of root lengths from the 84 hour seedlings. It was concluded from these results that cell length is a major determinant of root length. Within a single group of roots, cell length was almost the same at all the germination times studied; in other words the age of the root has no significant effect on cell length.

With respect to the cell diameter, the results clearly show that at early growth stages, i.e. at 36 and 60 hours germination, long roots had wider cells as compared with short roots, but at later growth stages (84 hours) the situation was reversed. The data regarding cell volumes show that at 36 and 60 hours, the elongated cells of long roots have a greater volume as compared with those of short roots, but this effect disappeared by 84 hours. By this time, the cell volume was almost the same in long and short roots. It is particularly interesting that the fast-growing roots (long roots) had a large cell volume (3.0×10^5 μm^3) and the slow growing roots (short roots) had a small cell volume (1.4×10^5 μm^3) at 36 hours, whereas at 84 hours they had similar and intermediate values (Ca 2.5×10^5 μm^3) (see Table 4.3). It is not clear why the cell volumes for the 84 hour seedlings in Table 4.7 were less than the corresponding 84 hour values in Table 4.3. The experiment presented in Table 4.7 was carried out some time after that in

Table 4.3 and it is possible that the vigour of the seeds had deteriorated in the intervening period of storage. Some similar results were obtained by Atwell (1993), who reported that, despite a large decrease in cell elongation in mechanically restricted roots of *Zea mays* L., cell volume can be unchanged due to the increase in radial cell expansion. It was also observed from our results that there was a significant increase in cell volume with increasing distance from the root tip. Similar results were found by MacLeod and McLachlan (1975) in *Vicia faba* roots, where cell volume increased basally along the root.

The number of cell files did not increase significantly with increase in distance from the tip. It is also clear from these results that the number of cell files decreased with increasing root length at all germination times. In other words short roots had a greater number of cell files and long roots had fewer cell files especially at early growth stages, i.e. at 36 and 60 hours germination. It is also noted that the age of the root had a significant effect on the number of cell files. Younger seedling roots contained more cell files as compared with older ones.

Root diameter is thus clearly dependent upon both the number of cell files and cell diameter. At early growth stages (36 and 60 hours), the two factors work in the opposite direction to each other. The file number tends to make the short roots wider, but cell diameter has the effect of reducing root diameter in short roots compared with long roots. At 84 hours, cell width and file number work in the same direction. They both have the effect of making short roots wider than long roots. These differences at 84 hours are much less than at 36 hour however. The situation regarding root length appears to be more straight forward. Only one factor, cell length, appears to determine

this root dimension. The relationships between cell dimensions and root dimensions will be picked up again in Chapter 8.

CHAPTER FIVE

Chapter Five

Radiolabelled Thymidine Incorporation and DNA Levels

5.1 Introduction

Root growth consists of two basic processes, cell division and cell elongation. An integral part of the first process is the synthesis of DNA (Maksymowych, *et al.*, 1986). In primary roots, DNA synthesis and cell division are restricted to the meristematic regions. These meristematic regions are located in the root apex and in more proximal parts of the roots where lateral root initiation is taking place.

The root meristem is a truly embryonic growth centre from which all root cells are ultimately derived and their cells can be considered as proliferative (Van't Hof, 1968). Meristems are complex structures made up of sub-populations of cells that differ in nuclear volume (Rasch *et al.*, 1967) and in the duration of their mitotic cycle (Murin, 1964; Webster and Davidson, 1968). The cells are highly cytoplasmic and have no clearly-defined central vacuole (Barlow, 1987; Clowes, 1976). In different regions of the meristem they are generally distinguishable from each other in appearance however (Gray and Scholes, 1951; Clowes, 1961). It has also been reported that the rates of DNA synthesis, which are related to rates of cell division, differ in different regions of the meristem (Clowes, 1956a, 1956b, 1958).

The most prominent feature of the root meristem of plants is the quiescent centre (Clowes, 1958), which has a rate of synthesis of DNA far below that of the rest of the meristem (Clowes, 1959). The cells within this quiescent centre can re-populate the meristem when the root tip is subjected to stress (Clowes, 1970; Barlow and Adam,

1989). It is known that treatments which suppress cell proliferation in meristematic cells generally, somehow induce DNA synthesis and mitosis in the cells of the quiescent centre. Such treatments include exposure to radiation (Clowes, 1972a), low temperature (Clowes and Stewart, 1967) or colchicine (Davidson, 1961), short periods of carbohydrate starvation (Webster and Langenauer, 1973), or removing the root cap (Clowes, 1972b). The quiescent centre can also regenerate whole plants when dissected and incubated *in vitro* (Feldman, 1976). Hence the quiescent centre comprises a reservoir of founder cells (Barlow, 1978).

In plants generally, cell division is a primary determinant for many aspects of development. The patterns of cell division within the root are central in determining its future structure (Barlow, 1987) and root growth ultimately depends on the rate of cell proliferation in the apical meristem. The number of cell divisions occurring in the meristem is amazingly high. Van't Hof (1967) reported that cell production per hour is about 6,500 for sunflower, 11,200 for broad bean, 17,500 for corn and between 10,900 and 13,700 for pea.

One intriguing aspect of plant cell division is that it is often asymmetric (Davidson, 1991). It follows that the cytoplasmic environment of each daughter cell is often quantitatively different and they do not continue to cycle at the same rate. For example, in pea, 90% of daughter cells have different cycle times (Webster, 1979). At the margin of the meristem, such asymmetric division may result in cells which have different levels of competence to respond to transportable morphogens (hormones, etc) from other parts of the plant.

Any attempt to regulate cell division must take account of the so-called cell cycle, which comprises the G_1 (pre-synthetic interphase), S (DNA synthetic phase), G_2 (post-synthetic interphase) and M (mitosis phase) phases (Quastler and Sherman, 1959). Specific biochemical reactions occur during each of these stages, and certain RNAs and proteins (including enzymes) are needed to progress from one stage to the next (Rost, 1977). The metabolic events occurring during the cell cycle need to be supplied with a constant source of oxygen, nutrients and the means to synthesize chemical energy (ATP) (Rost, 1977).

It has been reported that the cycle time of cells varies from one region of the root meristem to another (Clowes, 1963; Thompson and Clowes, 1968) and from one species to another. For example, Barlow and MacDonald (1973) reported that cell cycle duration in the root apical meristem of *Zea mays* varies significantly, depending on the meristem region. Van't Hof (1967) determined the number of hours required for cell cycle progression in pea, sunflower and broad bean root meristems. In three cultivars of pea, the value ranged from 12 to 14 hours. G_1 ranged from 3 to 5 hours, S was 4.5 hours, G_2 was 3.0 to 3.3 hours, and M was from 1.2 to 1.4 hours. The cell cycle duration in sunflower was similar to that of pea, except that S was slightly longer (5.5 hours) and G_2 was somewhat shorter (1.3 hours). The cell cycle duration for broad beans was 17 to 18 hours, with S showing the largest individual difference (9.0 hours). M was still rather short (1.9 hours).

Amano *et al.* (1959) reported that thymidine serves specifically as a precursor of DNA, and it presumably is incorporated only by cells which are synthesizing DNA during the S phase. Because of this, the incorporation of radiolabelled thymidine in to DNA

provides a highly specific method for measuring the rate of cell division. In present study therefore, radiolabelled (Me-^{3}H) thymidine was applied to cotton seedling roots to measure its incorporation into DNA. Also, total DNA levels were determined. Because the DNA content of cells is constant for a particular species, it can be used as a measure of the number of cells present in a tissue. Using these two methods, it was hoped to characterise any changes in cell division and cell numbers during the growth of the seedling root.

5.2 Methods

5.2.1 Selection of seedling root groups

Five methods were used for the selection of seedling root groups according to root length.

Method 5A: Batches of 100 seeds were germinated as described in Section 2.6. After 24, 36, 48, 60, 72, 84 and 96 hours germination, the seedlings were harvested. Ungerminated seeds were discarded and the remainder were arranged according to root length. Ten seedlings were selected from the centre of the root length distribution (ie. median roots). Two further sets of 10 seedlings were then selected, one of which shared a longer root length and the other a shorter root length. The roots were designated the 'long', 'medium' and 'short' roots.

Method 5B: In order to have a wider range of root lengths, a separate experiment was carried out in which 200 seeds were germinated (Section 2.6) for 84 hours. After harvesting, ungerminated seeds were discarded and the remainder were arranged according to root length. Two sets of 10 seedlings were then selected, one of

which shared a longer root length (8-10 cm) and the other a shorter root length (2-4 cm).

Method 5C: Batches of 100 seeds were germinated as described in Section 2.6. After 36, 48, 60, 72, 84 and 96 hours germination the seedlings were harvested and ungerminated seeds were discarded. Ten seedlings with long roots (above median length) were then selected at each germination period.

Method 5D: Batches of 100 seeds were germinated as described in Section 2.6. After 36, 48, 60, 72 and 84 hours germination, the seedlings were harvested. Ungerminated seeds were discarded and the remainder were arranged according to root length. Two sets of 10 seedlings were then selected, one of which shared a longer than median root length and the other a shorter than median root length. The roots were designated 'long', and 'short' roots.

Method 5E: Batches of 100 seeds were germinated (Section 2.6) for 36, 48, 60 and 72 hours. After harvesting, ungerminated seeds were discarded and the remainder were arranged according to root length. Two sets of 10 seedlings were then selected, one of which shared a longer than median root length and the other a shorter than median root length. The roots were designated 'long', and 'short' roots.

5.2.2 Measurement of uptake and incorporation of (Me-^{3}H) thymidine

The detailed procedure for the radiotracer experiment is described in Section 2.13. Two different experiments were carried out as follows:

1.a, Uptake and incorporation were measured in 1 cm tip segments cut from roots (selection Method 5A) that had been incubated with (Me-^{3}H) thymidine.

1.b, Uptake and incorporation were measured in 1cm tip segments cut from a wider range of roots (selection Method 5B) that had been incubated with (Me-^{3}H) thymidine.

2. Uptake and incorporation were measured in all the segments from roots (selection Method 5C) that had been incubated with (Me-^{3}H) thymidine.

5.2.3 Determination of DNA

The procedure for the determination DNA has been described in Section 2.14. Two different experiments were carried out as follows:

1.a, The DNA contents of whole roots were determined at different times of germination (selection Method 5D).

1.b, The DNA contents of whole roots were determined at 84 hours germination (selection Method 5B).

2. DNA was determined separately in the 1 cm tip segment and in the rest of the root at different germination times (selection Method 5E).

5.3 Results

5.3.1 Uptake and incorporation of (Me-^{3}H) thymidine in root tips

The results for uptake of (Me-^{3}H) thymidine in the first experiment in which the seedlings were selected into long, medium and short root groups (Method 5A; see Section 5.2.1) are presented in Table 5.1 and Figure 5.1A. It can be seen from these results that, at 24 hours, the uptake was 30.3, 21.9 and 21.3 x 10^3 dpm $(10 \text{ tips})^{-1}$ in

Table 5.1 (Me-^{3}H) thymidine uptake at different germination times.

Germin[a]	Long roots			Medium roots			Short roots		
period(h)	mean	±sd	±se	mean	±sd	±se	mean	±sd	±se
24	30.3	2.40	1.20	21.9	2.21	1.11	21.3	0.3	0.15
36	10.9	1.58	0.91	10.2	1.28	0.74	15.5	1.27	0.73
48	11.6	0.77	0.44	12.6	2.07	1.19	11.3	1.70	0.98
60	10.7	1.04	0.60	10.9	0.41	0.24	12.4	1.18	0.68
72	9.2	2.27	1.31	9.0	0.38	0.22	11.0	2.79	1.61
84	7.3	1.05	0.61	10.4	1.24	0.72	12.2	2.74	1.58
96	7.2	0.99	0.57	8.1	1.18	0.68	8.2	1.67	0.96

Each value is the mean uptake [dpm x10^3(10 tips)$^{-1}$] ±sd and ±se from either 3 or 4 separate experiments.

long, medium and short roots respectively. In fact, the highest uptake was measured at 24 hours germination and this reduced quickly to reach only 7.2, 8.1 and 8.2 x 10^3 dpm $(10 \text{ tips})^{-1}$ in the long, medium and short roots respectively at 96 hours germination. It is clear from these results that root age had a large and negative effect on uptake. At 24 hours, the seedling groups with long roots apparently took up more radioactivity than those with shorter roots. At 36 hours and beyond, however, there was a small but negative effect of root length on uptake. Further statistical analysis by ANOVA (in which the effects of seedling groups and germination times on uptake were investigated) confirmed that uptake was slightly dependent upon seedling group ($P = 0.05$) and that the effect of germination time was highly significant ($P < 0.001$). The interaction between seedling group and germination time ($P < 0.001$) reflected the differences in the effect of seedling group on uptake in relation to germination time.

The corresponding results for the incorporation of (Me-^{3}H) thymidine into DNA are presented in Table 5.2 and Figure 5.1B. The trend for these results is very similar to that for the uptake. Thus, at 24 hours germination, incorporation was 3962, 2204 and 1910 dpm $(10 \text{ tips})^{-1}$ in long, medium and short roots, respectively. The highest incorporation was measured at 24 hours germination and this reduced quickly to reach only 410, 372 and 399 dpm $(10 \text{ tips})^{-1}$ in long, medium and short roots respectively at 96 hour germination. As for uptake, the results show that root age had a large effect on incorporation. Root length had a much smaller and inconsistent effect except at 24 and 36 hours where the seedling group with long roots incorporated more (Me-^{3}H) thymidine than the shorter ones. Statistical analysis of the (Me-^{3}H) thymidine incorporation data, using ANOVA in which the effect of seedling groups and germination times were investigated, showed that the parameter was highly dependent upon both seedling group

Table 5.2 (Me-^{3}H) thymidine incorporation into DNA at different germination times.

Germin[a]	Long roots			Medium roots			Short roots		
period(h)	mean	±sd	±se	mean	±sd	±se	mean	±sd	±se
24	3962	736	368	2204	182	91	1910	109	54
36	2831	665	384	1803	687	397	2021	698	403
48	1460	546	315	1567	620	358	1333	586	338
60	722	214	124	597	127	73	612	260	150
72	527	61	35	558	185	107	581	189	109
84	442	91	53	545	19	11	530	41	23
96	410	6	3	372	49	28	399	78	45

Each value is the mean incorporation [dpm(10 tips)$^{-1}$] ±sd and ±se from either 3 or 4 separate experiments.

($P = 0.003$) and germination time ($P < 0.001$). The significant interaction between seedling group and germination time ($P = 0.001$) confirms that the effect of seedling group changed with germination time.

In order to get a more accurate assessment of the true incorporation, it was decided to calculate incorporation/ uptake ratios. These values are given in Table 5.3 and presented graphically in Figure 5.1C. The trend was similar to that for the straight incorporation values. At 24 hours germination, the I/U ratio was 0.130, 0.101 and 0.089 in long, medium and short roots respectively. The highest ratio was now measured at 36 hours germination (0.250, 0.180 and 0.130 in long, medium and short roots respectively), but this declined quickly again to reach only 0.057, 0.047 and 0.049 respectively at 96 hours germination. These results show again that root age had a large effect on the I/U ratio, while root length had a much smaller effect except at 24 and 36 hours germination. Nevertheless, statistical analysis of the data, using ANOVA in which the effect of seedling groups and germination times were investigated, showed that the I/U ratio was highly dependent upon both seedling group ($P = 0.005$) and germination time ($P < 0.001$). There was no significant interaction between seedling group and germination time ($P = 0.17$).

An experiment was carried out to look more carefully at the relationship between root length and the uptake and incorporation of (Me-^{3}H) thymidine. Two groups of roots (8-10 and 2-4 cm) from 84 hour seedlings (Method 5B; see Section 5.2.1) were used and uptake/incorporation was measured in the 1 cm tip segment. These roots were longer and shorter respectively than those used for the experiments reported in Tables 5.1 to 5.3. The results for this experiment are presented in Table 5.4. They show that uptake

Table 5.3 (Me-^{3}H) thymidine incorporation/uptake ratios at different germination times.

Germin^n	Long roots			Medium roots			Short roots		
period(h)	mean	±sd	±se	mean	±sd	±se	mean	±sd	±se
24	0.130	0.014	0.007	0.101	0.013	0.006	0.089	0.004	0.002
36	0.250	0.029	0.016	0.180	0.078	0.045	0.130	0.047	0.027
48	0.127	0.047	0.027	0.117	0.030	0.017	0.118	0.053	0.030
60	0.069	0.027	0.016	0.055	0.013	0.008	0.050	0.024	0.014
72	0.059	0.013	0.008	0.061	0.018	0.010	0.052	0.008	0.005
84	0.060	0.008	0.005	0.053	0.008	0.005	0.045	0.011	0.006
96	0.057	0.008	0.005	0.047	0.013	0.008	0.049	0.007	0.004

Each value is the mean I/U ratio ±sd and ±se from either 3 or 4 separate experiments.

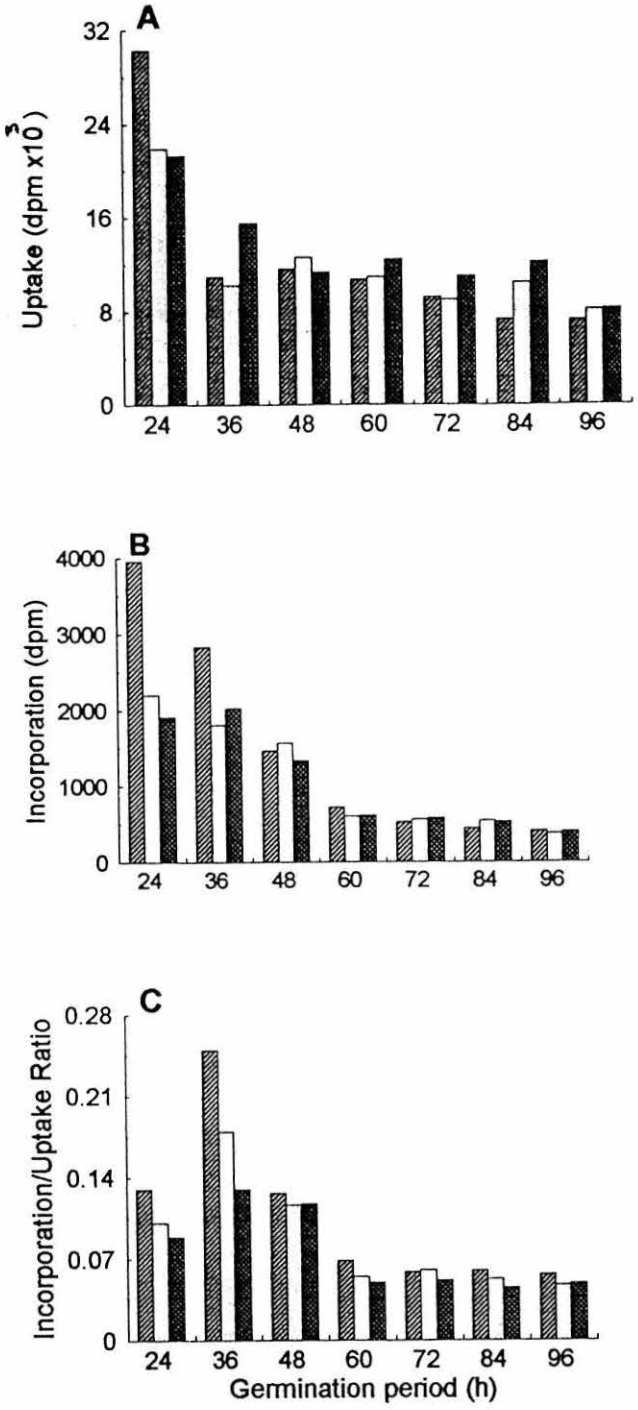


Fig. 5.1. (Me-^{3}H) thymidine uptake and incorporation at different germination times. A, uptake; B, incorporation; C, incorporation/uptake ratio; ▨, long roots; ▦, medium roots; ▩, short roots.

was 6.74 and 8.58 x 10^3 dpm (10 tips)$^{-1}$ for root lengths of 8-10 and 2-4 cm respectively. This suggests that short roots may have a greater uptake compared with the long roots, but the difference as assessed by a T-test, was insignificant (P = 0.13). The incorporation values for the 8-10 and 2-4 cm roots were 265 and 456 dpm (10 tips)$^{-1}$ respectively. Like the uptake data, these results indicate that incorporation was higher in short and less in long roots. On this occasion, statistical analysis (T-test) of the data showed that the difference was highly significant (P = 0.005). The incorporation/uptake ratios were 0.040 and 0.055 in 8-10 and 2-4 cm roots respectively. It was observed from these results that I/U ratio was higher in short than in the long roots, but the difference was insignificant (P = 0.07) statistically (T-test).

For the comparison of the uptake, incorporation and I/U ratios of all root lengths at 84 hours, data from Tables 5.1 to 5.4 were combined into one table (Table 5.5). In order to help comparison, the mean lengths of the roots from Table 5.1 to 5.3 are included. This table shows that uptake was 6.74, 7.29, 10.37, 12.17 and 8.58 x 10^3 dpm (10 tips)$^{-1}$ for root lengths of 8-10, 7, 6, 5 and 2-4 cm respectively. Like the earlier tables, it shows that the shorter roots had a greater uptake compared with longer roots (P = 0.008 using ANOVA), with the exception of the 2-4 cm roots. The incorporation values for the 8-10, 7, 6, 5 and 2-4 cm roots were 265, 442, 545, 530 and 456 dpm (10 tips)$^{-1}$ respectively. In other words, incorporation appears to increase slightly with increase in root length (P = 0.001 by ANOVA) up to a length of 6 cm. After that, it decreased sharply again. The incorporation/uptake ratios were 0.040, 0.060, 0.053, 0.045 and 0.055 in 8-10, 7, 6, 5 and 2-4 cm roots respectively; there is no clear-cut trend in this relationship between root length and the I/U ratio (P = 0.12 using ANOVA). Clearly, this combined set of data presents a confusing picture.

Table 5.4 (Me-^{3}H) thymidine uptake and incorporation at 84 hours germination.

		Root length (cm)	
		8-10	2-4
Uptake	Mean	6.74	5.58
	±sd	1.91	1.98
	±se	0.78	0.81
Incorporⁿ	mean	265	456
	±sd	99	91
	±se	41	37
I/U ratio	mean	0.040	0.055
	±sd	0.010	0.016
	±se	0.004	0.007

Each value is the mean uptake [dpm x10^3(10 tips)$^{-1}$], incorporation [dpm(10 tips)$^{-1}$] or I/U ratio ±sd and ±se from either 3 or 6 separate experiments.

Table 5.5 (Me-^{3}H) thymidine uptake and incorporation at 84 hours germination.

		Root length (cm)				
		8-10	7	6	5	2-4
Uptake	mean	6.74	7.29	10.37	12.17	5.58
	±sd	1.91	1.05	1.24	2.74	1.98
	±se	0.78	0.61	0.72	1.58	0.81
Incorporⁿ	mean	265	442	545	530	456
	±sd	99	91	19	41	91
	±se	41	53	11	23	37
I/U ratio	mean	0.040	0.060	0.053	0.045	0.055
	±sd	0.010	0.008	0.008	0.011	0.016
	±se	0.004	0.005	0.005	0.006	0.007

Each value is the mean uptake [dpm x10^3(10 tips)$^{-1}$], incorporation [dpm(10 tips)$^{-1}$] or I/U ratio ±sd and ±se from either 3 or 6 separate experiments.

5.3.2 Uptake and incorporation of (Me-^{3}H) thymidine in different root segments

The results for the uptake of (Me-^{3}H) thymidine in which one group of seedling roots was selected (Method 5C; see Section 5.2.1) are presented in Table 5.6 and Figure 5.2. They show that, at 36 hours, uptake was 9.9, 9.6 and 25.6 x 10^3 dpm (10 segments)$^{-1}$ in 1st, 2nd and 3rd segments respectively. This distribution of activity changed quickly as germination proceeded. Thus, uptake at 60 hours was 8.7, 9.9, 18.6, 26.5, and 24.4 x 10^3 dpm (10 segments)$^{-1}$ in the 1st through to the 5th segment. By 96 hours, uptake was 6.4, 7.9, 9.7, 16.1, 25.9, 29.4, 22.5, and 18.4 x 10^3 dpm (10 segments)$^{-1}$ in the 1st through to the 8th segment. It is clear from these results that uptake was low in the 1st (tip) segment and it increased with increase in segment number towards the base of root. In 36 hour roots, uptake occurred predominantly in the 3rd segment. This principle centre of activity remained predominant throughout germination and it moved further away from the tip as germination proceeded. At the same time, uptake in the distal segments decreased as germination proceeded. Statistical analysis using ANOVA, in which the effects of location (segment number from tip), seedling group and germination periods were investigated (using data from the 1, 2, 3 and 4 segment number to omit missing values) showed that uptake was strongly dependent upon location (root segment number) ($P < 0.001$), seedling group ($P < 0.001$) and upon germination period ($P < 0.001$). The fact that significant interactions were revealed between the effects of segment number and seedling group (< 0.001) and between segment number and germination period ($P < 0.001$) emphasises the complexity of the changes in (Me-^{3}H) thymidine uptake.

The results for the incorporation are presented in Table 5.7 and Figure 5.3. They show that, at 36 hours, incorporation was 1821, 530 and 1072 dpm (10 segments)$^{-1}$ in the 1st,

Table 5.6 (Me-^{3}H) thymidine uptake in different root segments.

Germin[a] period (h)	Root length (cm)		Segment number (from tip) 1	2	3	4	5	6	7	8
36	3	mean	9.9	9.6	25.6	-	-	-	-	-
		±sd	1.1	1.4	2.2	-	-	-	-	-
		±se	0.6	0.8	1.3	-	-	-	-	-
48	4	mean	10.7	15.7	32.7	36.0	-	-	-	-
		±sd	2.4	1.7	3.8	5.5	-	-	-	-
		±se	1.2	0.8	1.9	2.7	-	-	-	-
60	5	mean	8.7	9.9	18.6	26.5	24.4	-	-	-
		±sd	1.2	2.8	2.8	5.2	3.8	-	-	-
		±se	0.6	1.4	1.6	3.0	2.2	-	-	-
72	6	mean	8.2	15.4	18.9	18.7	26.3	21.7	-	-
		±sd	1.1	2.8	2.1	2.3	4.3	4.0	-	-
		±se	0.5	1.4	1.0	1.2	2.1	2.0	-	-
84	7	mean	8.3	11.3	14.7	19.5	23.9	22.0	16.5	-
		±sd	0.5	1.4	2.5	2.3	1.7	4.7	5.1	-
		±se	0.3	0.8	1.4	1.3	1.0	2.7	2.9	-
96	8	mean	6.4	7.9	9.7	16.1	25.9	29.4	22.5	18.4
		±sd	1.4	0.7	1.2	2.3	5.6	3.5	3.5	2.7
		±se	0.8	0.4	0.7	1.3	3.2	2.0	2.0	1.5

Each value is the mean uptake [dpm x10^3(10 segments)$^{-1}$] ±sd and ±se from either 3 or 4 separate experiments.

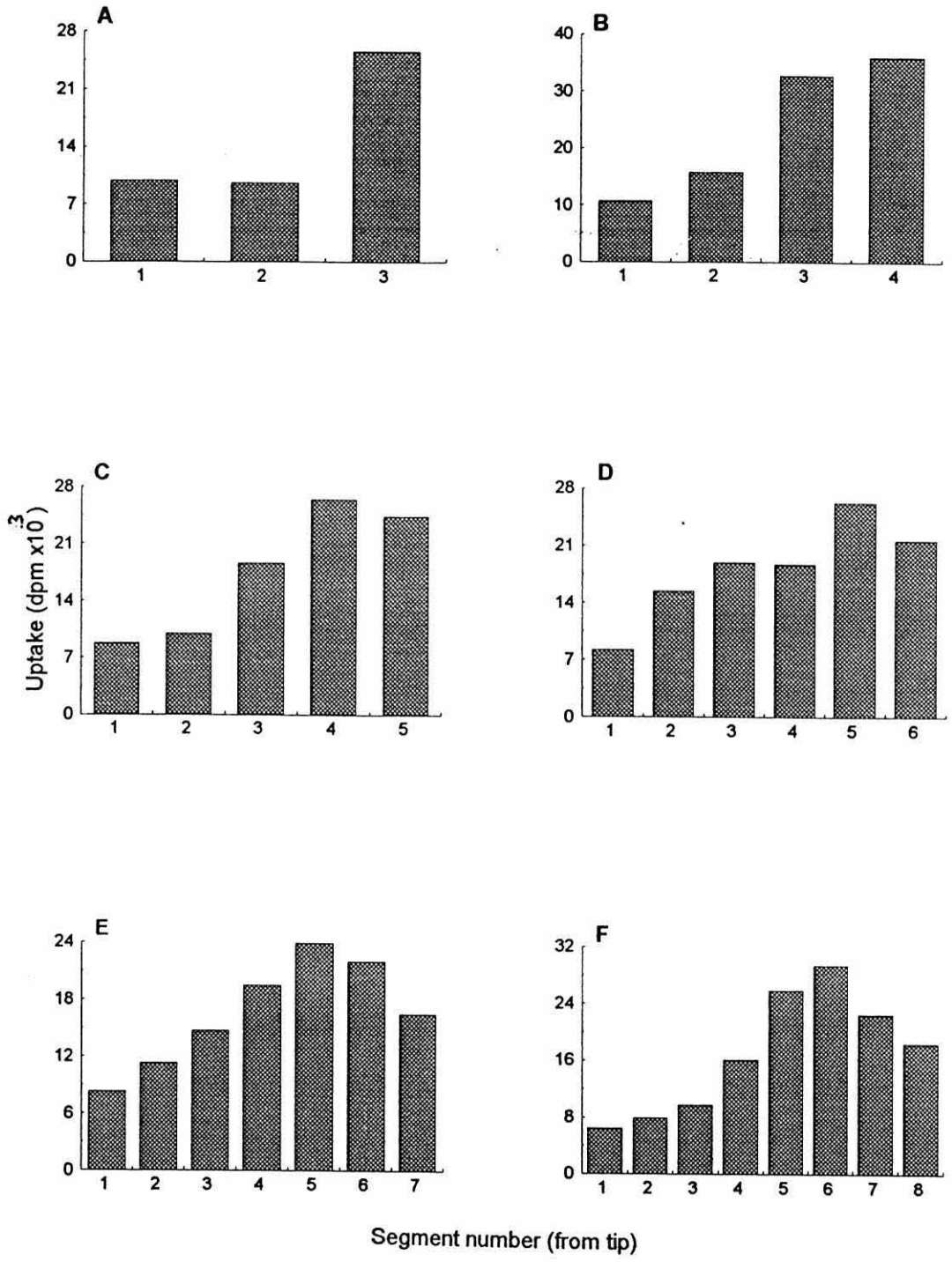


Fig. 5.2. (Me-^{3}H) thymidine uptake in different root segments. A, 36 hours; B, 48 hours; C, 60 hours; D, 72 hours; E, 84 hours; F, 96 hours.

Table 5.7 (Me-^{3}H) thymidine incorporation into DNA in different root segments.

Germin[a] period (h)	Root length (cm)		Segment number (from tip) 1	2	3	4	5	6	7	8
36	3	mean	1821	530	1072	-	-	-	-	-
		±sd	206	57	162	-	-	-	-	-
		±se	119	33	94	-	-	-	-	-
48	4	mean	1129	439	1560	1038	-	-	-	-
		±sd	359	104	357	108	-	-	-	-
		±se	179	52	179	54	-	-	-	-
60	5	mean	872	453	1116	1500	795	-	-	-
		±sd	151	58	180	194	223	-	-	-
		±se	76	29	104	112	129	-	-	-
72	6	mean	652	289	468	713	1213	563	-	-
		±sd	67	41	28	125	128	97	-	-
		±se	34	20	14	62	64	48	-	-
84	7	mean	362	389	512	831	994	694	347	-
		±sd	55	176	119	311	191	226	44	-
		±se	32	102	69	180	110	131	25	-
96	8	mean	269	108	201	356	521	857	521	426
		±sd	18	10	58	87	240	40	98	141
		±se	10	6	34	50	139	23	57	82

Each value is the mean incorporation [dpm(10 segments)$^{-1}$] ±sd and ±se from either 3 or 4 separate experiments.

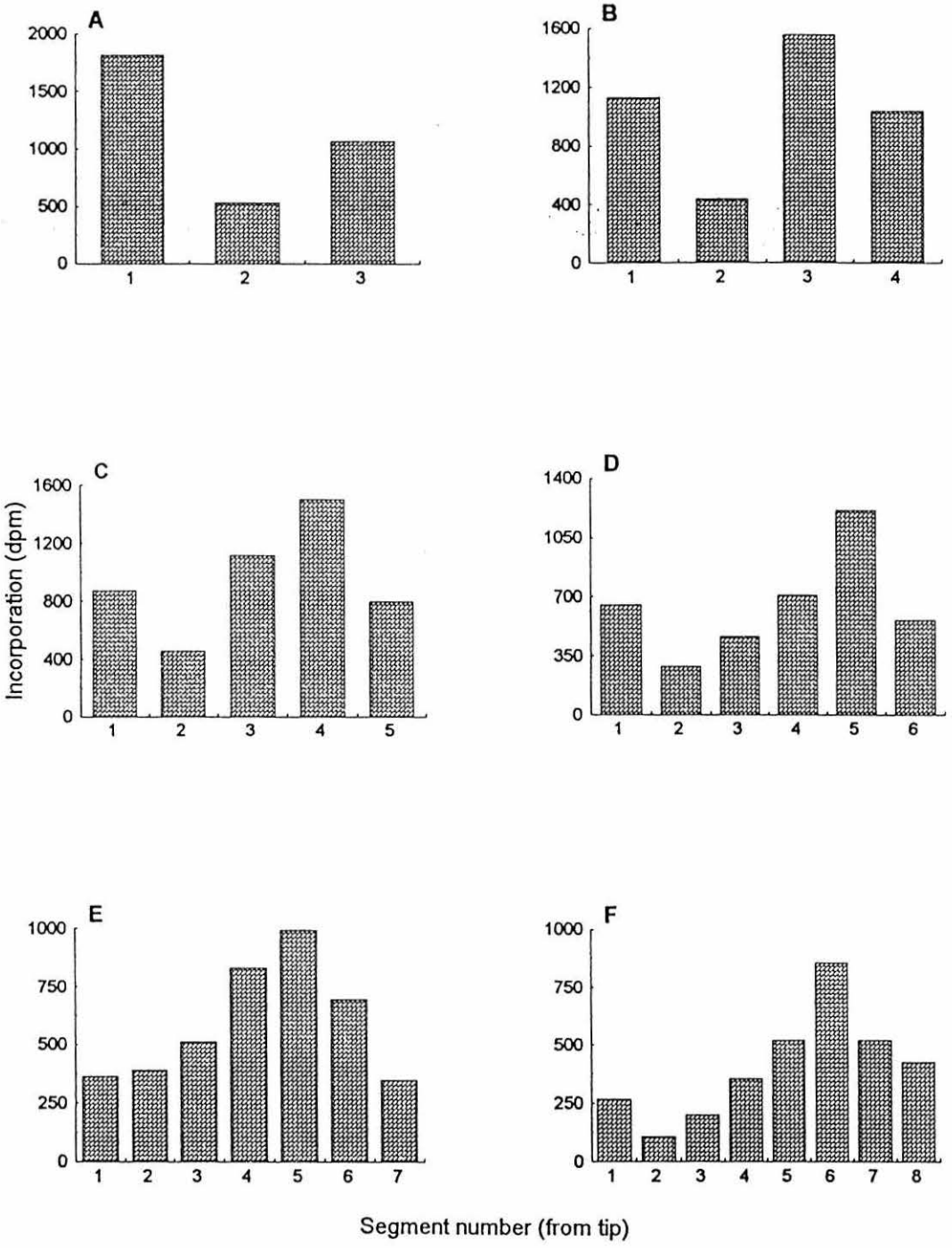


Fig. 5.3. (Me-^{3}H) thymidine incorporation in different root segments. A, 36 hours; B, 48 hours; C, 60 hours; D, 72 hours; E, 84 hours; F, 96 hours.

2nd and 3rd segments respectively. This distribution of activity also changed quickly as germination proceeded. Thus, incorporations of 872, 453, 1116, 1500 and 795 dpm (10 segments)$^{-1}$ in the 1st through to the 5th segment respectively were recorded at 60 hours germination. By 96 hours, incorporation was 269, 108, 201, 356, 521, 857, 521, and 426 dpm (10 segments)$^{-1}$ in the 1st through to the 8th segment. It is concluded from these results that incorporation in 36 hours roots occurred predominantly in the 1st segment, but another active centre of incorporation was present further up the root in the 3rd segment. This second region of activity progressively became the predominant one and it moved away from the tip as germination proceeded. At the same time, incorporation in the tip region declined. Statistical analysis using ANOVA, in which the effects of location (segment number from tip), seedling group and germination periods were investigated (using data from the 1, 2, 3 and 4 segment number to omit missing values) showed that incorporation was strongly dependent upon location (root segment number) ($P < 0.001$), seedling group ($P < 0.001$) and upon germination period ($P < 0.001$). The fact that significant interactions were revealed between the effects of segment number and seedling group ($P < 0.001$) and between segment number and germination period ($P < 0.001$) emphasises the complexity of the changes in (Me-^{3}H) thymidine incorporation (cf. uptake data, above).

The data for the incorporation/uptake ratios are presented in Table 5.8 and in Figure 5.4. They show that, at 36 hours, the I/U ratio was 0.183, 0.055 and 0.042 in 1st, 2nd and 3rd segments respectively. As for uptake and incorporation separately, this pattern of activity changed as germination proceeded. Thus, I/U ratios of 0.103, 0.048, 0.060, 0.057, and 0.032 in the 1st through to the 5th segment respectively were recorded at 60 hours. By 96 hours, the ratios were 0.043, 0.013, 0.020, 0.023, 0.021, 0.029,

Table 5.8 (Me-^{3}H) thymidine incorporation/uptake ratios in different root segments.

Germin^n period (h)	Root length (cm)		Segment number (from tip) 1	2	3	4	5	6	7	8
36	3	mean	0.183	0.055	0.042	-	-	-	-	-
		±sd	0.015	0.008	0.004	-	-	-	-	-
48	4	mean	0.112	0.028	0.048	0.029	-	-	-	-
		±sd	0.051	0.004	0.009	0.002	-	-	-	-
60	5	mean	0.103	0.048	0.060	0.057	0.032	-	-	-
		±sd	0.025	0.014	0.004	0.007	0.008	-	-	-
72	6	mean	0.081	0.019	0.025	0.039	0.047	0.027	-	-
		±sd	0.012	0.005	0.003	0.008	0.010	0.008	-	-
84	7	mean	0.044	0.034	0.035	0.042	0.041	0.031	0.022	-
		±sd	0.009	0.012	0.007	0.013	0.006	0.006	0.006	-
96	8	mean	0.043	0.013	0.020	0.023	0.021	0.029	0.023	0.023
		±sd	0.013	0.003	0.004	0.007	0.011	0.004	0.003	0.006

Each value is the mean I/U ratio and ±sd from either 3 or 4 separate experiments.

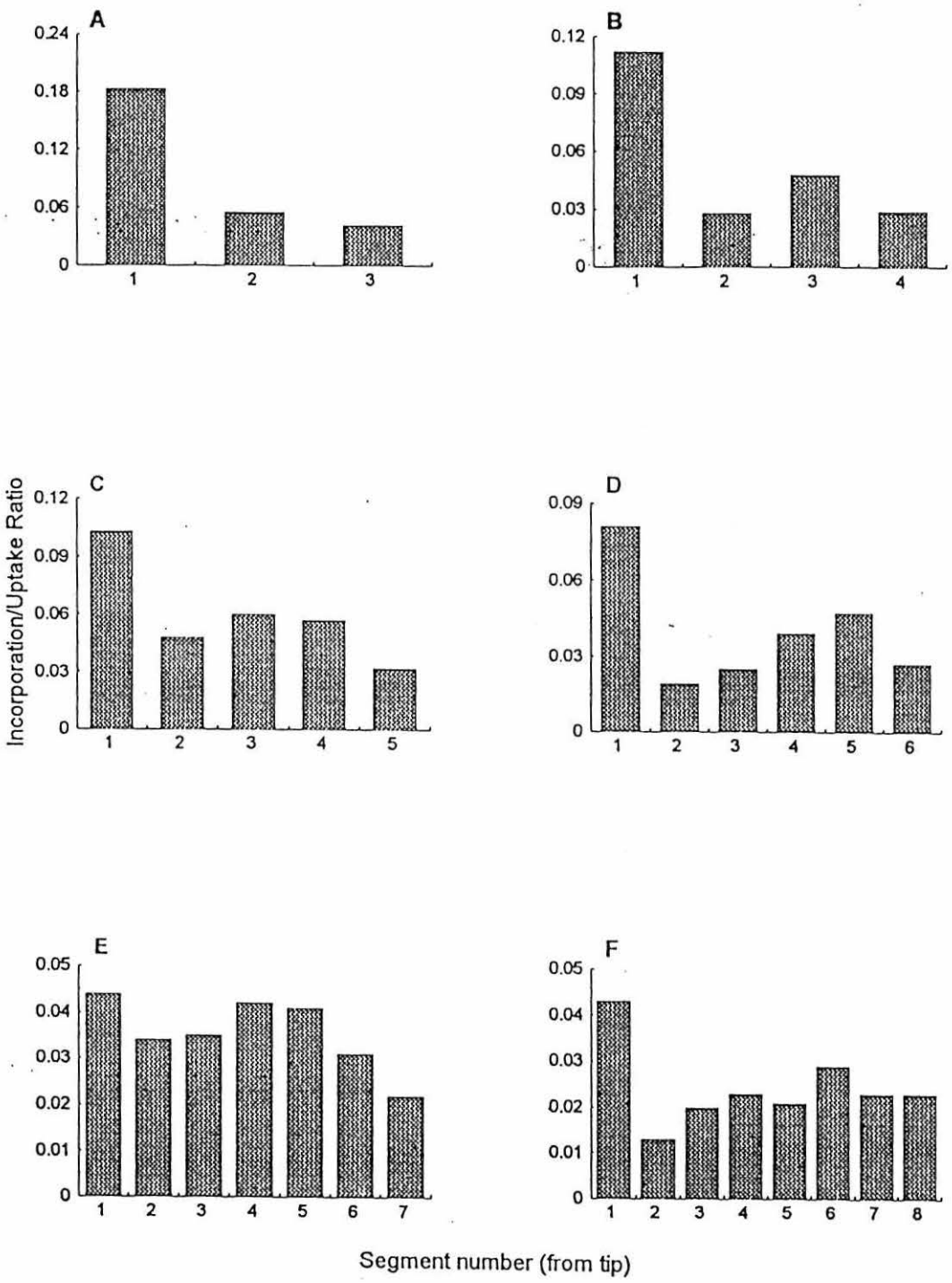


Fig. 5.4. (Me-^{3}H) thymidine incorporation/uptake ratio in different root segments. A, 36 hours; B, 48 hours; C, 60 hours; D, 72 hours; E, 84 hours; F, 96 hours.

0.023, and 0.023 in the 1st through to the 8th segment. It can be seen that the I/U ratio in 36 hour roots was highest in the 1st segment, and that a second region of activity became apparent further up the root as germination proceeded. There was a particularly marked decline in the ratio for the distal segments as germination proceeded. In the first (tip) segment, for example, the ratio decreased from 0.183 at 36 hours to 0.043 at 96 hours. The data for the incorporation/uptake ratios therefore present a similar picture to that obtained from the straightforward incorporation data. Statistical analysis using ANOVA, in which the effects of location (segment number from tip), seedling group and germination periods were investigated (using data from the 1, 2, 3 and 4 segment number to omit missing values) showed that I/U ratio was strongly dependent upon location (root segment number) ($P < 0.001$), seedling group ($P < 0.001$) and upon germination period ($P < 0.001$). In addition, there were significant interactions between the effects of segment number and seedling group ($P < 0.001$) and between segment number and germination period ($P < 0.001$).

5.3.3 DNA levels in whole roots and in root tips

The results from the DNA analyses of whole roots (selected by Method 5D; see Section 5.2.1) at different times of germination are presented in Table 5.9 and Figure 5.5. This table shows that the DNA contents at 24 hours germination were 344 and 368 μg (10 roots)$^{-1}$ in the long and short roots respectively. These values decreased markedly with increasing germination time. At 96 hours, the contents of the long and short roots were only 105 and 137 μg (10 roots)$^{-1}$ respectively. It is concluded that the younger roots contained much more DNA as compared with older roots. The differences between long and short roots were much less, however, although short roots appear to contain more DNA than the long roots. When the data were analyzed statistically by ANOVA, short

Table 5.9 DNA levels in whole roots at different germination times.

Germin[a]	Long roots			Short roots		
period(h)	mean	±sd	±se	mean	±sd	±se
24	344	30.5	7.6	368	23.6	5.9
36	317	17.6	4.4	342	40.0	10.0
48	257	82.0	20.5	307	45.4	11.4
60	173	15.3	3.8	218	22.8	5.7
72	140	7.4	1.9	194	36.3	9.1
84	105	13.0	3.3	137	15.8	4.0

Each value is the mean DNA content [μg(10 roots)$^{-1}$] ±sd and ±se from 4 separate experiments with 4 replicate analyses in each experiment.

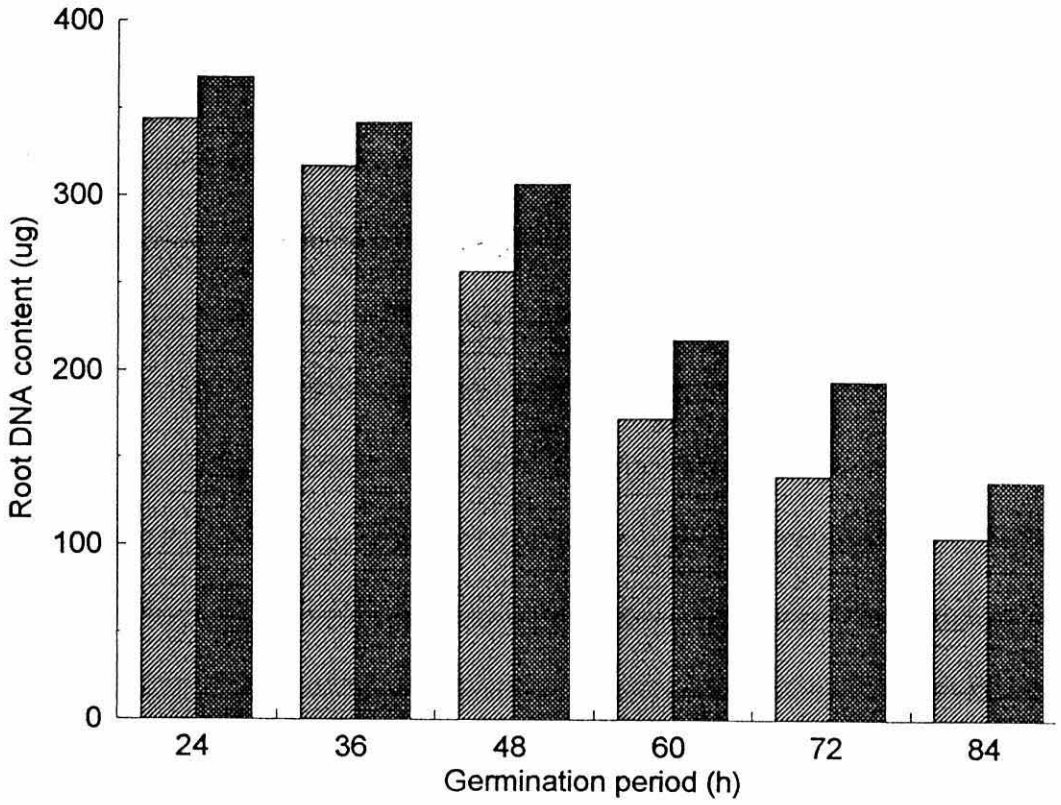


Fig. 5.5. DNA levels in whole roots at different germination times. ▨, long roots; ▩, short roots.

roots were always found to contain significantly more DNA than long roots ($P = 0.001$) and root DNA levels were negatively related to the age of the seedlings ($P < 0.001$).

An experiment was carried out to look more carefully at the relationship between root length and the DNA content. Two groups of roots (8-10 and 2-4 cm long) from 84 hour seedlings were used (Method 5B; see Section 5.2.1). These roots were deliberately selected to be longer and shorter than the roots used for the experiment reported in Table 5.9. The results for the experiment are presented in Table 5.10. The table shows that the mean DNA levels for 8-10 and 2-4 cm roots were 91 and 246 μg (10 roots)$^{-1}$. These results show that the DNA content was more in the short roots and less in the long roots. The difference was surprisingly large compared with the differences seen in Table 5.9. Statistical analysis (T-test) of the data in Table 5.10 showed that the difference between the two groups of roots was highly significant ($P < 0.001$).

For a better overview of the relationship between root length and DNA content, the values for the 84 hour long and short roots from the previous experiment (Table 5.9) were fitted together with the values in Table 5.10. The combined data are presented in Table 5.11. The mean lengths of the roots from Table 5.9 are included in order to make the comparisons easier. This table shows that the DNA levels for 8-10, 7, 5 and 2-4 cm roots were 91, 105, 137 and 246 μg (10 roots)$^{-1}$. It is concluded that the DNA content of the roots decreased with increasing root length. These results complement the results in (Table 5.9), which merely suggested that DNA content was higher in short roots as compared with long roots. Statistical analysis (ANOVA) of the data in Table 5.11 showed a negative relationship between DNA content and root length which was highly significant ($P < 0.001$).

Table 5.10 DNA levels in whole roots at 84 hours germination.

	Root length (cm)	
	8-10	2-4
mean	91	246
±sd	12.0	17.8
±se	3.0	4.5

Each value is the mean DNA content [μg(10 roots)$^{-1}$] ±sd and ±se from 4 separate experiments with 4 replicate analyses in each experiment).

Table 5.11 DNA levels in whole roots at 84 hours germination.

	Root length (cm)			
	8-10	7	5	2-4
mean	91	105	137	246
±sd	12.0	13.0	15.8	17.8
±se	3.0	3.3	4.0	4.5

Each value is the mean DNA content [μg(10 roots)$^{-1}$] ±sd and ±se from 4 separate experiments with 4 replicate analyses in each experiment.

A final experiment was carried out in which DNA levels were determined in 1 cm tip segments and separately in the remaining part of the root (Method 5E; see Section 5.2.1). The results from this experiment are presented in Table 5.12 and Figure 5.6. They show that the DNA contents for the tip segment of long roots were 88.9, 74.4, 75.7 and 49.9 μg (10 tips)$^{-1}$ at 36, 48, 60 and 72 hours respectively. The corresponding values for the tip segment of short roots were 394.1, 96.8, 76 and 62.6 μg (10 tips)$^{-1}$. The DNA values for the remaining root behind the tip for long roots at 36, 48, 60 and 72 hours were 249.4, 211.1, 132.1 and 123.4 μg (10 roots)$^{-1}$. At the same time, the DNA values for the remaining part of the short roots were 304.9, 211.1 and 164.4 μg (10 roots)$^{-1}$ at 48, 60 and 72 hours, respectively. These results confirm the conclusions drawn from Tables 5.9 and 5.10 that the DNA content was higher in short roots as compared with long roots ($P = 0.006$ using ANOVA) and that it decreased with increasing germination time ($P < 0.001$ by ANOVA). Further, these differences between long and short roots can be seen both in the tip segment of the roots and in the greater length of the root behind the tip. It was also noted that the DNA content was less in the tip than in the remaining part of the root throughout germination.

5.4 Summary and Discussion

Since in the experiments described the incorporation of ^{3}H-thymidine was almost the same in the tips of roots of all lengths at later germination times, it can be assumed that the rate of cell division was similar in long and short roots. This conclusion is supported by Gifford and Kurth (1982), who reported that apical cells of *Equisetum scirpoides* Michx. are as active mitotically in long roots as in short root lengths. There was a difference in incorporation values between long and short roots at 24 and 36 hours germination, however, indicating that the rate of cell division during early germination

Table 5.12 DNA levels in root tips and in the remaining roots at different germination times.

Germin[a] period (h)		Long roots mean	±sd	±se	Short roots mean	±sd	±se
36	T	88.9	16.02	4.00	394.1	56.70	14.18
	BT	249.4	28.16	7.04	-	-	-
48	T	74.4	19.80	4.95	96.8	11.24	2.81
	BT	211.1	93.06	23.27	304.9	21.30	5.33
60	T	75.7	11.56	2.89	76.0	18.03	4.51
	BT	132.1	12.27	3.07	211.8	73.64	18.41
72	T	49.9	5.74	1.44	62.6	12.40	3.10
	BT	123.4	17.06	2.27	164.4	23.91	5.98

Each value is the mean DNA content [μg(10 T or BT)$^{-1}$] ±sd and ±se from 4 separate experiments with 4 replicate analyses in each experiment. T, 1cm root tip; BT, remaining root behind tip.

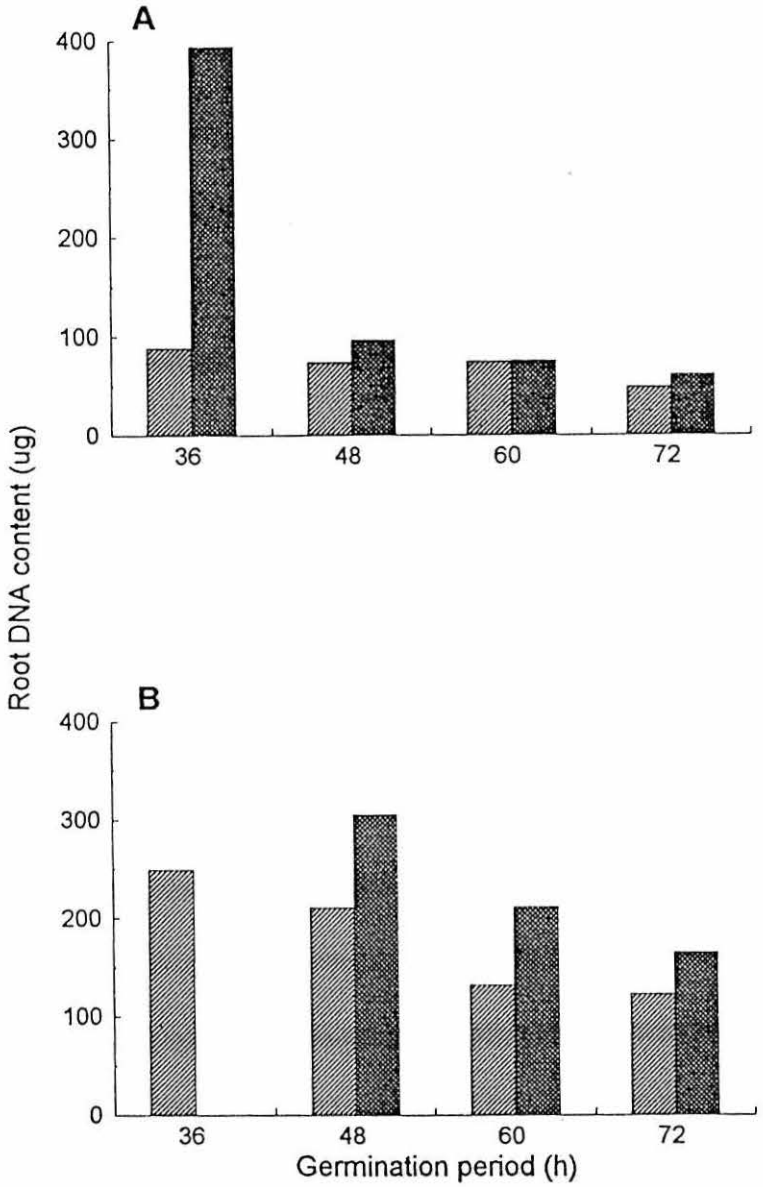


Fig. 5.6. DNA levels in the root tips and in the remaining root at different germination times. A, root tip; B, remaining root; ▨, long roots; ▩, short roots.

was greater in long roots as compared to short root. This difference is probably not a significant direct contributor to the differences in root elongation, however, because short roots contained slightly more DNA than long ones did (Table 5.9); The reverse of what would be expected from the incorporation data. The present results also show that incorporation values were higher in younger (24-48h) roots than in older (60-96h) roots. These results are in agreement with the results of Hecker *et al.* (1979); they reported that cell division in the *Agrostemma githago* L. seedling root tip is mainly confined to the earliest part of germination.

Incorporation was also active in the segments further away from the tip, which suggests that another meristemic region is also present in the cotton root. When the seedlings were left to grow beyond 96 hour, lateral roots appeared in this region (see Plate 3.3), indicating that the (Me-^{3}H) thymidine incorporation in the upper segments of the root is a reflection of the initiation of these lateral roots.

In the experiment to study incorporation in a wider range of root lengths (Table 5.5) a rather confusing set of results were obtained. This is probably due to the fact that the data were collected from two separate experiments which were conducted several months apart. Some of the observed differences might, therefore, be due to deterioration of the seed during storage. Nevertheless, it is safe to say that root length has only a small or even no effect on (Me-^{3}H) thymidine incorporation at 84 hours germination.

The amount of total DNA was slightly different in long and in short roots. Similar results were reported by Gifford and Kurth (1982) using a microspectrophotometric methods for the measurement of DNA. They also showed that there was no consistent

difference in DNA in apical tissue or in the other meristematic tissue of roots of different lengths. Their results support our view that cell division is not a major factor in determining the rate of root growth. This view is also supported by Green (1976), who reported that cell division does not result in extension; rather cell division provides the raw materials for subsequent cell expansion, but it does not itself drive growth. Furthermore, the amount of DNA was greater in younger and less in older roots. When this observation is put together with the thymidine incorporation data, it suggests that the actual rate of cell division is small compared with the total number of cells present in the root at the beginning of germination. It also indicates that there is a loss or death of significant numbers of cells from the root during elongation.

Similar results to the present ones have been reported for other species. Birdsall and MacLeod (1990) reported that, in onion roots, apical meristem activity (in terms of mitotic index, the percentage of cells undergoing DNA synthesis and cell doubling time) was maximal within a few days of emergence in both the primary and adventitious roots, but it gradually decreased during subsequent growth. Similarly, Bernhardt *et al.* (1984); reported that DNA synthesis in cotyledons reaches its maximum after 24 hour germination, thereafter decreasing to a lower level. To summarise the present results, they suggest that cell elongation has a greater importance than cell division for root elongation in cotton seedlings. On the other hand, a secondary region of cell division in the proximal region of the root is probably of great importance for the initiation of lateral root growth.

CHAPTER SIX

Chapter Six

Soluble and Insoluble Peroxidase Activities

6.1 Introduction

The root is an important organ of the plant and it plays a major role in the plant's growth and development. The growth of the root is dependent on its elongation rate which is the result of cell enlargement. When a plant cell enlarges, changes must take place in the cell wall to accommodate the expansion. During this cell wall extension, the bonds which hold the various wall components together need to be broken and reformed. These reactions are believed to be catalysed by a variety of cell wall enzymes, such as peroxidases, pectinesterases, galactosidases, arabinosidases, glucosidases and xylosidases (see Section 1.11).

Peroxidase is one of the most prominent among the cell wall enzymes and evidence is available which indicates that it plays an important role in cell wall extension by modifying the plastic characteristics of the walls (Masuda and Pilet, 1983; Wieser and Pilet, 1984). The enzyme has been found in wheat root cell walls, particularly the outer epidermal cell wall (Smith and O'Brien, 1979). In maize, peroxidase activity has also been found in the epidermis, hypodermis and endodermis of mature roots (Augeri *et al.*, 1991). An investigation of seven species of grasses revealed peroxidase activity in all cell types of the root (Avers and Grimm, 1959). Thus, it seems to be widely occurring.

Evidence exists that peroxidase in cell walls is concerned with the polymerisation of phenolic compounds (see Section 1.11). Lagrimini (1991); Chabbert *et al.* (1992); Mader and Fussl (1982) obtained several transgenic lines of tobacco with significant

over expression or down regulation of a specific anionic peroxidase supposed to be involved in lignification. They further reported that wound-induced lignification occurred 24-48 hours sooner in plants over expressing the anionic peroxidase. Lamport (1986) has also reported that peroxidases are involved in lignification and the crosslinking of extensin monomers and feruloylated polysaccharides. Further evidence has been presented favouring the involvement of peroxidase in the polymerization of monoligonals in differentiating xylem tissues (Harkin and Obst, 1973). In the elongating region of maize roots, where the peroxidase isozyme is associated with the hemicellulose fraction of the cell wall (Grison and Pilet, 1985), it may produce binding between hemicellulose and phenolic substances as observed in tobacco by Fry (1979). Peroxidase may also cause wall tightening following osmotic stress, but so far no isozyme has been found which correlates with this process (MacAdam *et al.*, 1992).

At the more molecular level, peroxidase catalyses the formation of covalent cross links between phenolic cell wall components, for example dityrosine bridges between the tyrosine sub-units of extensin (Fry, 1987) and diferulate an intramolecular bond of pectin (Wallace and Fry, 1993). It has been also reported that the peroxidase associated with horseradish cell walls catalyses the formation of H_2O_2 in the presence of NADH (Gross, 1977). This H_2O_2 is the likely oxidising agent needed to initiate the polymerisation of the phenolic residues.

Although many general investigations of plant peroxidase have been reported, only a few definite biochemical functions of particular isoperoxidases are known (Espelie *et al.*, 1986; Nakano and Asada, 1987). In horseradish root, for example, peroxidase occurs in seven isozymes forms that differ not only in electrophoretic mobility but also

in their catalytic properties (Shannon, 1968). Schloss *et al.* (1987) have further revealed that all of the peroxidase isozymes are secretory proteins, because they have to be transported from the site of synthesis in the cytoplasm to the sites of function, the extra cytoplasmic spaces (cell wall and vacuole).

In tobacco there are 12 isozymes classified into three groups; the anionic, the moderately anionic and the cationic. Each group appears to have a different function in the cell. The cationic isozymes are situated in the central vacuole (Mader, 1986), where they catalyse the synthesis of H_2O_2 from NADH and water (Mader *et al.*, 1980). These isozymes have also an indoleacetic acid-oxidase activity in the absence of H_2O_2 (Grambow and Schwich, 1983). They may also provide H_2O_2 to other peroxidase isozymes (Mader *et al.*, 1980), but their actual function in the plant is unclear. The moderately anionic peroxidase isozymes are located in the cell wall and they possess moderate activity towards lignin precursors. They are also strongly accumulated following wounding in tobacco stem (Lagrimini and Rothstein, 1987). The function of the anionic isoperoxidases are better understood. These isozymes are also associated with the cell wall and they have a high *in vitro* activity in the polymerisation of the lignin-precursor cinnamyl alcohol (Mader *et al.*, 1977). They therefore appear to function in the lignification process.

Because of the obvious importance of peroxidase in cell wall metabolism, it was decided to determine peroxidase (both soluble and cell wall) activities in our cotton seedling roots. Although some of the other cell wall enzymes might be more important than peroxidase in the cell elongation process (see Section 1.11), they are often more difficult to assay. Also, the necessary substrates for these other enzymes were not easily

available and shortage of time restricted our study to one enzyme. Peroxidase should ideally be assayed using ferulic acid as substrate, because ferulic acid occurs naturally complexed to carbohydrates in cell walls. This was tried in some preliminary experiments, but the levels of activity were too low to measure accurately. Instead, guaiacol was used because it gave higher activities and it was a convenient substrate to use.

6.2 Methods

6.2.1 Selection of seedling root groups

Three methods were used for the selection of seedling root groups according to root length.

Method 6A: Batches of 100 seeds were germinated for 24, 36, 48, 60, 72 and 84 hours germination as described in Section 2.6. Upon harvesting, their root lengths were measured (Section 2.8) and they were divided into four quartiles according to length. The 25 longest roots (first quartile) and the next 25 roots (second quartile) were retained for the peroxidase assay. The third and fourth quartiles were of poor quality and many were ungerminated; they were therefore discarded. The roots were dissected to obtain 2 cm root tips, except the short roots at 24 hours which were less than 2 cm long in total. These were weighed (fresh weight) and they were then used for the peroxidase (soluble and cell wall) determinations.

Method 6B: Batches of 400 seeds were germinated for 48 hours (Section 2.6). After harvesting, ungerminated seeds or seedlings with very short roots were discarded

and 200 well-germinated seedlings were selected for study. Their root lengths were measured (Section 2.8) and they were divided into 8 groups (quantiles) (25 roots in each group) according to root length. Two centimetre tips were dissected from the retained roots, their fresh weight was determined and they were then used for peroxidase (soluble and cell wall) determinations.

Method 6C: Batches of 600 seeds were germinated for 48 hours as described in Section 2.6. After harvesting, two groups of roots, long (4 cm) and short (2 cm), were selected with 25 roots of the same length in each group. The roots were then dissected so that the first and second segments were 0.5 cm long and the other (third, fourth and fifth) segments were of 1 cm in length. The cut segments were given numbers starting from the growing tip end. The segments with same number were grouped together for fresh weight and enzyme activity determinations.

6.2.2 Determination of peroxidase activity

The detailed procedure for this experiment is described in Section 2.15.

6.3 Results

6.3.1 Root lengths and root tip fresh weights at different germination times

Table 6.1 and Figure 6.1A contain the results for root length in the first and second quartiles (Method 6A; see Section 6.2.1) used for peroxidase analysis. The length of both groups of roots increased with increasing germination time, as expected. The first quartile roots were also longer than the second quartile, as expected. At 24 hours germination, for example, the root lengths for the first and second quartiles were 1.29

and 0.78 cm. The lengths measured at 84 hours germination were 6.87 and 4.80 cm. These data are entirely as expected and they compare well with those from the experiments described in Chapter 3.

The fresh weights of 2 cm root tips from the first and second quartiles are presented in Table 6.2 and Figure 6.1B. Root tip fresh weights for the first quartile were 0.61, 0.81, 0.50, 0.39, 0.31 and 0.28 g (25 roots)$^{-1}$ at 24, 36, 48, 60, 72 and 84 hours germination respectively. The corresponding values for the second quartile were 0.41, 0.87, 0.86, 0.55, 0.50 and 0.45 g (25 roots)$^{-1}$. These results show the tip fresh weights increased between 24 and 36 hours and then decreased as germination proceeded. This decrease in root tip fresh weights after 36 hours was more prominent in the first quartile. It was also observed that the second quartile had a higher tip fresh weight compared with first quartile at all times except at 24 hours when the opposite was true. Statistical analysis of the tip fresh weight data, using ANOVA in which the effect of seedling group (quartile) and germination time were investigated, showed that the parameter was highly dependent upon both seedling group and germination time ($P < 0.001$ in each case). There was also a highly significant interaction between seedling group and germination time ($P < 0.001$).

6.3.2 Peroxidase activities in root tips at different germination times

The results of an experiment in which seedling roots were selected into two groups (quartiles) (Method 6A; see Section 6.2.1) for soluble peroxidase activity in the root tips are presented in Table 6.3. They show that the activity calculated on a per root basis (Figure 6.2A) decreased in both quartiles as germination time increased, although the decrease in the second quartile was delayed until after 36 hours. The first quartile

Table 6.1 Root length at different germination times.

Germin^n	First quartile			Second quartile		
period(h)	mean	±sd	±se	mean	±sd	±se
24	1.29	0.23	0.023	0.78	0.19	0.019
36	3.04	0.41	0.041	2.30	0.55	0.055
48	3.73	0.46	0.046	2.47	0.46	0.046
60	5.01	0.79	0.079	3.82	0.61	0.061
72	5.89	0.80	0.080	3.99	0.52	0.052
84	6.87	1.05	0.105	4.80	0.88	0.088

Each value is the mean length (cm) ±sd and ±se from 4 separate experiments. In each experiment there were 25 roots in each quartile.

Table 6.2 Root tip fresh weight at different germination times.

Germin^n	First quartile			Second quartile		
period(h)	mean	±sd	±se	mean	±sd	±se
24	0.608	0.027	0.014	0.413	0.047	0.024
36	0.816	0.098	0.049	0.869	0.108	0.054
48	0.501	0.040	0.020	0.865	0.042	0.021
60	0.392	0.073	0.036	0.555	0.112	0.056
72	0.308	0.053	0.027	0.503	0.106	0.053
84	0.285	0.065	0.032	0.450	0.110	0.055

Each value is the mean fresh weight [g(25 roots)$^{-1}$] ±sd and ±se from 4 separate experiments. In each experiment there were 25 roots in each quartile.

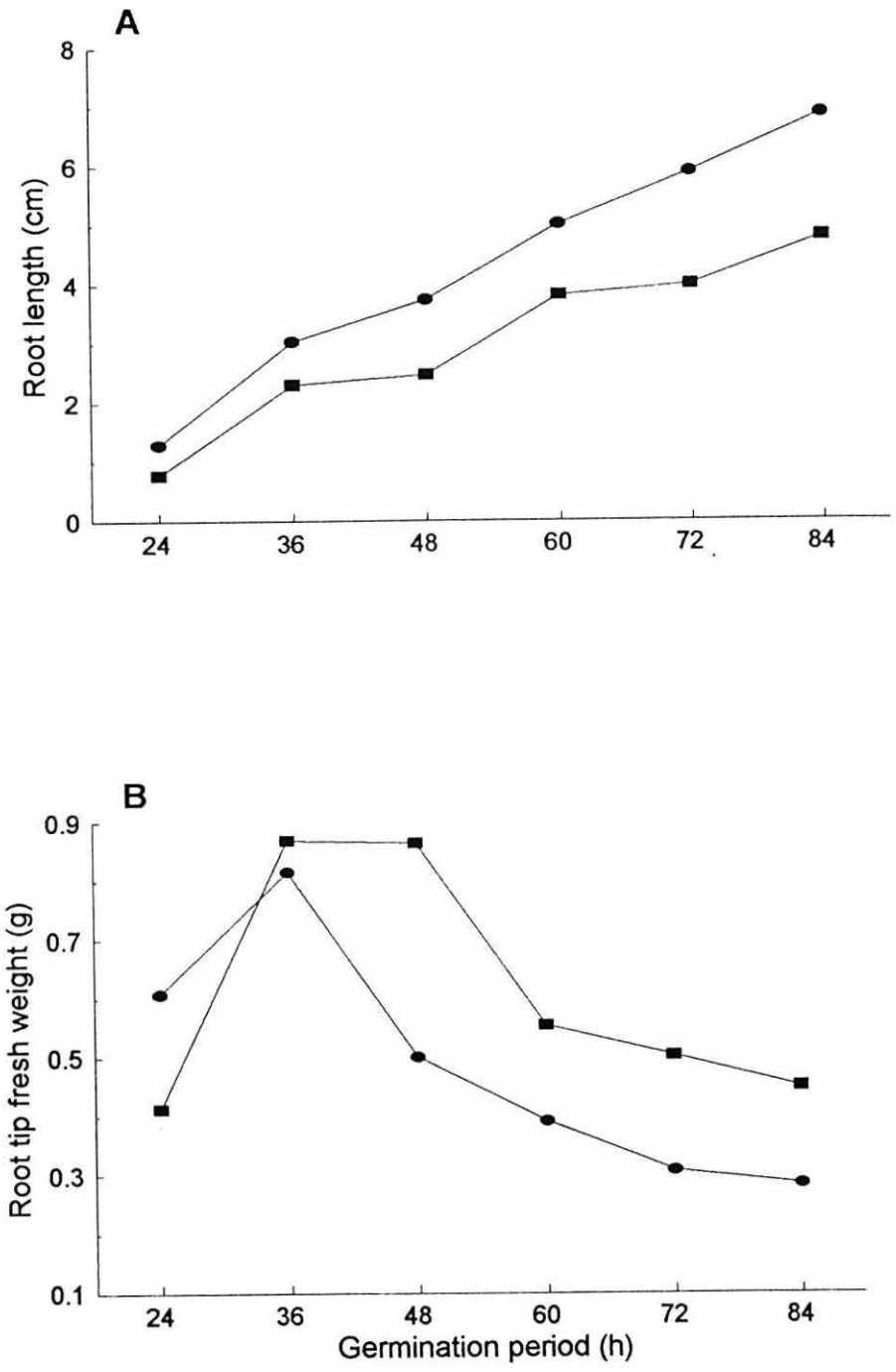


Fig. 6.1. Root length and tip fresh weight at different germination times. A, root length; B, root tip fresh weight; ●, first quartile; ■, second quartile.

showed higher activities than the second quartile. At 24 hours germination, for example, the long roots had an activity of 9.4 $\Delta A\ min^{-1}$ $(25\ roots)^{-1}$, while the group with the shorter roots had an activity of 6.9 $\Delta A\ min^{-1}$ $(25\ roots)^{-1}$. The values for 84 hour seedlings were 4.6 and 3.5 $\Delta A\ min^{-1}$ $(25\ roots)^{-1}$ in the first and second quartile, respectively. Statistical analysis of the soluble peroxidase activity in data, using ANOVA in which the effect of seedling group (quartile) and germination time (age of the root) were investigated, showed that the parameter was highly dependent upon both seedling group and germination time ($P < 0.001$ in each case).

The enzyme activities are also presented on a per gram fresh weight basis in Table 6.3 and Figure 6.2B. The mean activity values for the first quartile were 15.3, 9.9, 14.8, 14.3, 19.2 and 16.1 $\Delta A\ min^{-1}$ $(g\ fresh\ wt.)^{-1}$ at 24, 36, 48, 60, 72 and 84 hours, respectively. For the second quartile, the activities were 16.8, 8.8, 7.7, 8.3, 9.4 and 8.5 $\Delta A\ min^{-1}$ $(g\ fresh\ wt.)^{-1}$. These data show that the enzyme activity for both quartiles decreased sharply between 24 and 36 hours. In the first quartile, the activity then increased again up to its original level and remained high through the rest of germination. In the second quartile, the activity which had decreased between 24 and 36 hours remained low throughout the rest of germination. ANOVA was undertaken even though the variance of the results increased substantially with germination time. The analysis confirmed that both the root group and the age of the root had highly significant ($P < 0.001$ in each case) effects on the per gram soluble peroxidase activity and it showed that there was a significant interaction between root group and germination time ($P = 0.019$).

The results for the insoluble (cell wall) peroxidase activity in the root tips are presented

Table 6.3 Root tip soluble peroxidase activity at different germination times.

Germin[a] period (h)	Enzyme activity: ΔA min^{-1}(25 roots)$^{-1}$ First quartile mean	±sd	±se	Second quartile mean	±sd	±se	ΔA min^{-1}(g fresh wt.)$^{-1}$ First quartile mean	±sd	±se	Second quartile mean	±sd	±se
24	9.42	0.48	0.11	6.87	0.59	0.14	15.3	0.89	0.21	16.8	1.16	0.27
36	8.24	0.50	0.12	7.49	0.43	0.10	9.9	0.79	0.19	8.8	1.26	0.29
48	7.32	0.40	0.09	6.54	0.68	0.16	14.8	0.88	0.21	7.7	0.72	0.17
60	5.84	0.64	0.15	4.59	0.30	0.07	14.3	1.91	0.45	8.3	2.09	0.49
72	5.68	0.34	0.08	4.24	0.50	0.12	19.2	4.19	0.99	9.4	3.34	0.79
84	4.58	0.31	0.07	3.49	0.39	0.09	16.1	3.60	0.85	8.5	2.84	0.67

Each values is the mean activity ±sd and ±se from 3 separate experiments with 6 replicate analyses in each experiment.

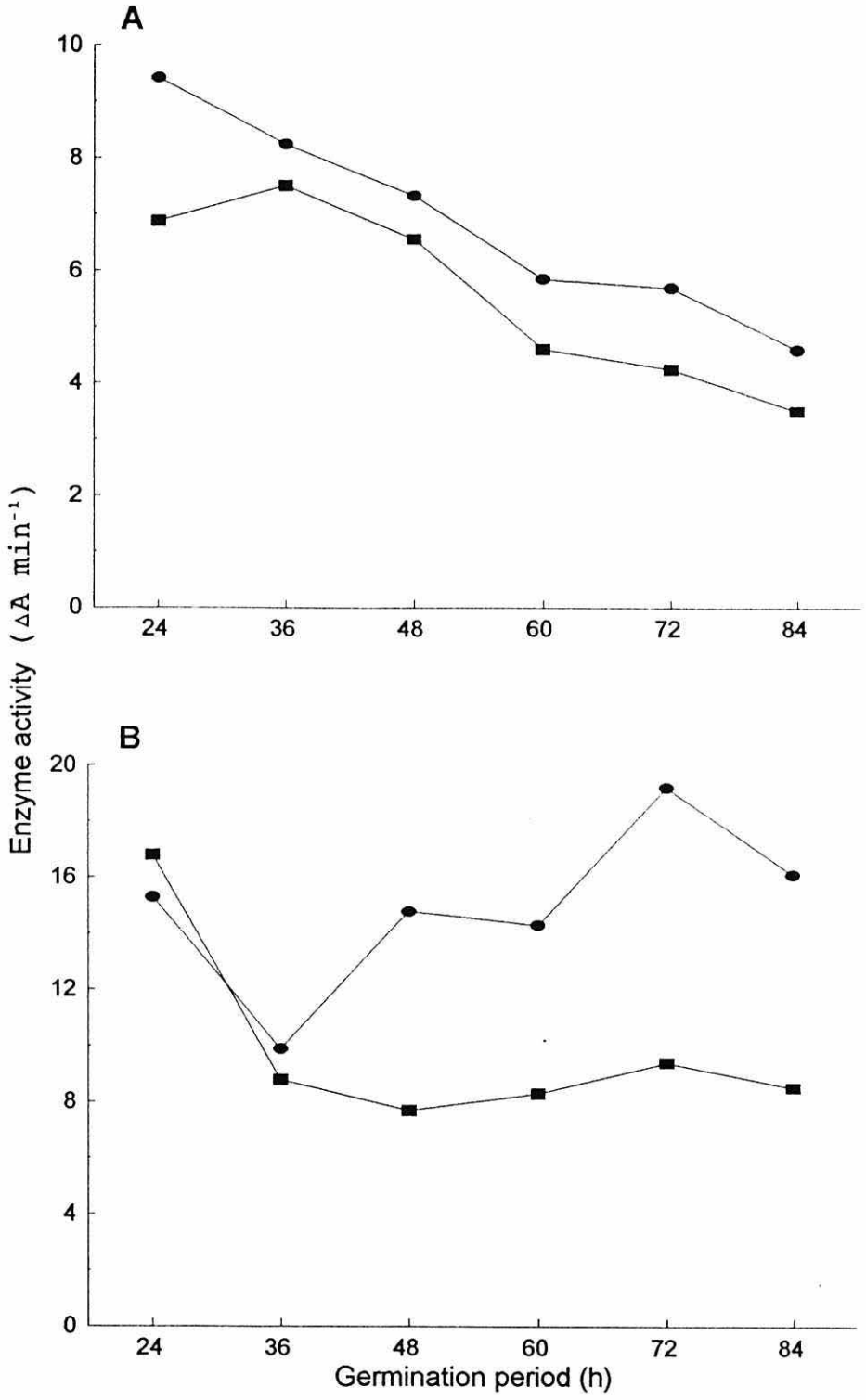


Fig. 6.2. Root soluble peroxidase activity at different germination times. A, per 25 roots; B, per gram fresh weight; ●, first quartile; ■, second quartile.

in Table 6.4 and Figure 6.3. On a 25 root basis (Figure 6.3A), the enzyme activity values for the first quartile were 2.0, 7.2, 6.9, 12.8, 12.7 and 11.1 $\Delta A\ min^{-1}$ $(25\ roots)^{-1}$ at 24, 36, 48, 60, 72 and 84 hours, respectively. For the second quartile the corresponding values were 1.6, 6.4, 5.8, 10.7, 11.7 and 13.5. These results show that the enzyme activity in both quartiles increased as germination time increased, except between 36 and 48 hours, when it dipped slightly. The first quartile showed higher activities than the second quartile, except at 84 hours where the activity was greater in the second quartile. Statistical analysis (using ANOVA) of the data showed that the root group had an insignificant effect ($P = 0.12$), whereas the age of the root had a highly significant effect ($P < 0.001$) on insoluble peroxidase activity. In addition, there was a significant interaction between root group and germination time ($P = 0.008$).

When the cell wall enzyme activities are expressed on a per gram fresh weight basis (Table 6.4 and Figure 6.3B), the values increased as germination proceeded for both the long and short root groups. The values for the first quartile were greater than those for the second quartile at all times except 24 hours. The mean values for the first and second quartiles were 3.3 and 3.8 $\Delta A\ min^{-1}$ $(g\ fresh\ wt.)^{-1}$ at 24 hours respectively, increasing to 39.3 and 32.4 $\Delta A\ min^{-1}$ $(g\ fresh\ wt.)^{-1}$ at 84 hours. The data were logged before being subjected to ANOVA in order to minimise the relationship between mean values and their variances. It was concluded from this analysis that both root length (seedling group) ($P = 0.001$) and the age of the roots ($P < 0.001$) had significant effects on the cell wall enzyme activity.

6.3.3 Root lengths and root tip fresh weights at 48 hours germination

In this experiment and in the peroxidase experiment described in the next section, 48

Table 6.4 Root tip insoluble (cell wall) peroxidase activity at different germination times.

Germin[a] period (h)	Enzyme activity: ΔA min^{-1}(25 roots)$^{-1}$ First quartile mean	±sd	±se	Second quartile mean	±sd	±se	ΔA min^{-1}(g fresh wt.)$^{-1}$ First quartile mean	±sd	±se	Second quartile mean	±sd	±se
24	2.0	0.21	0.05	1.6	0.26	0.06	3.3	0.23	0.05	3.8	0.22	0.05
36	7.2	1.37	0.32	6.4	1.26	0.29	8.7	1.89	0.44	7.4	0.74	0.17
48	6.9	0.77	0.18	5.8	0.50	0.12	13.9	1.97	0.46	6.9	0.59	0.14
60	12.8	1.23	0.29	10.7	0.78	0.18	31.3	2.74	0.65	19.2	3.87	0.91
72	12.7	1.14	0.27	11.7	0.82	0.19	42.7	7.64	1.80	25.1	5.69	1.34
84	11.1	0.31	0.07	13.5	0.87	0.20	39.3	8.03	1.89	32.4	9.25	2.18

Each values is the mean activity ±sd and ±se from 3 separate experiments with 6 replicate analyses in each experiment.

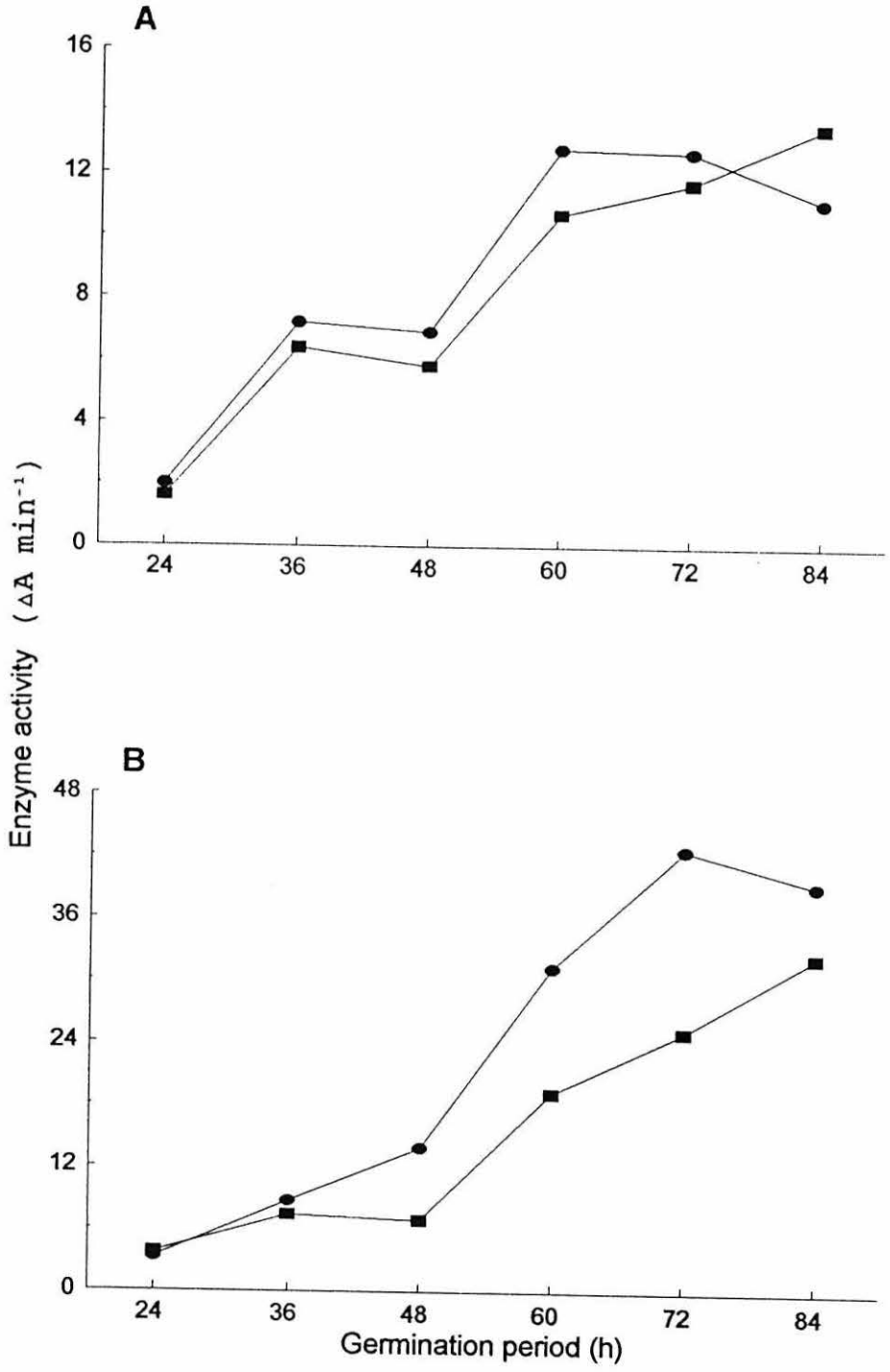


Fig. 6.3. Root insoluble (cell wall) peroxidase activity at different germination times. A, per 25 roots; B, per gram fresh weight; ●, first quartile; ■, second quartile.

hour seedlings were divided into eight groups (quantiles) according to their root length (Method 6B; see Section 6.2.1). This allowed a more detailed study of the relationship between root length and enzyme activity to be made.

Table 6.5 and Figure 6.4A show the root lengths of the eight quantiles studied. The lengths from the first through to the eighth quantile were 4.64, 4.00, 3.67, 3.42, 3.16, 2.91, 2.60 and 2.24 cm. The mean length of shortest roots was less than half that of longest roots. Statistical analysis of the data using ANOVA showed that the difference in root lengths between the quantiles was highly significant ($P < 0.001$).

The fresh weight of the 2 cm root tips from the various quantiles are also presented in Table 6.5 and in Figure 6.4B. They show that the fresh weights from the first through to the eighth quantile were 0.35, 0.38, 0.41, 0.49, 0.53, 0.59, 0.70 and 0.87 g $(25 \text{ roots})^{-1}$. Thus, the tip fresh weight increased as the length of the root decreased. The fresh weight of the tips from the shortest roots was almost double that of the longest. ANOVA showed that the negative relationship between root tip fresh weight and root length was highly significant ($P < 0.001$).

6.3.4 Peroxidase activities in root tips at 48 hours germination

The results of an experiment in which seedling roots were classified into 8 groups (quantiles) (Method 6B; Section 6.2.1) are presented in Table 6.6 and Figure 6.5A. They show that the soluble peroxidase activities were 8.75, 8.33, 8.14, 7.55, 7.76, 7.36, 7.52 and 7.93 $\Delta A \text{ min}^{-1}$ $(25 \text{ roots})^{-1}$ in the first through to the eighth quantile. It can be seen that the activity undergoes a progressive decrease with increase in quantile number. These changes were all rather small, however, and analysis of variance showed

Table 6.5 Root length and tip fresh weight at 48 hours germination.

	Quantile number							
	1	2	3	4	5	6	7	8
Root length:								
mean	4.64	4.00	3.67	3.42	3.16	2.91	2.60	2.24
±sd	0.44	0.20	0.18	0.16	0.18	0.23	0.26	0.26
±se	0.04	0.02	0.02	0.02	0.02	0.02	0.03	0.03
Root tip fresh weight:								
mean	0.35	0.38	0.41	0.49	0.53	0.59	0.70	0.87
±sd	0.021	0.008	0.04	0.04	0.04	0.06	0.13	0.10
±se	0.012	0.004	0.02	0.02	0.02	0.03	0.08	0.06

Each value is the mean root length (cm) or root tip fresh weight [g(25 roots)$^{-1}$] ±sd and ±se from either 3 or 4 separate experiments. In each experiment there were 25 roots in each quantile.

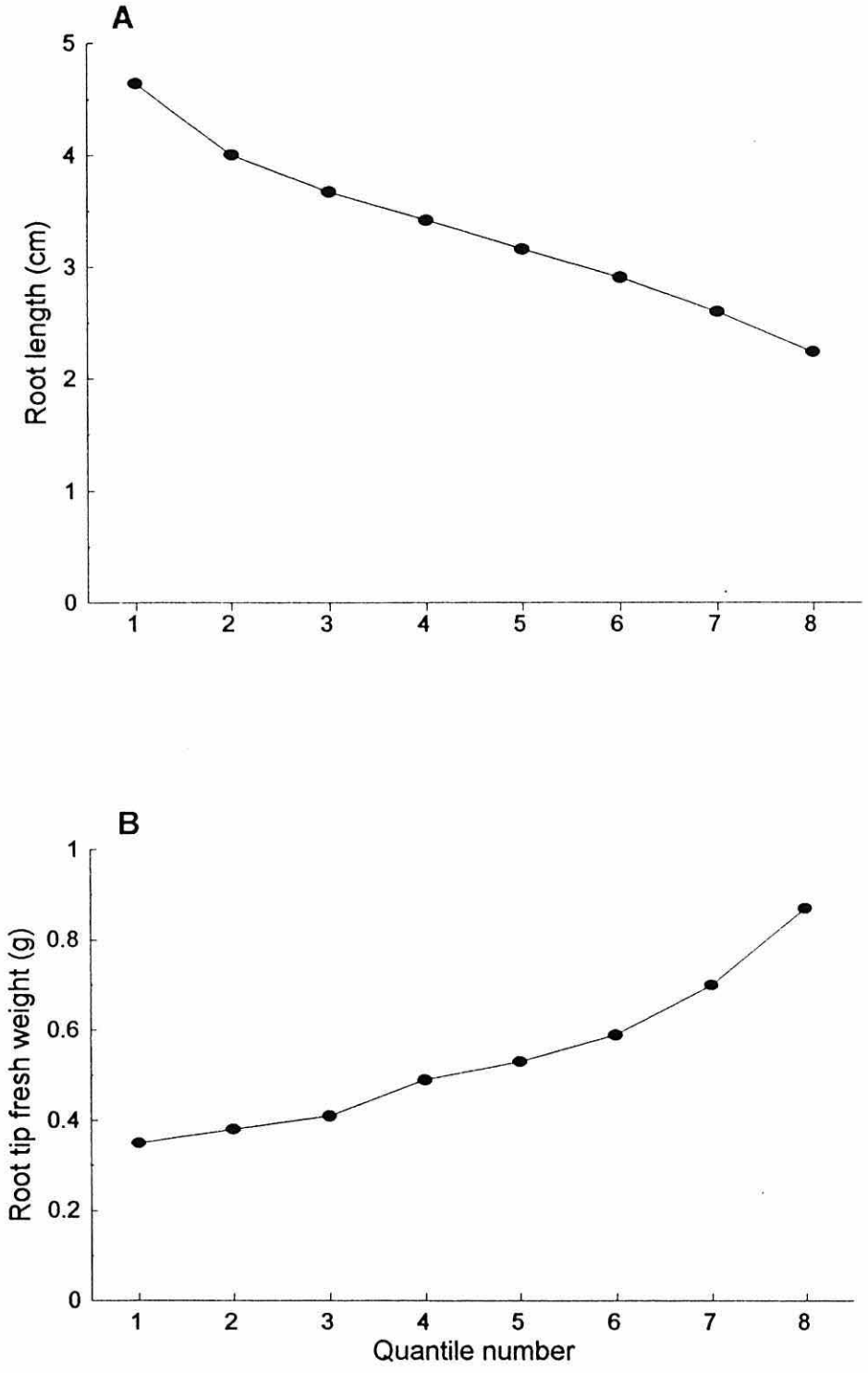


Fig. 6.4. Root length and tip fresh weight at 48 hours germination. A, root length; B, root tip fresh weight.

that the root group (quantile) had an insignificant effect ($P = 0.40$) on soluble peroxidase activity.

Differences in activity are more obvious when expressed on a per gram fresh weight basis. Here the activities were 25.4, 21.7, 19.8, 15.5, 14.4, 12.5, 10.9 and 9.19 ΔA min^{-1} (g fresh wt.)$^{-1}$ in the first through to the eighth quantile. In this case, the activities decreased progressively with decreasing root length ($P < 0.001$ using ANOVA). The decrease from the first to the eighth quantile was about 64%.

The results for the insoluble (cell wall) peroxidase (Table 6.6 and Figure 6.5B) show that the activities were 5.93, 6.42, 6.50, 7.15, 7.96, 8.58, 9.56 and 9.43 ΔA min^{-1} (25 roots)$^{-1}$ in the first through to the eighth quantile, respectively. Thus, there was about 59% increase from the first through to the eighth quantile. In other words, this activity was greater in short than in long roots ($P = 0.009$ by ANOVA).

When the cell wall peroxidase results were calculated on the basis of gram fresh weight, the trend was reversed. The activities were then 17.1, 16.8, 15.7, 14.7, 15.0, 14.5, 13.8 and 10.8 ΔA min^{-1} (g fresh wt.)$^{-1}$ for the first through to the eighth quantile. These results show that the enzyme activity on a per gram basis decreased progressively with decreasing root lengths ($P = 0.01$ using ANOVA). The decrease from the first to the eighth quantile was about 37%.

6.3.5 Fresh weights of different root segments at 48 hours germination

The experiment in this section and in the next section were designed to study the distribution of peroxidase activities along the length of seedling root. Two groups of

Table 6.6 Root tip peroxidase activities at 48 hours germination.

	Enzyme activity															
	$\Delta A\ min^{-1}(25\ roots)^{-1}$								$\Delta A\ min^{-1}(g\ fresh\ wt.)^{-1}$							
	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8
Soluble peroxidase:																
mean	8.75	8.33	8.14	7.55	7.76	7.36	7.52	7.93	25.4	21.7	19.8	15.5	14.4	12.5	10.9	9.19
±sd	0.87	0.92	0.94	0.45	0.68	0.53	0.68	0.55	3.58	1.99	2.66	1.48	1.21	0.65	1.13	1.33
±se	0.20	0.22	0.22	0.10	0.16	0.12	0.16	0.13	0.84	0.46	0.63	0.34	0.28	0.15	0.27	0.31
Insoluble (cell wall) peroxidase:																
mean	5.93	6.42	6.50	7.15	7.96	8.58	9.56	9.43	17.1	16.8	15.7	14.7	15.0	14.5	13.8	10.8
±sd	0.61	0.35	0.74	0.73	0.80	1.53	1.38	1.36	1.29	1.20	0.92	1.19	1.45	1.75	2.36	1.26
±se	0.14	0.08	0.17	0.17	0.19	0.36	0.32	0.32	0.30	0.28	0.21	0.28	0.34	0.41	0.55	0.29

Each value is the mean activity ±sd and ±se from 3 separate experiments with 6 replicate analyses in each experiment. Q1, Q2 etc., quantile number.

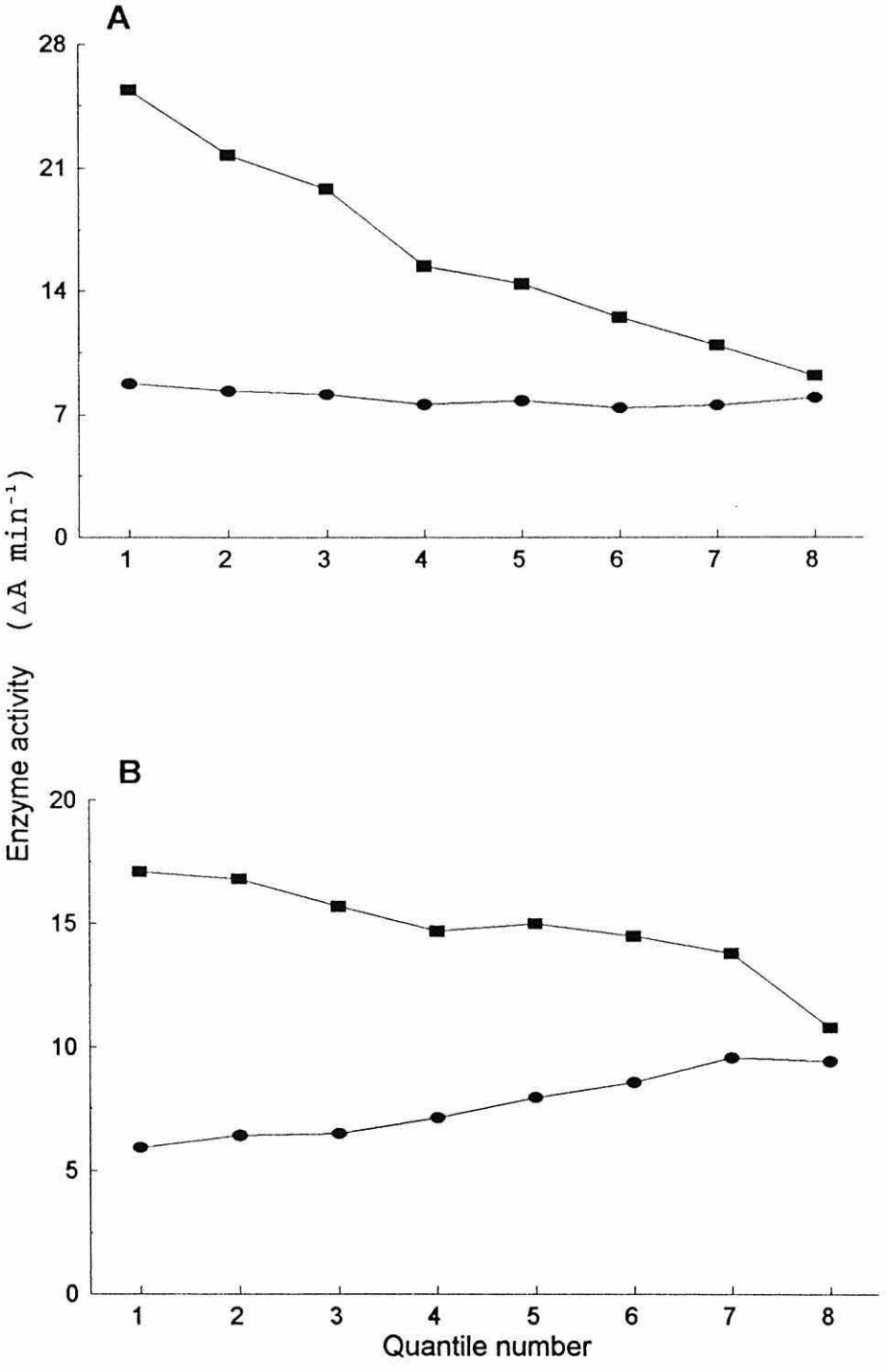


Fig. 6.5. Root tip peroxidase activities at 48 hours germination. A, soluble peroxidase; B, insoluble (cell wall) peroxidase; ●, per 25 roots; ■, per gram fresh weight.

seedlings with long (4 cm) and short (2 cm) roots (Method 6C; see Section 6.2.1) were studied.

The results for the long roots (Table 6.7 and Figure 6.6) show that the segment fresh weights were 0.06, 0.09, 0.30, 0.70 and 0.93 g $(25 \text{ roots})^{-1}$ in the first through to the fifth segment. The fresh weight values for the short roots were 0.07, 0.15 and 0.78 g $(25 \text{ roots})^{-1}$ in the first, second and third segments respectively. It can be seen from these results that, in both root groups, the segment fresh weight increased progressively with increasing segment number. It is important here to remember that the first and second segments were 0.5 cm long, while all the other segments were 1 cm long (see Section 6.2). Statistical analysis of the data, using ANOVA in which the effects of segment number and root group (root length) were investigated (using data selectively from the first, second and third segments to omit missing values), showed that segment fresh weight was highly dependent upon both segment number and root group ($P < 0.001$ in each case).

6.3.6 Peroxidase activities in different root segments at 48 hours germination

This experiment was concerned with comparing peroxidase activity in different segments along the root (Method 6C; see Section 6.2.1). The distribution of enzyme activity along the roots is summarized in Table 6.8 and in Figures 6.7 and 6.8. It can be seen from the data that soluble peroxidase activity in long roots was 4.29, 1.06, 2.45, 3.82 and 4.41 $\Delta A \text{ min}^{-1}$ $(25 \text{ roots})^{-1}$ in the first through to the fifth segment. The corresponding activities in the short roots were 3.19, 1.28 and 3.83 $\Delta A \text{ min}^{-1}$ $(25 \text{ roots})^{-1}$ in first, second and third segments, respectively. From these data it is obvious (see Figure 6.7A) that in long roots there was a high activity in the first segment followed

Table 6.7 Root segment fresh weights at 48 hours germination.

	Long roots					Short roots		
	S1	S2	S3	S4	S5	S1	S2	S3
mean	0.06	0.09	0.30	0.70	0.93	0.07	0.15	0.78
±sd	0.004	0.006	0.03	0.08	0.10	0.001	0.013	0.032
±se	0.002	0.003	0.017	0.046	0.057	0.000 7	0.007	0.018

Each value is the mean fresh weight [g(25 roots)$^{-1}$] ±sd and ±se from 3 separate experiments. In each experiment there were 25 roots in each group of roots.
S1, S2 etc., segment number from the tip end.

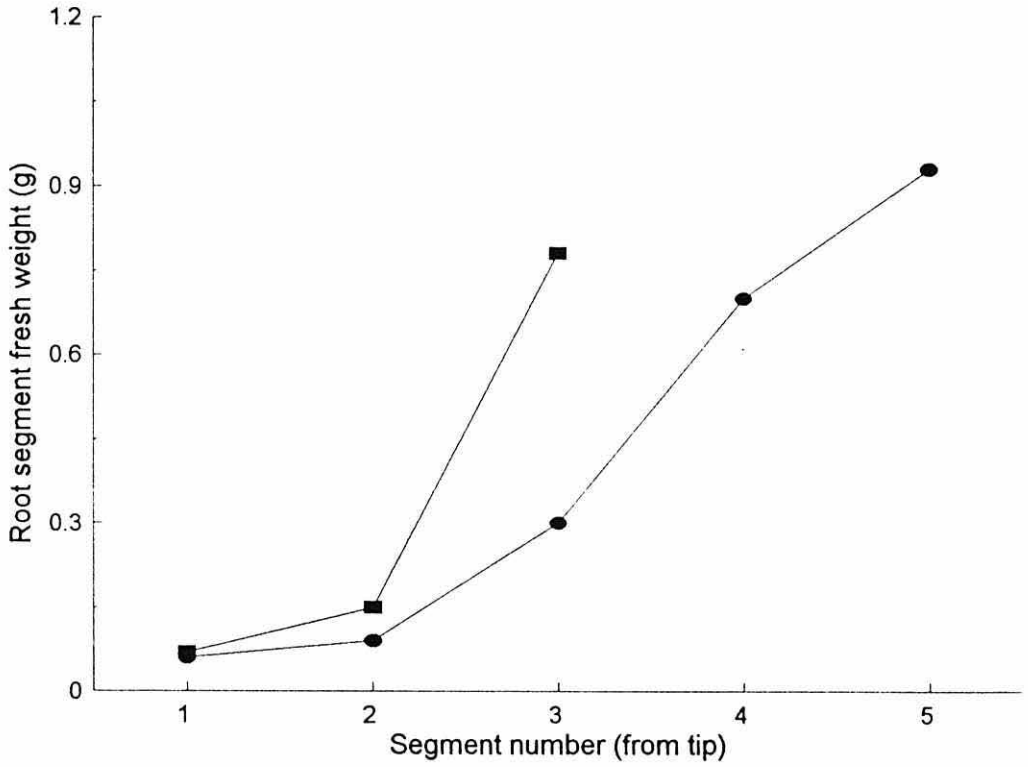


Fig. 6.6. Root segment fresh weights at 48 hours germination. ●, long roots; ■, short roots.

by a much lower activity in the next segment then gradually increasing activity through to the last segment. The short roots showed the same pattern of enzyme distribution; that is, high activity in first segment followed by lower activity in second segment and higher activity in the last segment. The fact that the first two segments were shorter than other segments does not fundamentally change these patterns. Statistical analysis of the data, using ANOVA in which the effects of segment number and root group (root length) were investigated (using data selectively from the first, second and third segments to omit missing values), showed that soluble peroxidase activity was highly dependent upon segment number ($P < 0.001$) while the seedling group had an insignificant effect ($P = 0.31$) on the soluble peroxidase activity.

The picture changed somewhat when the activities were calculated on the basis of per gram fresh weight. Then, the values for the long roots were 76.9, 11.8, 8.9, 5.9 and 4.8 $\Delta A\ min^{-1}$ (g fresh wt.)$^{-1}$ in the first through to the fifth segment. The activities for the short roots were 48.9, 8.5 and 4.9 $\Delta A\ min^{-1}$ (g fresh wt.)$^{-1}$ in first, second and third segments respectively. It is observed from these results (see Figure 6.7B) that, in long roots, the activity was high in the first segment, decreased sharply in the second segment and then decreased more slowly through the third, fourth and fifth segments. There was about 94% decrease in activity from the first through to the last segment. The short roots showed the same pattern of enzyme distribution; that is, high activity in the first segment, decreasing sharply in the second and third segments. The activity in the third segment was reduced by about 90% compared with the first segment. Further, the activities in the short roots were always lower than those for corresponding segments in the long roots. Statistical analysis of these data was carried out using Student's T-test, since the combination of unbalanced experimental design and

differences in group variances rendered ANOVA inappropriate. The analysis showed that the differences in activities between the corresponding segments of long and short roots were significant ($P = 0.01$).

The values for insoluble (cell wall) peroxidase activity in the long roots were 2.28, 0.46, 1.29, 1.92 and 0.83 $\Delta A\ min^{-1}(25\ roots)^{-1}$ in the first through to the fifth segment (Table 6.8 and Figure 6.8A). The activities for short roots were 2.63, 0.96 and 1.85 $\Delta A\ min^{-1}\ (25\ roots)^{-1}$ in first, second and third segments respectively. There was thus a high activity in first segment, lower activity in second segment followed by higher activity again in the third and fourth segments and again a lower activity in the last segment along the length of the long roots. The short roots showed the same general pattern of enzyme distribution. That is, high activity was evident in the first segment followed by lower activity in the second segment, then higher activity in the last segment. These distribution patterns are not altered fundamentally by the fact that the first two segments are only half as long as the other segments. Statistical analysis of the data, using ANOVA in which the effects of segment number and root group (root length) were investigated (using data selectively from the first, second and third segments to omit missing values), showed that both the segment number ($P < 0.001$) and the seedling group ($P = 0.008$) had highly significant effects on the cell wall peroxidase activity.

As expected, the pattern for insoluble peroxidase distribution changed when the data were calculated on the basis of per gram fresh weight (Table 6.8 Figure 6.8B). For the long roots, the activities were 40.4, 4.99, 4.67, 2.94 and 0.87 $\Delta A\ min^{-1}(g\ fresh\ wt.)^{-1}$ in the first through to the fifth segments. The activities for short roots were 40.2, 6.40

Table 6.8 Peroxidase activities in different root segments at 48 hours germination.

	Enzyme activity														
	$\Delta A\ min^{-1}(25\ roots)^{-1}$								$\Delta A\ min^{-1}(g\ fresh\ wt.)^{-1}$							
	Long roots					Short roots			Long roots					Short roots		
	S1	S2	S3	S4	S5	S1	S2	S3	S1	S2	S3	S4	S5	S1	S2	S3
Soluble peroxidase:																
mean	4.29	1.06	2.45	3.82	4.41	3.19	1.28	3.83	76.9	11.8	8.86	5.85	4.80	48.9	8.48	4.94
±sd	0.64	0.19	0.33	0.40	0.83	0.49	0.08	0.31	12.9	1.93	0.58	0.78	1.18	8.34	0.79	0.34
±se	0.15	0.04	0.08	0.09	0.19	0.11	0.02	0.07	3.05	0.45	0.14	0.18	0.28	1.96	0.19	0.08
Insoluble (cell wall) peroxidase:																
mean	2.28	0.46	1.29	1.92	0.83	2.63	0.96	1.85	40.4	4.99	4.67	2.94	0.87	40.2	6.40	2.36
±sd	0.31	0.18	0.16	0.28	0.19	0.48	0.05	0.28	3.16	1.75	0.40	0.50	0.13	7.74	0.62	0.29
±se	0.07	0.04	0.04	0.06	0.04	0.11	0.01	0.06	0.74	0.41	0.09	0.12	0.03	1.82	0.15	0.07

Each value is the mean activity ±sd and ±se from 3 separate experiments with 6 replicate analyses in each experiment. S1, S2 etc., segment number from the tip end.

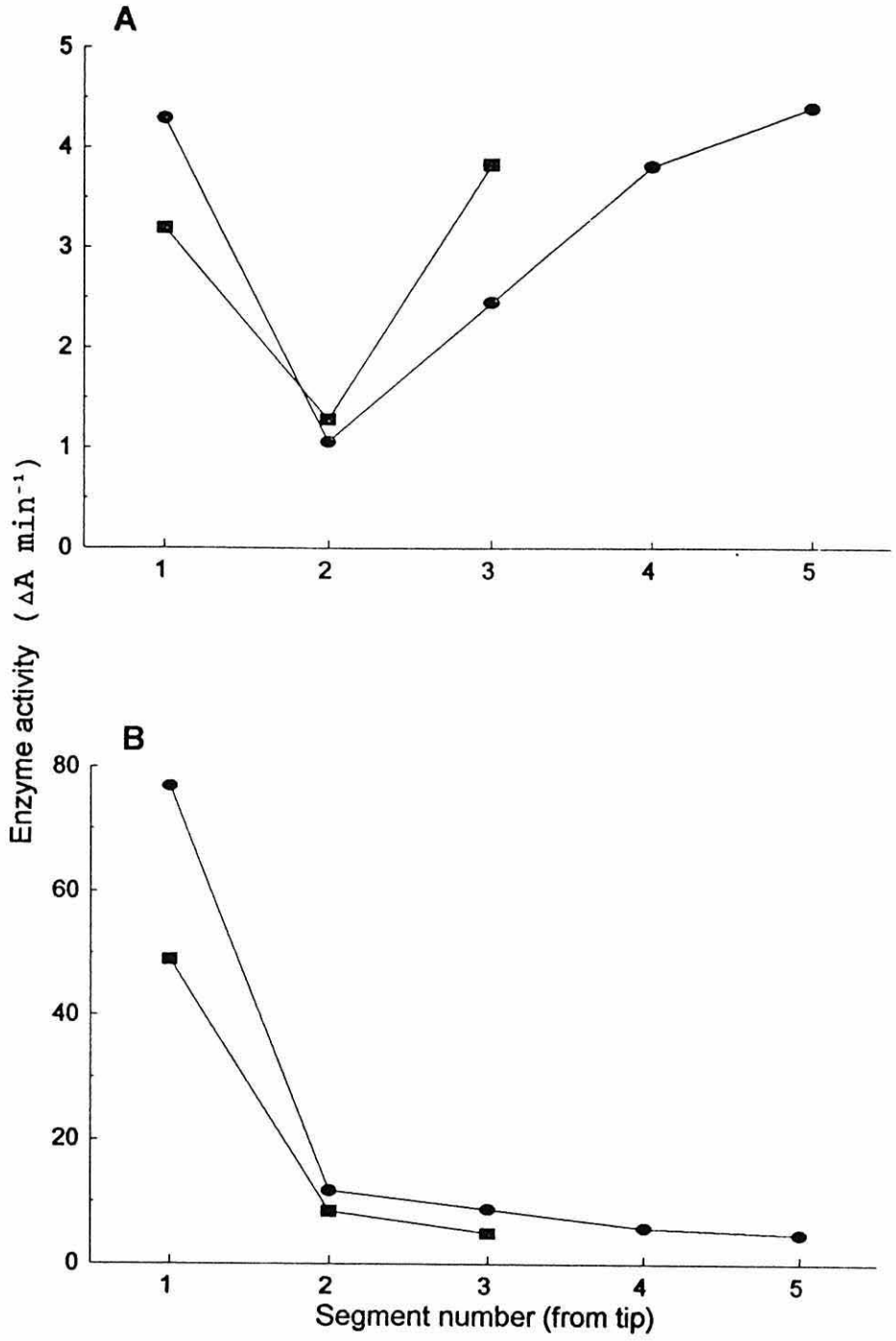


Fig. 6.7. Soluble peroxidase activity in different root segments at 48 hours germination. A, per 25 roots; B, per gram fresh weight; ●, long roots; ■, short roots.

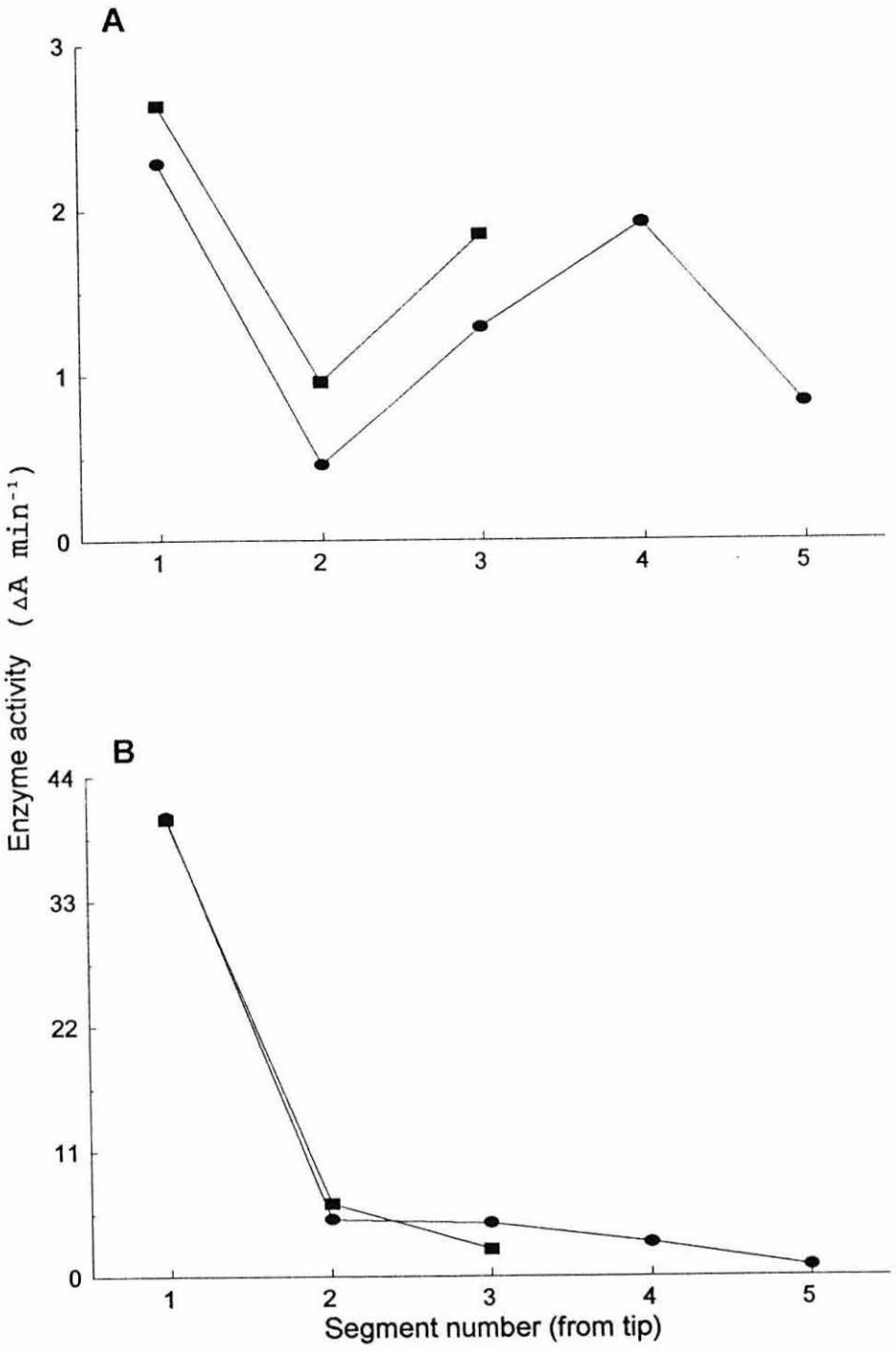


Fig. 6.8. Insoluble (cell wall) peroxidase activity in different root segments at 48 hours germination. A, per 25 roots; B, per gram fresh weight; ●, long roots; ■, short roots.

and 2.36 $\Delta A\ min^{-1}$ (g fresh wt.)$^{-1}$ in first, second and third segments respectively. These results show that, in long roots, the activity was high in the first segment, decreased sharply in the second segment and then decreased more slowly through the third, fourth and fifth segments. The short roots showed the same pattern of enzyme distribution; that is high activity in the first segment, decreasing sharply in the second and third segments. The activities in the segments of the short roots were not very different from those in the corresponding segments of the long roots; this was particularly true for the first (tip) segment. Statistical analysis of these data was carried out using Student's T-test, since the combination of unbalanced experimental design and differences in group variances rendered ANOVA inappropriate. The analysis showed that the differences in activities between the corresponding segments of long and short roots were significant ($P = 0.01$) except for the first segment ($P = 0.94$).

6.4 Summary and Discussion

Seedling roots, which are the subject of present study, have been used as an experimental material to study the distribution of peroxidase activity. Using the present extraction procedure, two sites of peroxidase localization can be identified, the cell wall and the cytoplasm plus vacuole (Schloss *et al.*, 1987). These are considered to contain particulate (cell wall) and soluble enzyme activities respectively.

The first experiment was based on the root tips from two seedling groups (first and second quartiles). Their results show that the soluble peroxidase activity calculated on a per root basis decreased in both quartiles as germination time increased. The first quartile showed higher activities than the second quartile. On a per gram fresh weight basis, the picture was different. Here the enzyme activity for both quartiles decreased

sharply between 24 to 36 hours. In the first quartile, the activity then increased again up to its original level and remained high through the rest of germination. In the second quartile, the activity remained low throughout the rest of germination. Similar soluble peroxidase activity results have been reported before for cotton seedling roots (Zainon, 1993).

The insoluble (cell wall) peroxidase activity, when calculated on a per root basis, showed the opposite pattern to that of the soluble enzyme. In both quartiles, activity increased as germination time increased, except between 36 and 48 hours. On a per gram fresh weight basis, the values showed large increases as germination proceeded for both the long and short root groups. Whether the data were expressed on a per root or a per gram basis, the values for the first quartile were greater than those for the second quartile. It was concluded from these studies that both root length and the age of the roots had significant effects on both the soluble and the cell wall enzyme activities.

The second experiment was based on eight root groups (quantiles) of the same age but with different root lengths. The results from this experiment show that the fresh weight of 2 cm root tips increased as the length of the root decreased. The fresh weight of the tips from the shortest roots was almost double that of the longest group. Soluble peroxidase activity on per root basis did not vary much according to root length. The activity on a per gram fresh weight basis decreased progressively with decreasing root length however.

The cell wall peroxidase activity on a per root basis was greater in short than in long

roots; this is the opposite way round to the corresponding soluble peroxidase pattern, but again the change was not very great. The activity on a per gram fresh weight basis decreased progressively with decreasing root lengths. This decreasing pattern is now similar to the pattern for the soluble enzyme when it is expressed on a per gram fresh weight basis. It is clear that both the soluble and the insoluble enzyme patterns, when they are expressed on a per gram basis, are dictated by the change in the fresh weight of the root tip.

The third experiment was concerned with the distribution of peroxidase activity along the length of the root. The results expressed on a per root basis show that, in long roots, there was a high soluble peroxidase activity in the first segment followed by a much lower activity in the next then gradually increasing activity through to the last segment. The short roots showed generally the same pattern of enzyme distribution. The activity on per gram fresh weight basis shows that, in long roots, it was high in the first segment, decreased sharply in the second segment and then decreased more slowly through the third, fourth and fifth segments. The short root again showed the same pattern of enzyme distribution, but the activities were lower than those for the corresponding segments in long roots due to the greater fresh weight of the short root segments. The distribution of the insoluble (cell wall) peroxidase activity was very similar to that of the soluble enzyme. The cell wall enzyme activities were also of the same order as the soluble enzyme activities, a general observation that agrees with the results of the earlier experiments (Cf. Tables 6.3, 6.4, 6.6)

The distribution of enzyme activity along the cotton seedling roots reflects the localization of root elongation. Thus, there was always a high level of activity in the

root tip, where elongation takes place, followed by lower activity in the region just behind the tip. A second region of high activity in the more proximal region of the root may similarly be associated with the cell division and elongation that occurs as part of lateral root initiation and growth. It is known that lateral roots appear later in this part of the root (see Section 3.3.7).

In all of the experiments described in this Chapter, there were large changes in the fresh weights of the root segments as well as changes in peroxidase activities. These two variables interact with each other, so that the results for the enzyme activities can give different pictures depending upon how they are presented (on a per segment or a per gram fresh weight basis). This situation will be discussed further in Chapter 8.

CHAPTER SEVEN

Chapter Seven

Effects of Hormones on Root Growth and Peroxidase Activities

7.1 Introduction

In the experiments described in the earlier chapters, large variations in root growth rate were noted, which occurred even within a single seed lot (see Chapter 3). The differences in root growth rate were dependent upon the differences in the extent of cell elongation (see Chapter 4). To obtain a better understanding of this variability, a study was also carried out to see if peroxidase activity could be related to the elongation process (see Chapter 6). Another parameter that is worth studying is the effect of plant growth substances on these processes. Endogenous plant growth substances (hormones) are essential for plant development, including root growth, and root growth is quite sensitive to applied hormones (Torrey, 1976). Seedling roots contain auxin, cytokinins, gibberellins, abscisic acid and ethylene (Torrey, 1976). At least one of these hormones, auxin, is known to be involved in cell elongation (Scott, 1972).

Auxin (indole acetic acid) synthesis occurs in the shoot tips, young leaves, buds, and in root meristem. Batra *et al.* (1975) proposed that auxin, which is transported acropetally in the roots, stimulates cell elongation in the zone proximal to the apical meristem. Exogenously supplied auxin at low concentrations (10^{-7} - 10^{-12} M) can promote the elongation in many species. At higher concentration (1-10 μM), elongation is almost always inhibited (Pilet *et al.*, 1979). This inhibition has long been assumed to be caused by ethylene, because auxin can stimulate many types of plant cells to produce ethylene and, in most species, ethylene retards elongation of roots and stems. Nevertheless, results reported by Eliasson *et al.* (1989) show that applied indoleacetic acid can inhibit

the elongation of pea seedling roots, but it fails to affect ethylene production from the same root soon after they are excised. This and other results indicate that indoleacetic acid inhibits the growth of pea roots by a mechanism that is independent of ethylene.

Indoleacetic acid is also known to stimulate cell division in the tip of the primary root and in lateral roots (Davidson and MacLeod, 1966, 1968) as well as in the vascular cambium (Torrey and Loomis, 1967). The results of experiments on *Vicia faba* root tips, in which cell division was inhibited by colchicine (Davidson *et al.*, 1965; Davidson and MacLeod, 1966), indicate that reactivation and reorganization of new meristem is stimulated by application of indoleacetic acid. The significance of this point is that auxin is influential in the induction of meristematic activity in the root, but it is not synthesized as a result of it. A further implication of these results is that auxin supplied from the base of the root is active in promoting cell division. It should be noted, in addition, that colchicine-induced inhibition is closely correlated with alteration in the polarity of root cells (MacLeod, 1966). It follows, therefore, that cell polarity changes may alter the polarity and functioning of the auxin transport system, which in turn may account for the inhibition of cell division by colchicine and loss of tissue organization.

It is highly likely that cytokinins are also involved in the cell division process in the root tip (Van't Hof, 1968). Cytokinins enhance cell division in carrot root tissues, for example, and the synthesis of cytokinins occurs in the cambial regions of the root (Chen *et al.*, 1985). In fact, roots are a major site for the synthesis of the hormone (Skene, 1975; Torrey, 1976). It would seen reasonable, therefore, that significant interactions between the auxins and cytokinins go on in this region. If the availability of auxin or cytokinin is altered in some way, so as to create an imbalance, then an alteration in

growth and development would be expected.

Gibberellins are also synthesized in the roots. Exogenous gibberellins have little effect on root growth, however, but they inhibit adventitious-root formation. This group of hormones can be detected in the xylem exudates of roots, when the stems are excised and root pressure forces the xylem sap out. Inhibitors of gibberellin synthesis decrease the amount of gibberellins in these exudates. Repeated excision of part of the root system causes marked decreases in the concentrations of gibberellins in the shoot, suggesting either that much of the shoot's gibberellin supply arises from the root via the xylem or that repeatedly excised roots can not supply water and mineral nutrients in the sufficient amounts to maintain the ability of the shoot to synthesize its own gibberellins.

Other growth substances such as abscisic acids may also be involved in root development. It has been found that water-stressed roots produce increased amounts of abscisic acid and that this abscisic acid is transported through the xylem to the leaves, where it causes stomata to close thereby aiding drought protected. Of course, because photosynthesis nearly stops, shoot growth is restricted. This further reduces water loss, but the growth of deeper roots can continue until they become too dry.

Because of these established roles of the plant hormones, it was decided to study their effects on root growth and peroxidase activities in the cotton seedling. The studies of these effects is the main objective of the experiments described in this chapter.

7.2 Methods

7.2.1 Selection of seedling root groups

Batches of 100 seeds were germinated for either 24 or 36 hours. After this period, groups of 10 seedlings with the longest roots were selected from each batch. They were reincubated either with distilled water (control) or with hormones for a further 12 to 48 hours. After every 12 hours, groups of seedlings were harvested and their root lengths were measured (Section 2.8). In separate experiments, 2 cm tips were dissected from the roots, their fresh weights were determined and they were used for peroxidase determination (Section 2.15).

7.2.2 Determination of hormone effects on root growth and peroxidase activity

The detailed procedures for these experiments are described in Section 2.16.

7.3 Results

7.3.1 Effect of gibberellic acid on root growth

Five different concentrations of GA_3 (10^{-4} to 10^{-8} M) were used to study their effects on root growth. Six groups of 10 seedlings selected from seedlings germinated for 36 hours were used for the experiment. One group was employed as the control (reincubated with distilled water) and the remaining 5 groups were reincubated with different concentrations of GA_3. All the seedling groups were reincubated for a further 36 hours. After this time, they were harvested and their root lengths were measured. A zero time control (seedlings at the end of the 36 hours germination period and before reincubation) was not employed in this experiment.

The results for the experiment are presented in Table 7.1 and Figure 7.1A. They show

that the root lengths of the control seedlings and seedlings incubated with 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M GA_3 were 4.27, 4.18, 4.51, 4.45, 4.38 and 4.19 cm respectively. It can be seen that root elongation was apparently stimulated by GA_3 at 10^{-5} to 10^{-7} M, but it was inhibited at 10^{-4} and 10^{-8} M. The differences between treatments were very small, however, and statistical analysis (ANOVA) of the data showed that the hormone treatments had an insignificant effect on root growth ($P = 0.23$).

It was therefore decided to study the effect of 10^{-6} M GA_3 on root growth following different periods of hormone treatment. Ten groups of 10 seedlings were selected from the 36 hour germinated seedlings. Two groups were used immediately for root measurement (0 hour control). From the remaining eight groups, four were used as no-hormone controls (reincubated with distilled water), while four were reincubated with 10^{-6} M GA_3. The eight groups were reincubated for 6, 12, 24 or 36 hours. At the end of each period, two groups (one control and one hormone treated) were harvested and their root lengths were measured.

The results presented in Table 7.2 and Figure 7.1B, show that the root length of the control at zero time was 2.50 cm. After 6, 12, 24 and 36 hours, the values for the distilled water controls were 3.09, 3.69, 4.96 and 6.43 cm respectively. The corresponding values for hormone-treated seedlings were 3.11, 3.73, 5.22 and 6.92 cm. Obviously, the root lengths of the control and the hormone-treated seedlings increased with time, but the GA_3-treated seedlings also grew faster than the controls. ANOVA indicated that the hormone had a significant effect ($P = 0.002$) and the treatment period had a highly significant effect on root growth ($P < 0.001$). It also revealed a significant interaction between the effects of hormone and time ($P = 0.01$).

Table 7.1 Effect of gibberellic acid on root growth.

	Control	Gibberellic acid				
		10^{-4}M	10^{-5}M	10^{-6}M	10^{-7}M	10^{-8}M
mean	4.27	4.18	4.51	4.45	4.38	4.19
±sd	0.62	0.44	0.66	0.74	0.68	0.53
±se	0.16	0.11	0.17	0.19	0.18	0.14

Each value is the mean root length (cm) ±sd and ±se from 3 separate experiments with 5 replicate analyses in each experiment. Germinating seedlings were harvested at 36 hours and reincubated for a further 36 hours either with distilled water (control) or with hormone.

Table 7.2 Effect of 10^{-6} M gibberellic acid on root growth.

	Treatment period									
	0h	6h		12h		24h		36h		
	C	C	T	C	T	C	T	C	T	
mean	2.50	3.09	3.11	3.69	3.73	4.96	5.22	6.43	6.92	
±sd	0.34	0.46	0.45	0.58	0.60	0.75	0.87	1.10	1.14	
±se	0.08	0.11	0.11	0.14	0.14	0.18	0.21	0.26	0.27	

Each value is the mean root length (cm) ±sd and ±se from 3 separate experiments with 6 replicate analyses in each experiment. Germinating seedlings were harvested at 36 hours and reincubated for up to 36 hours either with distilled water (control) or with hormone. C, control; T, hormone-treated.

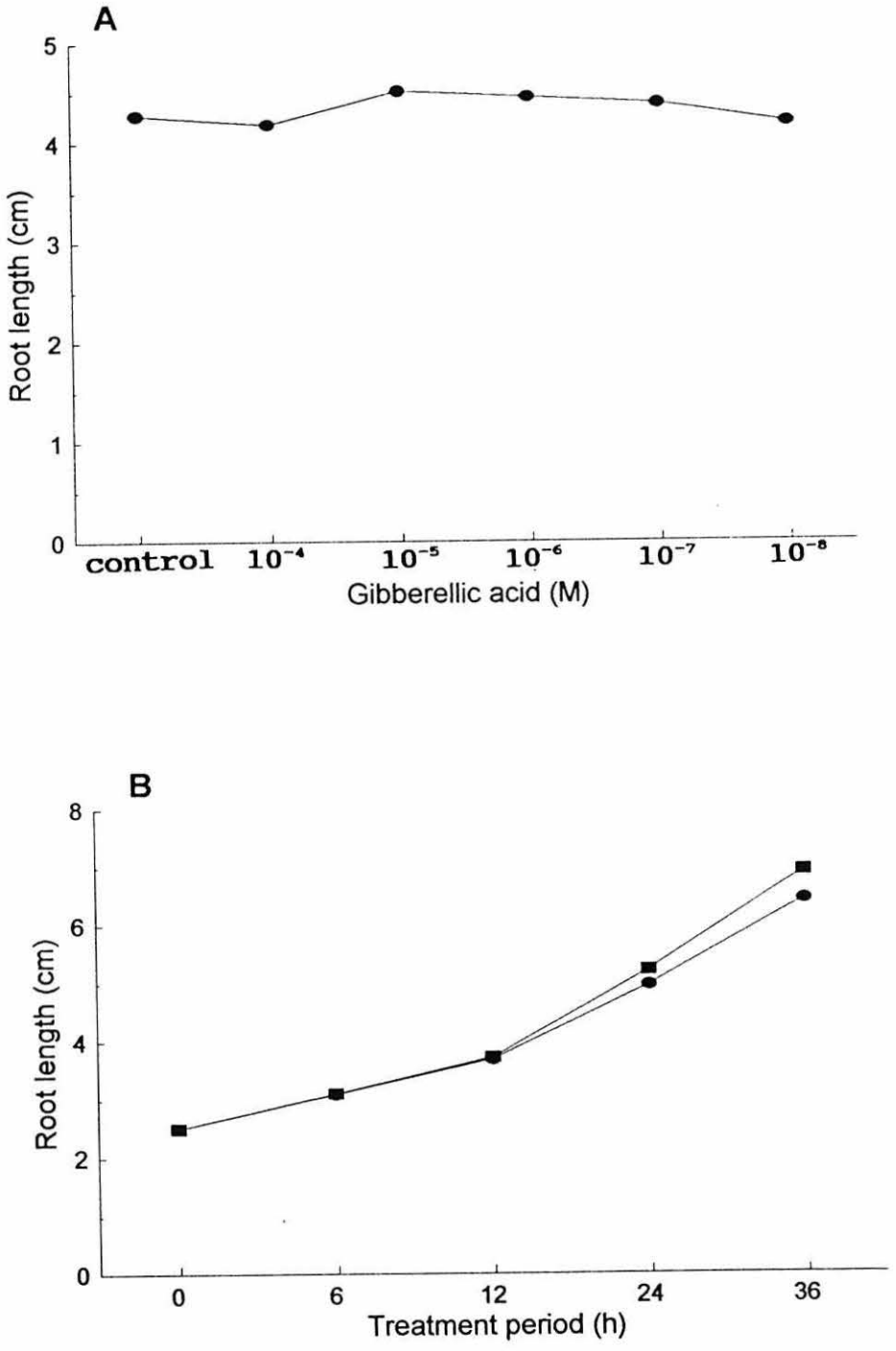


Fig. 7.1. Effect of gibberellic acid on root growth. A, effect of different concentrations; B, effect of different treatment periods; ●, control; ■, hormone-treated.

7.3.2 Effect of abscisic acid on root growth

In this experiment, three different concentrations of ABA (10^{-5} , 10^{-6} and 10^{-7} M) were used to study their effects on root growth. For each ABA concentration, ten groups of 10 seedlings were selected from different sets of 24 hour germinated seedlings. The root lengths of two groups were measured immediately (0 hour control). From the remaining 8 groups, 4 were used as no-hormone controls (reincubated with distilled water), while 4 were reincubated with ABA. These groups were reincubated for 12, 24, 36 or 48 hours. At the end of each period, two groups (one control and one hormone-treated) were harvested and their root lengths were measured.

The results (Table 7.3 and Figure 7.2) show that the root length of the control seedlings at zero time was 1.0 cm. This length increased upon further incubation. At 24 hours, the values for three control lots of seedlings were 3.67, 3.69 and 3.69 cm. The corresponding values for the 10^{-5}, 10^{-6} and 10^{-7} M ABA-treated seedlings were 3.94, 3.76 and 3.80 cm respectively. Thus, the root lengths were slightly higher in the hormone-treated seedlings as compared with their controls (The design of the experiment did not provide a universal for all hormone treatments). This situation changed as incubation time increased however. By the end of the experiment at 48 hours, the values for controls were 7.65, 7.65 and 7.67 cm. The corresponding 10^{-5}, 10^{-6} and 10^{-7} M ABA-treated seedling values were 6.95, 7.04 and 7.63 cm respectively. Now, the root lengths of the controls were actually higher than those of the hormone-treated seedlings. The differences between control and hormone-treated roots were always very small. Analysis of variance (ANOVA) showed that the treatment period had a highly significant effect ($P < 0.001$), but the hormone-treatment had an insignificant effect ($P = 0.12$) on root growth.

Table 7.3 Effect of abscisic acid on root growth.

ABA Conc. (M)		Treatment period								
		0h	12h		24h		36h		48h	
		C	C	T	C	T	C	T	C	T
10^{-5}	mean	1.00	2.06	2.15	3.67	3.94	5.30	5.31	7.65	6.95
	±sd	0.14	0.20	0.19	0.24	0.29	0.41	0.49	0.77	0.75
	±se	0.04	0.05	0.05	0.06	0.07	0.11	0.13	0.20	0.19
10^{-6}	mean	1.00	2.07	2.09	3.69	3.76	5.33	5.24	7.65	7.04
	±sd	0.14	0.22	0.31	0.23	0.58	0.46	0.77	0.80	0.98
	±se	0.04	0.06	0.08	0.06	0.15	0.12	0.20	0.21	0.25
10^{-7}	mean	1.00	2.06	2.01	3.69	3.80	5.33	5.49	7.67	7.63
	±sd	0.14	0.23	0.26	0.26	0.36	0.43	0.48	0.82	0.73
	±se	0.04	0.06	0.07	0.07	0.09	0.11	0.12	0.21	0.19

Each value is the mean root length (cm) ±sd and ±se from 3 separate experiments with 5 replicate analyses in each experiment. Germinating seedlings were harvested at 24 hours and reincubated for up to 48 hours either with distilled water (control) or with hormone. C, control; T, hormone-treated.

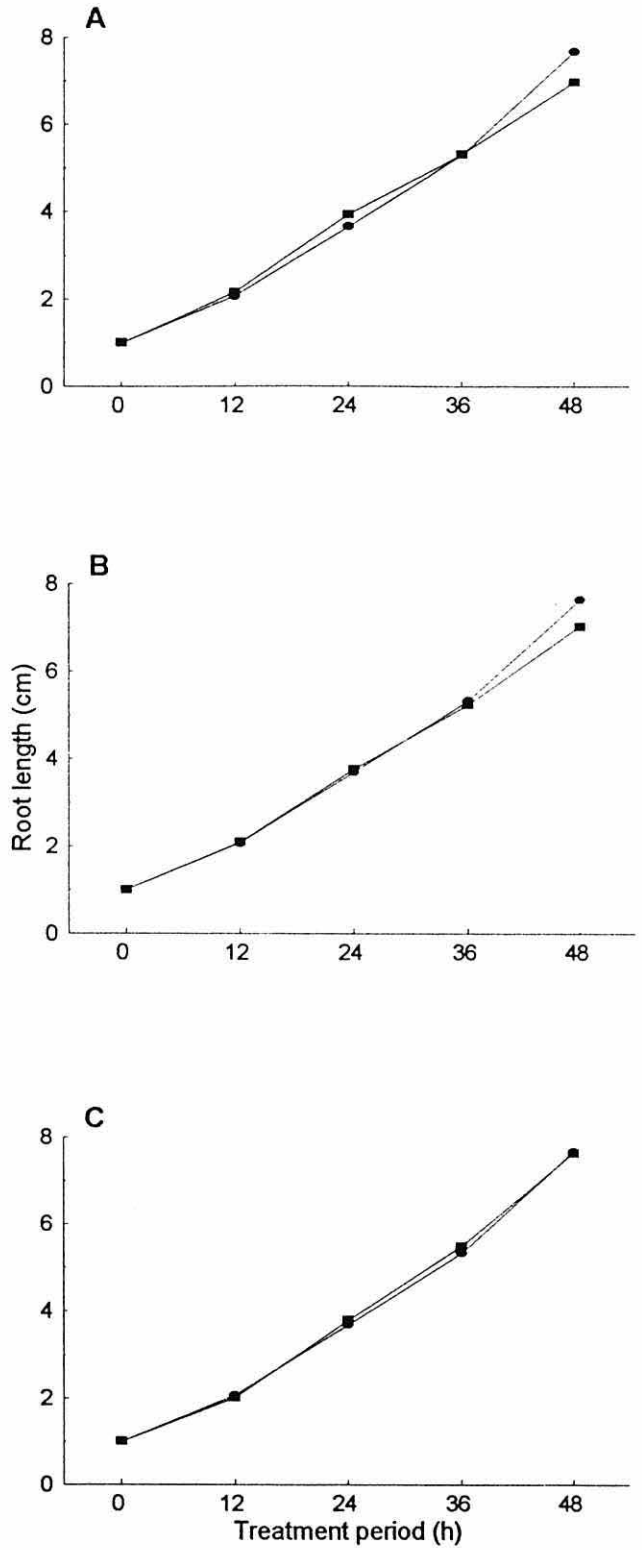


Fig. 7.2. Effect of abscisic acid on root growth. A, 10^{-5}M; B, 10^{-6}M; C, 10^{-7}M; ●, control; ■, hormone-treated.

7.3.3 Effect of indole acetic acid on root growth

Five different concentrations of IAA (10^{-3}, 10^{-4}, 10^{-6}, 10^{-8} and 10^{-10} M) were used to study their effects on root growth. The design of this experiment was exactly the same as the one for ABA and the results for the IAA experiment are presented in Table 7.4 and Figure 7.3. The root lengths of all the controls at zero time were 1.0 cm. Upon reincubation, the higher concentrations of IAA (10^{-3} and 10^{-4} M) inhibited root growth. At these concentrations, the root lengths of the IAA-treated seedlings were markedly less than those of the controls at all incubation times. In the case of 10^{-3} M IAA, for example, the control values for 12, 24, 36 and 48 hours were 2.13, 3.77, 5.49 and 7.76 cm, while the corresponding values for the hormone-treated seedlings were 1.41, 2.45, 3.78 and 5.61 cm.

In contrast to the high concentrations, lower concentrations of IAA (10^{-6}, 10^{-8} and 10^{-10} M) promoted root growth. In the case of 10^{-8} M IAA, for example, the control values at 12, 24, 36 and 48 hours were 2.12, 3.53, 5.09 and 6.67 cm. The corresponding values for the hormone-treated seedlings were 2.25, 3.93, 5.78 and 7.68 cm respectively. The differences between control and hormone-treated values were small at 12 and 24 hours, but they increased later. The overall ANOVA showed that both the hormone treatment ($P = 0.002$) and the treatment period ($P < 0.001$) had a highly significant effect on root growth. It was also confirmed that the hormone effect was strongly dependent upon its concentration (interaction $P < 0.001$).

7.3.4 Effect of indole acetic acid on root tip fresh weight

After consideration of the root growth results, it was decided to concentrate on one hormone (IAA) and to study its effects further at root growth inhibitory (10^{-3} M) and

Table 7.4 Effect of indole acetic acid on root growth.

IAA Conc. (M)		Treatment period								
		0h	12h		24h		36h		48h	
		C	C	T	C	T	C	T	C	T
10^{-3}	mean	1.00	2.13	1.41	3.77	2.45	5.49	3.78	7.76	5.61
	±sd	0.14	0.34	0.18	0.55	0.29	0.78	0.42	1.03	0.61
	±se	0.04	0.09	0.05	0.14	0.07	0.20	0.11	0.27	0.16
10^{-4}	mean	1.00	2.15	1.81	3.79	3.17	5.53	4.83	7.81	6.75
	±sd	0.14	0.38	0.21	0.59	0.37	0.79	0.64	1.04	0.82
	±se	0.04	0.10	0.05	0.15	0.10	0.20	0.16	0.27	0.21
10^{-6}	mean	1.00	2.12	2.15	3.58	3.73	5.08	5.47	6.65	7.21
	±sd	0.14	0.43	0.20	0.56	0.54	0.72	0.83	0.96	1.22
	±se	0.04	0.11	0.05	0.14	0.14	0.19	0.21	0.25	0.32
10^{-8}	mean	1.00	2.12	2.25	3.53	3.93	5.09	5.78	6.67	7.68
	±sd	0.14	0.49	0.32	0.52	0.63	0.74	0.82	0.94	1.04
	±se	0.04	0.13	0.08	0.13	0.16	0.19	0.24	0.24	0.27
10^{-10}	mean	1.00	2.13	2.21	3.53	3.69	5.11	5.46	6.69	7.23
	±sd	0.14	0.48	0.27	0.52	0.52	0.77	0.83	0.95	1.21
	±se	0.04	0.12	0.07	0.13	0.13	0.20	0.21	0.25	0.31

Each value is the mean root length (cm) ±sd and ±se from 3 separate experiments with 5 replicate analyses in each experiment. Germinating seedlings were harvested at 24 hours and reincubated for up to 48 hours either with distilled water (control) or with hormone. C, control; T, hormone-treated.

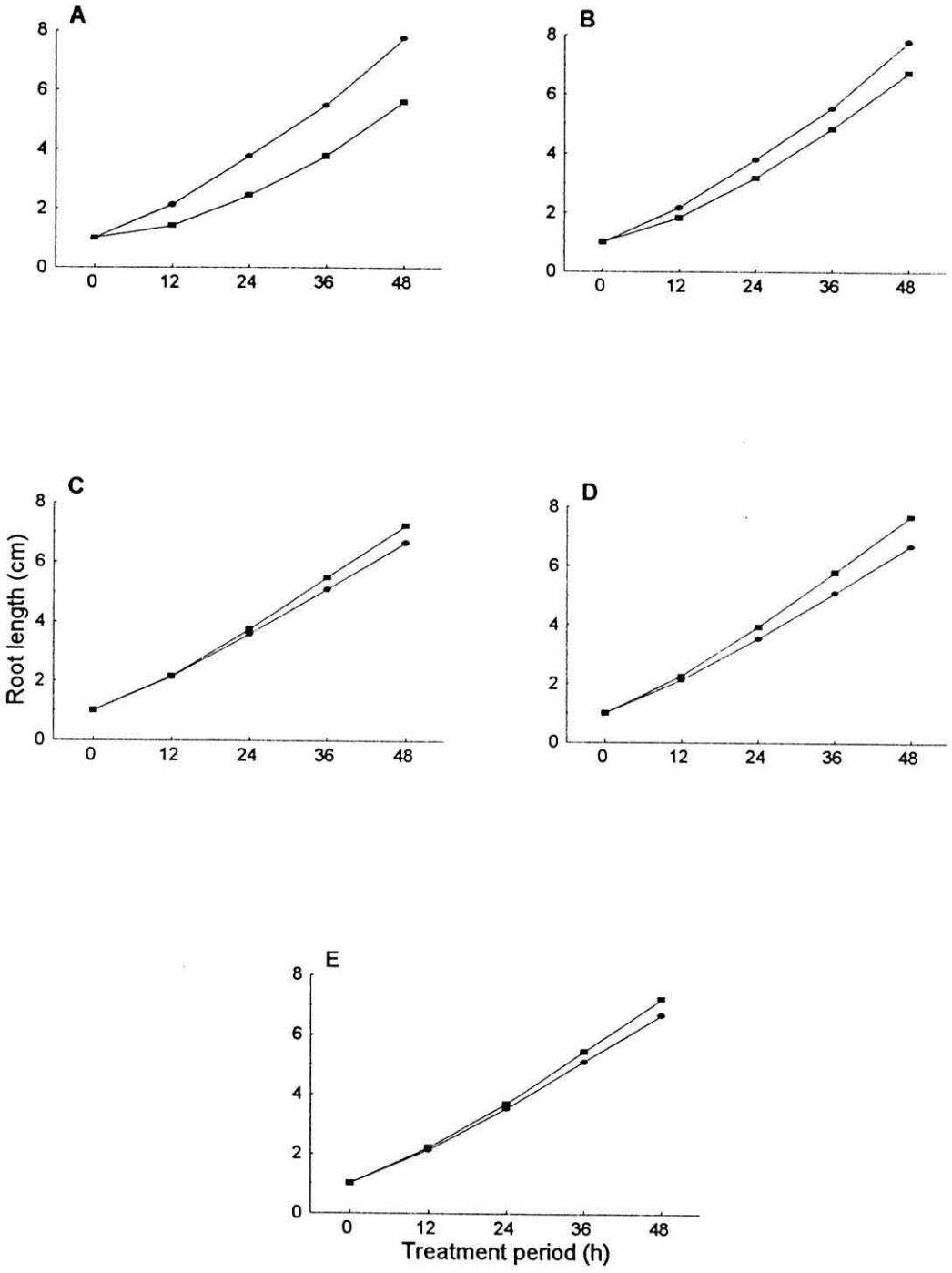


Fig. 7.3. Effect of indole acetic acid on root growth. A, 10^{-3}M; B, 10^{-4}M; C, 10^{-6}M; D, 10^{-8}M; E, 10^{-10}M; ●, control; ■, hormone-treated.

growth promotory (10^{-8} M) concentrations. These further studies were targeted at the root tips and the first experiment was to determine the effects of the hormones on the tip fresh weight.

The general design of the experiment was exactly the same as for the root length measurements above. In this experiment, 2 cm root tips were dissected from the harvested roots and their fresh weights were determined. The results are presented in Table 7.5 and Figure 7.4. They show that, for the experiment with the lower concentration of IAA (10^{-8} M), the root tip fresh weight of the control seedlings at zero time was 0.11 g (10 tips)$^{-1}$. This value had almost tripled at 12 hours, but it then decreased progressively with increase in incubation time. Thus, after 12, 24, 36 and 48 hours the control values were 0.30, 0.24, 0.16 and 0.14 g (10 tips)$^{-1}$. The corresponding values for the hormone-treated seedlings were 0.29, 0.26, 0.16 and 0.14 g (10 tips)$^{-1}$. The values for the control and the treated seedlings were the same at 36 and 48 hours. The control values were higher at 12 hours and lower at 24 hours compared with the hormone-treated seedlings. These differences were always very small however. ANOVA showed (see Figure 7.4A) that hormone-treatment had an insignificant effect ($P = 0.85$), but that treatment period had a highly significant effect ($P < 0.001$) on root tip fresh weight.

For the higher concentration of IAA (10^{-3} M) (Table 7.5 and Figure 7.4B), the root tip fresh weight of the control at zero treatment time was 0.070 g (10 tips)$^{-1}$. In the controls, this value increased more than 3-fold up to 24 hours and then decreased slightly at 36 and 48 hours. A similar large (6-fold) increase in the hormone-treated values continued up to 36 hours before decreasing at 48 hours. After 12, 24, 36 and 48

Table 7.5 Effect of indole acetic acid on root tip fresh weight.

IAA Conc. (M)		Treatment period								
		0h	12h		24h		36h		48h	
		C	C	T	C	T	C	T	C	T
10^{-8}										
	mean	0.11	0.30	0.29	0.24	0.26	0.16	0.16	0.14	0.14
	±sd	0.014	0.026	0.017	0.011	0.024	0.009	0.013	0.023	0.002
	±se	0.008	0.015	0.009	0.006	0.013	0.005	0.007	0.013	0.001
10^{-3}										
	mean	0.07	0.19	0.15	0.24	0.37	0.19	0.42	0.17	0.19
	±sd	0.008	0.009	0.016	0.016	0.041	0.028	0.079	0.044	0.033
	±se	0.004	0.005	0.009	0.009	0.023	0.016	0.045	0.025	0.019

Each value is the mean fresh weight [g(10 roots)$^{-1}$] ±sd and ±se from 3 separate experiments. Germinating seedlings were harvested at 24 hours and reincubated for up to 48 hours either with distilled water (control) or with hormone. C, control; T, hormone-treated.

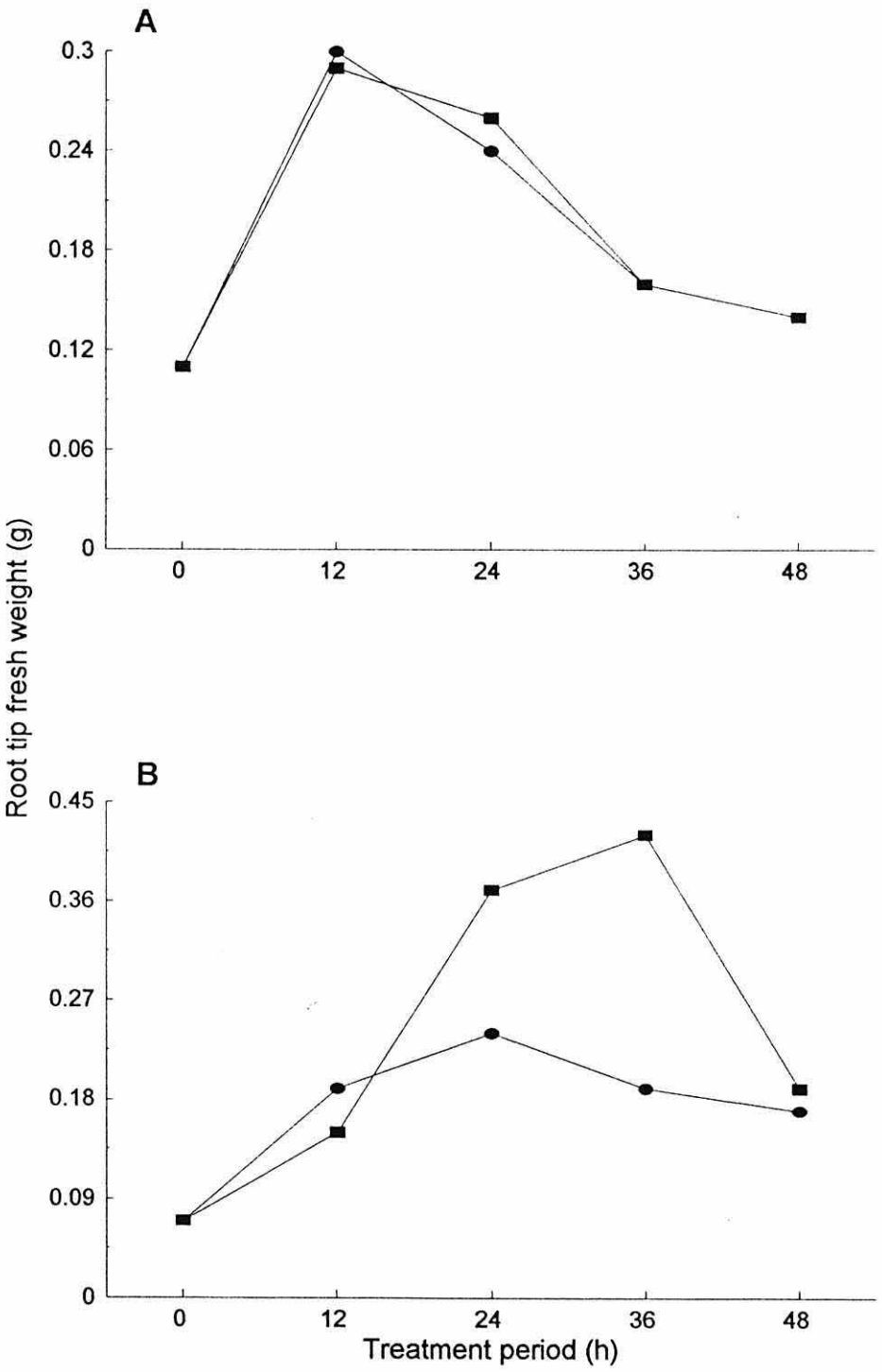


Fig. 7.4. Effect of indole acetic acid on root tip fresh weight. A, 10^{-8}M; B, 10^{-3}M; ●, control; ■, hormone-treated.

hours, the control values were 0.19, 0.24, 0.19 and 0.17 g (10 tips)$^{-1}$. The corresponding hormone-treated values were 0.15, 0.37, 0.42 and 0.19 g (10 tips)$^{-1}$. From these data it can be seen that the values for hormone-treated seedlings were higher than the controls at all incubation times except 12 hours, where the opposite was the case. The differences at 24 and 36 hours were quite large. ANOVA confirmed that both the hormone-treatment and treatment period had a highly significant effect ($P < 0.001$ in both cases) on root tip fresh weight.

7.3.5 Effect of indole acetic acid on peroxidase activities

The design of this experiment was the same as that described in Section 7.3.4 above up to the point where the root tips were dissected and weighed. After that, they were used directly for the determination of peroxidase activity. In fact, the same seedlings were used for both fresh weight and peroxidase assays.

The results for the effects of the lower concentration of IAA (10^{-8} M) on the soluble peroxidase are presented in Table 7.6 and Figure 7.5A. They show that the enzyme activity in the control at zero time was 0.99 ΔA min^{-1} (10 roots)$^{-1}$. After 12, 24, 36 and 48 hours the control values were 2.80, 2.68, 2.65 and 2.45 ΔA min^{-1} (10 roots)$^{-1}$. The corresponding hormone-treated seedlings values were 2.73, 2.85, 2.86 and 2.51 ΔA min^{-1} (10 roots)$^{-1}$. Thus, the control value had increased by about 3-fold at 12 hours, but it then decreased progressively with increase in incubation time. The hormone-treated values continued to increase from 12 to 36 hours, where the increase was about 3-fold. These data show that the soluble peroxidase activities expressed on a per root basis were higher in hormone-treated seedlings than in the controls at all incubation times except 12 hours, where the opposite was the case. The differences between control and

Table 7.6 Effect of 10^{-8} M indole acetic acid on root tip soluble peroxidase activity.

Enzyme activity ($\Delta A\ min^{-1}$)	Treatment period								
	0h	12h		24h		36h		48h	
	C	C	T	C	T	C	T	C	T
Activity (10 roots)$^{-1}$:									
mean	0.99	2.80	2.73	2.68	2.85	2.65	2.86	2.45	2.51
±sd	0.16	0.22	0.11	0.13	0.19	0.22	0.28	0.24	0.15
±se	0.04	0.05	0.03	0.03	0.04	0.05	0.07	0.06	0.04
Activity (g fresh wt.)$^{-1}$:									
mean	8.82	9.19	9.55	11.17	11.24	17.08	18.20	17.43	17.86
±sd	1.50	0.32	0.32	0.67	1.11	0.79	0.76	1.39	1.30
±se	0.35	0.08	0.08	0.16	0.26	0.19	0.18	0.33	0.31

Each value is the mean activity ±sd and ±se from 3 separate experiments with 6 replicate analyses in each experiment. Germinating seedlings were harvested at 24 hours and reincubated for up to 48 hours either with distilled water (control) or with hormone. C, control; T, hormone-treated.

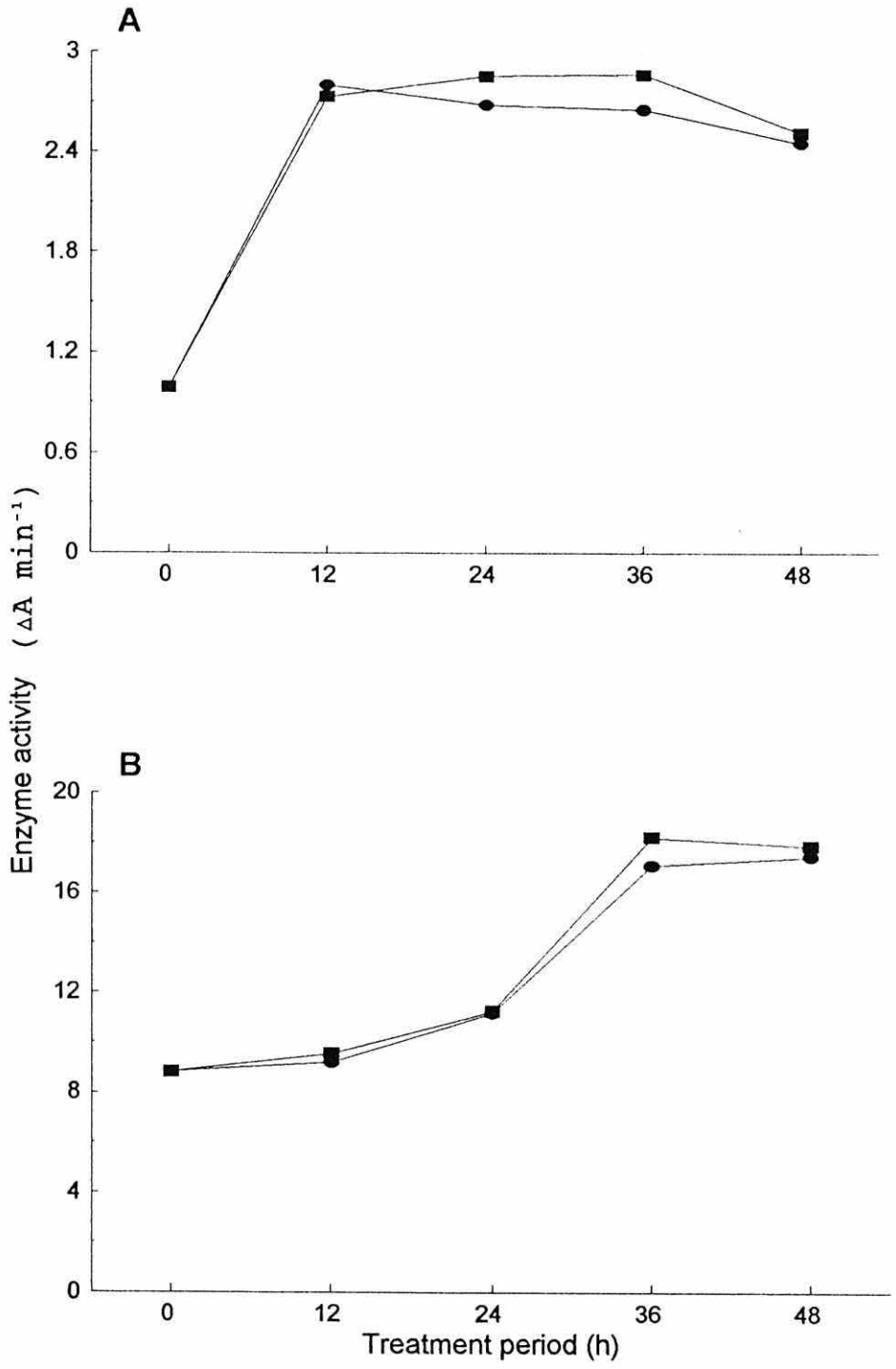


Fig. 7.5. Effect of 10^{-6}M indole acetic acid on soluble peroxidase activity. A, per 10 roots; B, per gram fresh weight; ●, control; ■, hormone-treated.

hormone-treated values were very small however. ANOVA confirmed that the hormone-treatment had an insignificant ($P = 0.25$) effect on peroxidase activity.

Because of the small fresh weight values (see Table 7.5), the values for the soluble peroxidase activity were higher when they were calculated on a per gram fresh weight basis (Table 7.6 and Figure 7.5B). The activity determined for the control at zero time was now 8.82 $\Delta A\ min^{-1}$ (g fresh wt)$^{-1}$. The control values for 12, 24, 36 and 48 hours were 9.19, 11.17, 17.08 and 17.43 $\Delta A\ min^{-1}$ (g fresh wt.)$^{-1}$. The corresponding values for hormone-treated seedlings were 9.55, 11.24, 18.20 and 17.86 $\Delta A\ min^{-1}$ (g fresh wt.)$^{-1}$. It can be seen that both sets of values increased with increase in incubation time, except between 36 and 48 hours where the treated seedlings showed a slight reduction. It was also noted that the soluble peroxidase activities expressed on a per gram fresh weight basis were higher in hormone-treated seedlings than in the controls at all incubation times. The differences were not large however. ANOVA again confirmed that hormone-treatment had an insignificant effect ($P = 0.16$) on enzyme activity.

The results for the effect of 10^{-8} M IAA on the insoluble (cell wall) peroxidase are presented in Table 7.7 and Figure 7.6. This activity was 0.25 $\Delta A\ min^{-1}$ (10 roots)$^{-1}$ in the control at zero time and it increased with increase in time both in the control and in the hormone-treated seedlings. Thus, the control values at 12, 24, 36 and 48 hours were 0.86, 1.59, 1.93 and 3.31 $\Delta A\ min^{-1}$ (10 roots)$^{-1}$. The corresponding hormone-treated seedling values were 0.96, 2.05, 2.61 and 3.51 $\Delta A\ min^{-1}$ (10 roots)$^{-1}$. It can be seen that the insoluble (cell wall) peroxidase activity expressed on a per root basis was significantly higher (see Figure 7.6A) in hormone-treated seedlings than in the controls at all incubation times ($P = 0.01$ using ANOVA on data which were logged in order

Table 7.7 Effect of 10^{-8} M indole acetic acid on root tip insoluble peroxidase activity.

Enzyme activity ($\Delta A\ min^{-1}$)	Treatment period								
	0h	12h		24h		36h		48h	
	C	C	T	C	T	C	T	C	T
Activity (10 roots)$^{-1}$:									
mean	0.25	0.86	0.96	1.59	2.05	1.93	2.61	3.31	3.51
±sd	0.07	0.17	0.11	0.18	0.34	0.22	0.24	0.50	0.38
±se	0.02	0.04	0.03	0.04	0.08	0.05	0.06	0.12	0.09
Activity (g fresh wt.)$^{-1}$:									
mean	2.19	2.82	2.37	6.66	7.99	12.48	16.70	23.53	24.92
±sd	0.38	0.39	0.25	0.95	0.85	0.95	1.10	1.04	2.42
±se	0.09	0.09	0.06	0.22	0.20	0.22	0.26	0.25	0.57

Each value is the mean activity ±sd and ±se from 3 separate experiments with 6 replicate analyses in each experiment. Germinating seedlings were harvested at 24 hours and reincubated for up to 48 hours either with distilled water (control) or with hormone. C, control; T, hormone-treated.

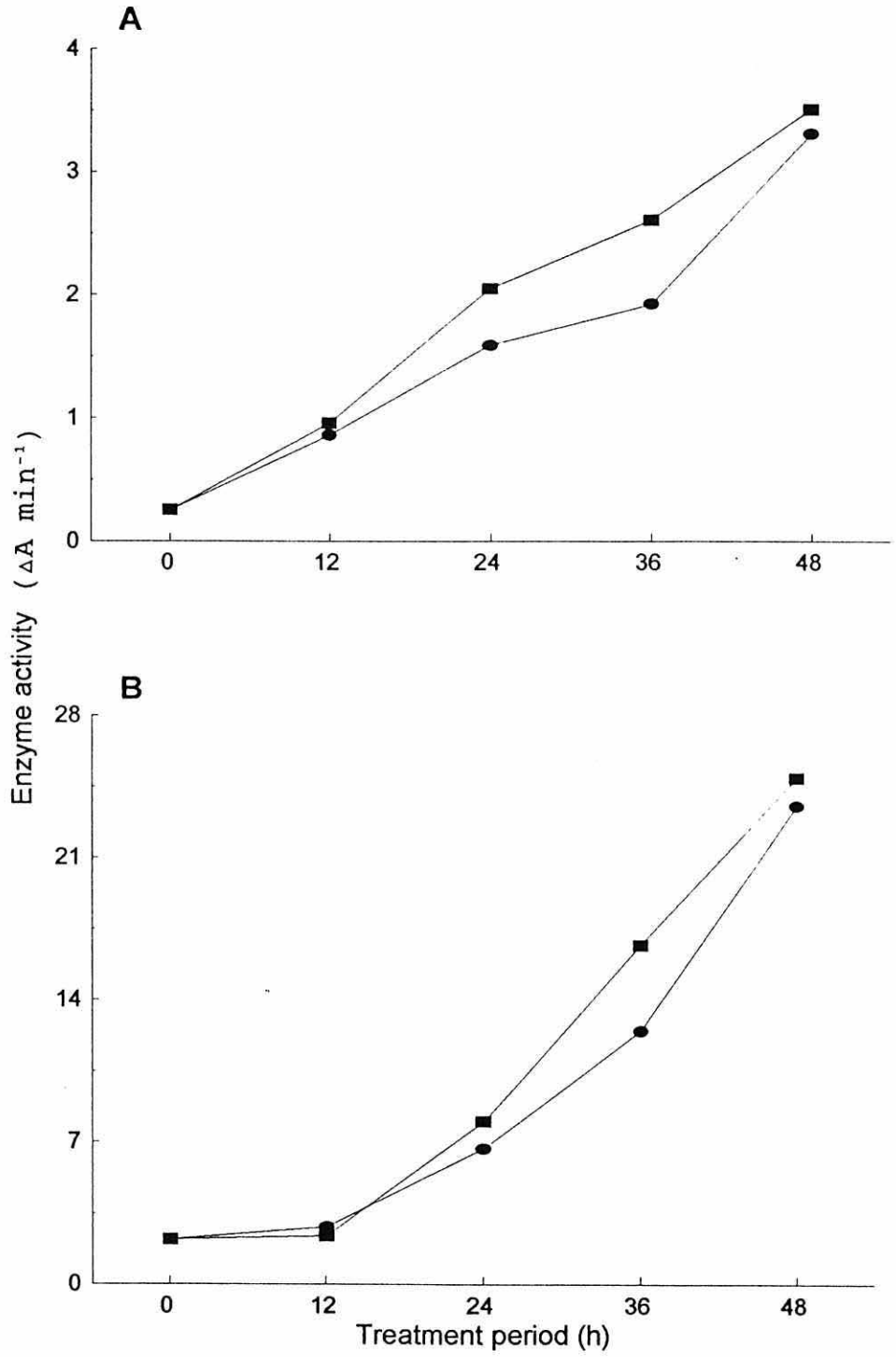


Fig. 7.6. Effect of 10^{-6}M indole acetic acid on insoluble (cell wall) peroxidase activity. A, per 10 roots; B, per gram fresh weight; ●, control; ■, hormone-treated.

to minimize the positive relationship between mean values and their variance).

The values were changed when they were calculated on the basis of per gram fresh weight (Table 7.7 and Figure 7.6B), but the general patterns of activities were similar. Thus, the activity for the control at zero time was 2.19 $\Delta A\ min^{-1}$ (g fresh wt.)$^{-1}$ and the control values for 12, 24, 36 and 48 hours were 2.82, 6.66, 12.48 and 23.53 $\Delta A\ min^{-1}$ (g fresh wt.)$^{-1}$. The corresponding hormone-treated values were 2.37, 7.99, 16.70 and 24.92 $\Delta A\ min^{-1}$ (g fresh wt.)$^{-1}$. It is clear from these results that activities increased progressively with increase in incubation time. It can also be seen that the activities in hormone-treated seedlings were higher than the controls at all incubation times except at 12 hours, where the opposite was the case. ANOVA (using logged data; see above) showed that the hormone-treatment had a highly significant effect ($P < 0.001$) on the cell wall peroxidase activity expressed on a per gram fresh weight basis.

The results for the experiment to study the effect of the higher concentration of IAA (10^{-3} M) on soluble peroxidase activity are presented in Table 7.8 and Figure 7.7. This peroxidase activity in the control at zero time was 0.65 $\Delta A\ min^{-1}$ (10 roots)$^{-1}$ and at 12, 24, 36 and 48 hours the values were 1.22, 2.54, 2.16 and 2.48 $\Delta A\ min^{-1}$ (10 roots)$^{-1}$. The corresponding hormone-treated seedling values were 1.35, 4.39, 3.73 and 2.96 $\Delta A\ min^{-1}$ (10 roots)$^{-1}$. Activity thus increased sharply up to 24 hours (see Figure 7.7A). After that, the hormone-treated values decreased progressively with increase in time, but the control values decreased at 36 hours and then increased again at 48 hours. These latter changes were rather small however. The activities in the hormone-treated seedlings were higher than the controls at all incubation times and statistical analysis of the data using ANOVA showed that enzyme activity was greatly affected by the hormone-

Table 7.8 Effect of 10^{-3} M indole acetic acid on root tip soluble peroxidase activity.

Enzyme activity	Treatment period								
	0h	12h		24h		36h		48h	
($\Delta A\ min^{-1}$)	C	C	T	C	T	C	T	C	T
Activity (10 roots)$^{-1}$:									
mean	0.65	1.22	1.35	2.54	4.39	2.16	3.73	2.48	2.96
±sd	0.08	0.17	0.23	0.09	0.29	0.22	0.15	0.12	0.39
±se	0.02	0.04	0.05	0.02	0.07	0.05	0.04	0.03	0.09
Activity (g fresh wt.)$^{-1}$:									
mean	9.16	6.56	9.48	10.60	11.87	11.82	9.07	15.60	16.05
±sd	0.87	0.73	1.76	0.70	1.29	1.41	1.89	2.81	0.84
±se	0.21	0.17	0.41	0.16	0.30	0.33	0.45	0.66	0.20

Each value is the mean activity ±sd and ±se from 3 separate experiments with 6 replicate analyses in each experiment. Germinating seedlings were harvested at 24 hours and reincubated for up to 48 hours either with distilled water (control) or with hormone. C, control; T, hormone-treated.

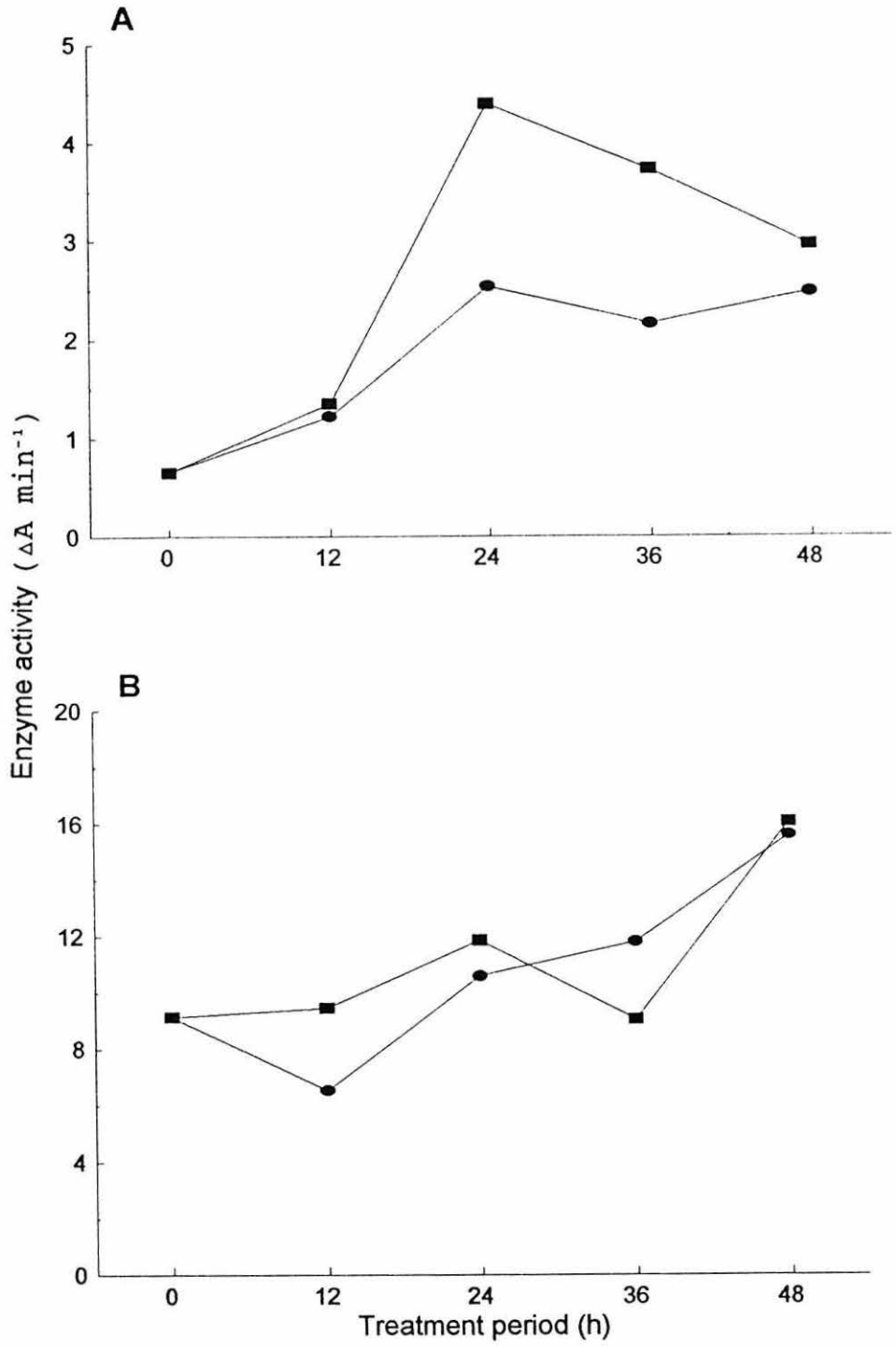


Fig. 7.7. Effect of 10^{-3}M indole acetic acid on soluble peroxidase activity. A, per 10 roots; B, per gram fresh weight; ●, control; ■, hormone-treated.

treatment ($P < 0.001$). Furthermore, there was a significant interaction between the effect of hormone-treatment and time ($P < 0.001$).

Although the values for the soluble peroxidase activity were higher when they were calculated on a per gram fresh weight basis (Table 7.8 and Figure 7.7B), the general pattern of the activities remained similar. The control activity values decreased at 12 hours and then increased progressively with increase in time throughout the germination period. The hormone-treated values increased with time up to 24 hours, decreased at 36 hours and then increased again at 48 hours. Thus, the activity for the control at zero time was 9.16 $\Delta A\ min^{-1}$ (g fresh wt.)$^{-1}$ and the control values for 12, 24, 36 and 48 hours were 6.56, 10.60, 11.82 and 15.60 $\Delta A\ min^{-1}$ (g fresh wt.)$^{-1}$. The corresponding values for the hormone-treated seedlings were 9.48, 11.87, 9.07 and 16.05 $\Delta A\ min^{-1}$ (g fresh wt.)$^{-1}$. It can be seen that the value for the hormone-treated seedlings were higher than the controls, except at 36 hours where the opposite was the case. ANOVA revealed, however, that the hormone-treatment had an insignificant effect ($P = 0.53$) on enzyme activity when the latter was expressed on a per gram basis.

Table 7.9 and Figure 7.8, contain the results for the effects of 10^{-3} M IAA on the insoluble (cell wall) peroxidase. They show that this peroxidase activity in the control roots at zero time was 0.17 $\Delta A\ min^{-1}$ (10 roots)$^{-1}$. The activity increased sharply up to 24 hours, but after that the increase was much smaller. Thus, the control values at 12, 24, 36 and 48 hours were 0.72, 2.10, 2.28 and 2.57 $\Delta A\ min^{-1}$ (10 roots)$^{-1}$. The corresponding hormone-treated values followed a similar pattern; they were 0.50, 2.17, 2.75 and 2.79 $\Delta A\ min^{-1}$ (10 roots)$^{-1}$. The activities in the hormone-treated seedlings were higher than the control values at all incubation times except at 12 hours, where the

Table 7.9 Effect of 10^{-3} M indole acetic acid on root tip insoluble peroxidase activity.

Enzyme activity ($\Delta A\ min^{-1}$)	Treatment period								
	0h	12h		24h		36h		48h	
	C	C	T	C	T	C	T	C	T
Activity (10 roots)$^{-1}$:									
mean	0.17	0.72	0.50	2.10	2.17	2.28	2.75	2.57	2.79
±sd	0.02	0.03	0.09	0.31	0.51	0.49	0.32	0.50	0.23
±se	0.005	0.007	0.02	0.07	0.12	0.12	0.08	0.12	0.05
Activity (g fresh wt.)$^{-1}$:									
mean	2.38	3.88	3.48	8.83	5.80	12.22	6.70	15.79	15.46
±sd	0.35	0.21	0.75	1.83	0.88	1.33	1.48	1.88	3.01
±se	0.08	0.05	0.18	0.43	0.21	0.31	0.35	0.44	0.71

Each value is the mean activity ±sd and ±se from 3 separate experiments with 6 replicate analyses in each experiment. Germinating seedlings were harvested at 24 hours and reincubated for up to 48 hours either with distilled water (control) or with hormone. C, control; T, hormone-treated.

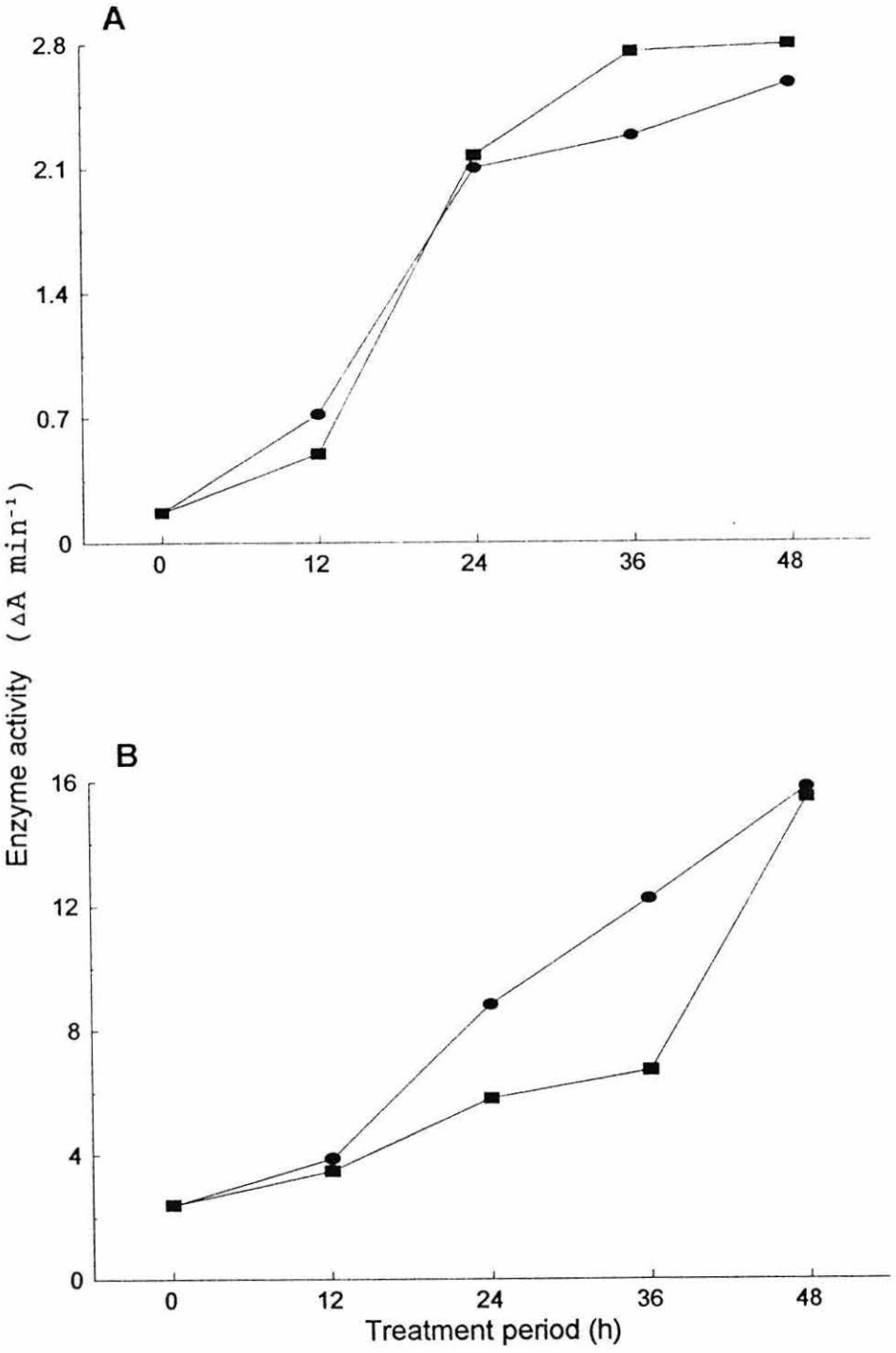


Fig. 7.8. Effect of 10^{-3}M indole acetic acid on insoluble (cell wall) peroxidase activity. A, per 10 roots; B, per gram fresh weight; ●, control; ■, hormone-treated.

opposite was the case (see Figure 7.8A). These differences were small and insignificant ($P = 0.42$ using ANOVA) however.

Because of the small fresh weights of the tip tissues, the enzyme activity values were much higher when they were calculated on the basis of per gram fresh weight (Table 7.9 and Figure 7.8B). The activity for the control at zero time was now 2.38 $\Delta A\ min^{-1}$ (g fresh wt.)$^{-1}$. This value increase with increase in incubation time. The control values for 12, 24, 36 and 48 hours were 3.88, 8.83, 12.22 and 15.79 $\Delta A\ min^{-1}$ (g fresh wt.)$^{-1}$. The corresponding hormone-treated seedlings values similarly increased to 3.48, 5.80, 6.70 and 15.46 $\Delta A\ min^{-1}$ (g fresh wt.)$^{-1}$. The activities expressed on per gram fresh weight basis were thus higher in the controls than in the hormone-treated seedlings, although the differences were small at 12 and 48 hours. Statistical analysis (ANOVA) of these data showed that hormone-treatment had a significant effect ($P = 0.003$) on enzyme activity and that there was a significant interaction between hormone-treatment and time ($P = 0.03$).

7.4 Summary and Discussion

In the experiments described the root length always increased with increasing incubation time as expected, and the general pattern of root growth was the same as seen in earlier studies (see Chapter 3). The effects of various hormones on root growth were very much as expected. Root elongation was slightly stimulated by 10^{-5} to 10^{-7} M GA_3. This was confirmed by a more detailed study using 10^{-6} M, which showed that although the effect was small it was statistically significant. The picture for ABA was more complex. Root growth was apparently stimulated up to 24 hours, but by 48 hours the situation had reversed. The hormone now apparently inhibited growth. Again, all of the effects were

small.

The effects of IAA were much more pronounced and statistically convincing. High concentrations of IAA (10^{-3} and 10^{-4} M) inhibited root growth. In contrast, low concentrations of IAA (10^{-6}, 10^{-8} and 10^{-10} M) promoted growth. These results are in agreement with those of many investigators working on several species (see eg. Yahalom *et al.*, 1991), They reported that IAA at a concentration of 10^{-8} M stimulated *Medicago polymorpha* L. root elongation, while concentrations exceeding 10^{-5} M were inhibitory. In the present study, only this hormone produced effects that could be used as the basis for further experiments.

The low (stimulating) concentration of IAA (10^{-8} M) had no effect on root tip fresh weight. The high (inhibitory) concentration (10^{-3} M), however, caused a large increase in the fresh weight compared with the control, especially at 24 and 36 hours. The effect had disappeared by 48 hours however. Significantly, shorter roots are already known to have fatter root tips (see Chapter 3). It is therefore not surprising that the inhibition of root growth by IAA lead to higher fresh weight values. It is perhaps more surprising that the stimulation of root growth by 10^{-8} M IAA was not accompanied by a reduction in the tip fresh weight.

Generally, the control soluble peroxidase activity, measured on a per root basis, increased with time up to 24 hours, but then decreased at 36 and 48 hours. On a per gram fresh weight basis, the control values increased with time all the way to 48 hours; this was due to the change (decrease) taking place in the fresh weight of the tip and not because of any large change in the actual enzyme activity. The control values for the

insoluble peroxidase, on a per root as well as on a per gram fresh weight basis, increased with time all the way to 48 hours; the increase was most rapid between 0 and 24 hours.

At the low concentration of IAA (10^{-8} M), the hormone had no effect on soluble peroxidase activity, but the insoluble (cell wall) peroxidase activity was increased. This is the opposite of what might be expected, because decreased peroxidase activity is expected when root growth is stimulated (see Chapter 6).

At the high concentration of IAA (10^{-3} M), soluble peroxidase activity on a per root basis was increased by hormone treatment up to 36 hours, but this effect had almost disappeared by 48 hours. The effect of the hormone was still evident when the data were recalculated on to a per gram basis. There was little effect on insoluble (cell wall) peroxidase activity. A small hormone-induced increase in activity was observed on a per root basis. On a per gram fresh weight basis, the activity was actually decreased by hormone action due to the effect of the hormone on the fresh weight of the tip. It is difficult to relate these changes in the cell wall enzyme activity to the observed effect (inhibition) of the 10^{-3} M IAA on root elongation.

CHAPTER EIGHT

Chapter Eight

General Discussion

The experiments described in this thesis were undertaken primarily to study the physiological and biochemical differences between slow and fast growing cotton seedling roots. In the physiological studies, the general parameters of root growth were measured, i.e. root length, fresh weight, dry weight, percentage moisture content, root diameter and segment fresh weight. Identification of the region of root elongation and the demonstration of lateral root initiation were also carried out. In histological studies, the lengths, diameters and volumes of the root cortical cells were measured and the number of cell files per root were recorded. In the biochemical studies, the uptake and incorporation of (Me-^{3}H) thymidine, total DNA levels and peroxidase activities were determined. Finally, the effects of hormones on root growth and the effects of indole acetic acid on peroxidase activities were investigated. It is clear from these studies that slow and fast growing roots behave differently with respect to many of the above mentioned properties.

Obviously, the fresh weight and dry weight of the seedling root increased with increasing germination time. The difference between the fresh weights of the long and short roots was greater than the difference between their corresponding dry weights however. This was because the long roots contained more water than the short roots did, at least during early germination. This conclusion was most clearly expressed in the data for percentage moisture content, which showed clear differences during early germination. The most likely explanation for these differences lies with different cell enlargement rates, leading to differences in cytoplasmic volume and so different water

contents. This was confirmed later when the cell dimensions were measured histologically.

The difference between short (slow growing) and long (fast growing) roots was not confined to their lengths. The short roots were consistently fatter than the long roots, particularly behind the tip region of roots from seedlings that were more than 36 hours old. The same difference could be seen throughout the length of the root (including the tip) during early germination, especially at 36 hours. It might be anticipated, therefore, that the histological studies would reveal significant differences in both cell length and cell diameter.

A clear-cut conclusion of the present study is that root elongation occurred exclusively in the tip region. This was confirmed in later experiments by measuring the cortical cell lengths. These histological experiments showed that most of the cell elongation takes place between 2.5 to 6.0 mm from the tip. In 60 and 84 hour seedlings at least, cell length continued to increase slightly between 6.0 and 7.5 mm from the tip, but not much thereafter. This indicates that, at 7.5 mm from the root tip, the cells were almost fully elongated. There was a considerable difference between the final cell lengths of long and short roots at all germination times; long roots had longer cells as compared with the cells of short roots. It was concluded from these experiments that cell length could be a major determinant of root length.

When the numbers of end-to-end elongated cortical cells in long and short roots were calculated, it was found that long roots contained more cells than short roots at early germination times (36h), but this difference disappeared at later germination times

(84h). Both roots then possessed almost the same number of elongated cells. These calculations took the form of a simple exercise in which the distance from end of the elongation zone (7.5 mm behind the tip) to the root-hypocotyl junction was divided by the cell length. At 36 hours, long roots had 68 end-to-end cells while short roots had 23 cells. At 60 hours there were 121 cells and 87 cells respectively in the long and short roots, while at 84 hours the values were 246 and 216. It can be confidently concluded from these data that final cell length is not the only determinant of root length. The rate of cell elongation also plays a significant part. During early germination, meristematic cells obviously pass more quickly into and through the elongation zone in long roots than they do in short roots. By 84 hours, however, the number of cells that had passed through this developmental process was almost the same. It appears that the meristematic tissues of the fast-growing and slow-growing roots have the same numbers of cells available for differentiation (elongation), but the rate of cell flux through the differentiation process initially proceeds more quickly in fast-growing roots than in slow-growing roots. Eventually, the supply of meristematic cells is used up, so that the slow-growing and fast-growing root end up in the same situation with an equal number of elongated cells.

The results from the cell diameter measurements showed that, at early growth stages i.e. at 36 and 60 hours germination, the newly elongated cortical cells in long roots were wider as compared with those in short roots. At later growth stages (84 hours), however, the situation was reversed. The data regarding cell volumes showed that at 36 and 60 hours the fully elongated cells of long roots also had a greater volume compared with those of short roots, but this effect disappeared by 84 hours. By this time, cell volume was almost the same in the long and short roots. It is particularly interesting that

at 36 hours the fast-growing (long) roots had a cell volume of 3.0 x10^5 μm^3 and the slow-growing (short) roots had a cell volume of 1.4 x10^5 μm^3, whereas at 84 hours they had similar and intermediate values (Ca 2.5 x10^5 μm^3). Thus, the interaction between cell elongation and cell widening changes as seedling root growth proceeds.

The number of cell files did not change significantly with increase in distance from the tip to the end of the elongation zone. Clearly, the cell file number is determined at a very early point in root morphogenesis when the various types of cell (cortex, xylem, phloem, etc) are differentiating just behind the meristem. On the other hand, it is clear that the number of cell files was inversely correlated with root length at all germination times. In other words, short roots had a greater number of cell files than the long roots did. Also, younger seedling roots contained more cell files as compared with older ones, so that the number of files being formed decreased as root growth proceeded from 36 hours up to 84 hours. Obviously, the file number must also play a part in determining the root diameter.

As mentioned above, at early growth stages (36 and 60 hours) the cortical cell diameter in the distal part of the root is greater in long roots than in short roots. The effect of this should be make the long roots wider. The long roots have fewer cell files, however, which has the opposite effect; it reduces root diameter. Actually, the effect of the file number is greater than that of cell diameter, so that the long roots are narrower than the short roots. At 84 hours, the distal part of the short roots has both wider cells and more cell files. That is, both parameters act in the same direction, so that the short roots are wider than long roots. This difference at 84 hours is much less than at 36 hours however. Generally, it is concluded that the final root dimensions and shape are

dependent on a complex interaction of cell length, cell diameter, the number of cells per file and the number of cell files per root. The consequence of these interactions could actually be seen with the naked eye in the form of the degree of tapering of the roots, where long roots were more tapered than short roots were.

A second area of elongation was observed in the hypocotyls after 48 hours of germination. This elongation facilitates in the emergence of cotyledons above the ground during later germination. Finally, it has been demonstrated that lateral root initiation occurred away from the root tip over a fairly extended range up to the root-hypocotyl junction. The function of these lateral roots is to penetrate the soil and to facilitate the uptake of water and nutrients which are essential for plant growth and development.

At early (24 and 36 hours) germination times, the incorporation of (Me-^{3}H) thymidine into DNA was more in long than in short roots, indicating that the rate of cell division during early germination was greater in long roots than in short roots. At later germination times (beyond 36 hours), the incorporation was almost the same in the tips of roots of all lengths. It can be assumed from this observation that the rates of DNA synthesis and cell division at these later times were similar in long and short roots. The present results also show that the thymidine incorporation values were markedly higher in younger (24-48h) roots than in older (60-96h) roots. In other word, the rate of cell division declines quickly as germination and early root growth proceeds. Also it was observed that older (beyond 48h) roots showed signs of damage (browning of the tip) which could be one reason for the decline in the rate of cell division. It was probably not a major reason however, because thymidine incorporation was decreasing well before any tip damage could be seen. The most significant decrease was observed

between 24 and 36 hours. Seedling roots from 24 hours germination incorporated much more thymidine than those from 36 hours, even though the short roots at 24 hours were half of the length of those at 36 hours. This suggests that cell division starts in the radicle of the seed immediately following water imbibition and before germination. It then decreases progressively as germination proceeds. Unfortunately, it was not possible to tell the difference between slow-growing and fast-growing roots before 24 hours, so that meaningful measurements of (Me-^{3}H) thymidine incorporation could not be carried out at these times. The observed higher rate of DNA synthesis in long roots compared with short roots during this early germination period suggests that a faster rate of cell division might contributes to the faster growth of the long roots. The histological data for cell dimensions and cell flux through the elongation processes indicate that this is not the case however. The differences between the rates of cell division appear to be unimportant.

Incorporation was also active in the segments further away from the tip, which suggests that another meristemic region or regions are also present in the cotton seedling root. When the seedlings were left to grow beyond 96 hour, lateral roots appeared in these regions, indicating that the (Me-^{3}H) thymidine incorporation in the upper segments of the root is a reflection of the initiation of these lateral roots.

Besides the differences in DNA synthesis, the amount of total DNA was also different in long and in short roots. Short roots contained more DNA than long roots. This picture was similar for whole root as well as for the root tip and the remaining part of the root behind the tip. More striking, however, was the finding that the amount of DNA was much greater in young and less in older roots. This pattern in the whole roots

was also reflected in the root tip and in the remaining part of the root behind the tip. The large decline in DNA levels was presumably due to the death or loss of mature cells in the older roots. Cell death is known to be associated with xylem tissue formation and the differentiation of phloem also involves the loss of nuclei from the sieve elements. Cells may also be lost from the root cap by a sloughing off process. The latter is known to occur from roots growing through soil, but it is less expected in the present cotton seedlings which were grown between the layers of the rolled paper. Nevertheless, the amount of DNA lost during the growth of the cotton seedling roots in the present experiments is surprising. The presence of more DNA in short roots than in long roots during the early stages of germination is also surprising. It presumably means that the short roots have more cells than long roots do. This is the opposite of what might be expected. At least the finding supports the conclusion from the histological study that differences in root growth rate are not due to differences in the total number of meristematic cells available for elongation. Moreover, as mentioned above, the differences between the rates of thymidine incorporation in long and short roots, and any differences in cell division that it may reflect, are also probably not important to the process of early root growth. It seems that cell elongation and cell flux through the elongation process are the prime determinants of root growth rates.

The observation that root elongation in cotton occurs exclusively in the tip region agrees with results from many other species. The present finding that cell elongation is mainly responsible for root growth and that cell division is not an important contributor is not true for all species however. For example, Maksymowych *et al.* (1986) reported that, up to 4 mm distance from the tip, maize roots grew by both cell division and cell elongation. The region between 4 and 9 mm from the tip grew only by cell elongation

however. This finding is also supported by the earlier work of Erickson and Sax (1956) and Green (1976), who observed that, away from the root cap in maize, the frequently of cell division decreases and cells then increase in length. It has also been shown by Demchenko (1984) that the developing metaxylem elements of wheat roots undergo rapid cell elongation coupled with extensive vacuolation. Ivanov (1987) found that the cessation of mitoses and the onset of this rapid cell elongation coupled with vacuolation are separate developmental processes. It must be remembered in this context that cell division by itself cannot result in root growth (Green, 1976); it only promotes root growth by providing new cells for the subsequent elongation process.

Our observation in cotton of complex interactions between cell elongation, cell widening and cell volume are supported by the earlier work of MacLeod and McLachlan (1975), they reported that in *Vicia faba* roots, cell volume increased basally along the root in all of the tissues of the root, but this was not a result of cell expansion taking place uniformly in all dimensions in the epidermis, cortex and stele. In other words, the degree of cell elongation and cell widening varied along the root. Thus, while increase in cell length was the major factor involved in increasing the cell volume in the stele, the corresponding dimensions in the epidermis and the cortex were cell length and width. They further noted that cell volume was greater in the cortex than other tissues. Atwell (1993) reported that, despite a large decrease in cell elongation in mechanically restricted roots, cell volume can be unchanged due to an increase in radial cell expansion. Maeshima (1990) noted that, in mungbean, the major part of the increase in cell volume is accounted for by enlargement of the vacuolar compartment of the cell. Our histological study has shown that this is also true for cotton.

Relatively little work has been reported on differences in root growth rates in plants growing under optimal environmental conditions. A bigger literature is available for plants subjected to stress. Whalen and Feldman (1988) reported that inhibition of cortical cell elongation was the primary contributor to the inhibition of root elongation in abiotically stressed plants. Fraser *et al.* (1990), studying maize seedling roots, found that cortical cell length was decreased by 30% in water-stressed roots compared with controls. In studies on corn roots subjected to water deficit (Sharp *et al.*, 1988), variability was noted in the sensitivity of the elongation region to the stress. This variability in the sensitivity of the elongating cells was considered responsible for a variability in overall root growth. Thomson and Atwell (1989) reported that the final length of epidermal cells and the total number of cells displaced from the elongation zone per hour (cell flux) were reduced by 20 and 44% respectively in wheat roots grown at 0.055 mM O_2 (partially anaerobic) compared with roots grown at 0.115 or 0.26 mM O_2. In roots exposed to 0.01 mM O_2, cell degradation was evident in the root apices after 48h, presumably due to a lack of ATP required for the maintenance of metabolism.

Cramer *et al.* (1986) and Zhong and Lauchli (1993a) reported that the elongation of the primary root of cotton seedlings is severely inhibited by high levels of salinity and this inhibition can be mitigated by supplying the roots with additional Ca^{2+}. It has been suggested that the protective effect of supplemental Ca^{2+} is related to the improved Ca^{2+} status of the tissue and the maintenance of Na/K selectively (Cramer *et al.*, 1987). Zhong and Lauchli (1993b) reported that, in the primary root of cotton, high salinity also leads to a decrease in the cellulose content of the tissue and that supplemental Ca^{2+} overcomes the inhibitory effect of high Na^{+} on the cellulose content. An anatomical

study by Kurth *et al.* (1986) revealed that the cortical cell size as well as the rate of cell production in the cotton roots were affected by high concentrations of NaCl and/or $CaCl_2$.

Barley (1962) reported that the increase in maize root radius produced by mechanical stress is due to an increase in cell diameter and not to an increase in cell number (i.e. cell division) across the root radius. Wilson *et al.* (1977) also found that mechanical impedance increased the diameter of barley roots, this being largely due to an increased thickness of the cortex. This greater root width was largely due to the greater cross-sectional area of the cortical cells (Barley, 1965; Goss, 1974). Similar results have been reported by Eavis (1972). The situation is rather similar to the situation in the slower growing cotton roots in our present study, especially at later germination times (84 hours) where shorter roots possessed wider cells. Pritchard (1994) proposed that an increase in cell radius following a cessation of cell elongation could result from two mechanisms. Elongation could be stopped by a tightening of the cell wall in the longitudinal direction. This stopping of elongation growth might lead to an increase in turgor if solute import is not regulated. Once turgor exceeds the minimum required for expansion in the radial direction, the cell will swell. Alternatively, radial swelling could also occur if the cell walls loosen in the radial direction. The first mechanism would imply that a rise in turgor would accompany cell swelling, while second would allow radial swelling in the absence of turgor change.

In general, the literature on plant responses to water deficit or high salinity reports that roots also become thicker under these stress conditions (Sharp *et al.*, 1988). For example, Fraser *et al.* (1990) revealed that slower growth rates were seen in water-

stressed roots of maize. Kurth *et al.* (1986) and Zhong and Lauchli (1993a) reported that thickening of cotton roots occurred under high concentration of either NaCl or $CaCl_2$. Solomon *et al.* (1989) found that, in pea roots, high Ca^{2+} abolished the salt-induced root thickening.

The relevance of the literature on stress responses in cell enlargement processes is not immediately obvious for the present work on cotton. In present study, cotton seeds were germinated under normal (non-stressed) conditions. Seedling vigour plays an important role in seed germination and seedling growth however. Heydecker (1972) has pointed out that seeds that produce seedling with faster growth rates are better ones, because they can sustain their growth under unfavourable growing conditions and ultimately produce higher yield. Dalians (1982), working on cotton, also reported that the speed of radicle emergence provides a good prediction of seedling performance in the field. Thus, the differences that we have observed between slow and fast growing cotton roots appear to be similar in nature to the reported differences between roots grown in normal and stressed environments. These observations have important practical implication, because the establishment of vigorously growing seedling is one of the pre-requisites of cotton production (Christiansen and Rowland, 1981). A farmer will fail to achieve his prime goal of harvesting a good crop, he is faced with non-uniform seedling emergence due to poor seed quality.

The two halves of longitudinally bisected maize roots curve inwards when they are reincubated. The maximum degree of inward bending is located in the region of maximal growth and involves the inhibition of cellular elongation (see Pritchard, 1994). The growth of the inner tissues of the root (i.e. the cortex, endodermis and stele) are

affected most by the bisection. This implies that inner tissues are responsible for the regulation (modulation) of the growth of the root. Removal of the epidermis of wheat roots with *n*-diamylacetic acid does not effect the process (Burstrom, 1950). Thus, it appears that the epidermis has no major role in controlling root growth. The restricting layer(s) must lie deeper in the root. Longitudinally bisected cortical sleeves prepared from the growing zone of wheat still bend inwardly. During the preparation of these cortical sleeves, the root divides along the outer radial wall of the endodermis. Therefore, the site of growth restriction is not in the stele but in the inner layer of the cortex or the outer wall of the endodermis. This conclusion was strengthened by measurements of the tensiometric extensibility of cortical and stelar cells in the decelerating zone of maize roots (the proximal end of the elongation zone of the root where the cell elongation rate is decreasing). The cortical tissue was less extensible (both plastic and elastic) than the corresponding stelar tissue (Pritchard and Tomos, 1993). Significantly, there are more cells and therefore more walls per cross-sectional area in the stele compared with the cortex. This would be expected to decrease the extensibility of the stele compared with that of the cortex (expressed on a unit area basis and assuming that the cell walls in each tissue have the same properties). It is more important to note, however, that the cortex as a whole occupies a greater cross-sectional area of the root than the stele (roughly five times). This difference could result in greater extensibility of the stele. It is the relative extensibilities of the two whole tissues that matters most. To summarise this complex picture, the expansion of the whole root would appear to be regulated (modulated) by the cells of the cortex, and in particular those of the inner layer of the cortex. Growth is apparently driven by the middle cortical cell files (Fraser *et al.*, 1990). This situation suggest that, in cotton, the difference between long and short root could be due to one of three reasons; in short roots either

the elongation force in the middle files is less, or the resistance to growth in inner files is more, or a mixture of two effects is present.

A closer look at the literature on the mechanism of cell enlargement and root growth is important. The subject has been recently reviewed by several authors (Taylor *et al.*, 1994; Ferris and Taylor, 1994; Van *et al.*, 1994; Hildalgo *et al.*, 1991; Tang *et al.*, 1992). Lockhart (1965) and Ray *et al.* (1972) were among the first to recognize that cell enlargement begins with a reduction or relaxation of wall stress. As a consequence, turgor pressure and water potential are reduced and water is drawn into the cell. The result is that the cell enlarges by uptake of water initiated by the yielding of the wall. A weakening of the load-bearing network in the wall is needed to produce this reduction of turgor pressure and thereby to cause the uptake of water. Cosgrove (1993) has also summarised the view that wall expansion in growing cells arises from wall stress relaxation followed by water uptake. Significantly, our results for cotton reveal that long (fast-growing) roots contain more water than short roots especially during early germination. In the light of the available literature, therefore, it is possible that the cells of the long roots take up more water because their cell walls undergo a greater relaxation. Maintenance of cell expansion during steady-state growth also requires a continual uptake of solutes by the growing cells. Without this the cell contents would be diluted by water uptake. This would lead to a reduction in osmotic pressure inside the cell and hence a corresponding loss of turgor pressure. In steady state conditions, the water flow into the cell must be balanced by an equivalent solute influx (Steudle, 1985). Thus, the variable growth of cotton roots might be due to variable rates of solute transport into the cortical cells of the elongation zone. During the early stages of germination and root growth these solutes are derived from the cotyledons, so that the

poor root growth might be caused by slow mobilisation of stored nutrients in the cotyledon and/or slow transport of the mobilisation products to the root.

Cell enlargement is brought about largely by changes in cell wall properties (Taiz, 1984). The cell wall consists of cellulose microfibrils, hemicelluloses and pectin (Cassab and Varner, 1988; Fry, 1988; Carpita and Gibeaut, 1993). Root cells generally expand more in the longitudinal than the radial direction (Green, 1976). The orientation of the cellulose microfibrils provides the structural basis of this anisotropy, since they are relatively extensible in the longitudinal direction. They can reorientate and/or separate during expansion in the transverse orientation however (Taiz, 1984; Lloyd and Barlow, 1982; Preston, 1988). Extensible (or loose) walls would be expected to have a microfibril orientation at right angles to the direction of expansion (transverse). Conversely, an orientation parallel to the direction of expansion (oblique) would restrict expansion. When roots were restricted by high soil strength, the swelling of the outer cortical cells was associated with a more oblique microfibril orientation (Veen, 1982).

The cell wall thins during the acceleration phase of enlargement (the loosening phase), only thickening again after growth stops (Scott *et al.*, 1956; Jensen and Ashlan, 1960; Dever *et al.*, 1968; Smith and O'Brien, 1979). This suggests that root growth is not restricted by the rate of synthesis of wall components. However, in maize the hemicellulose component of the wall is laid down during the acceleration phase (Roberts and Butt, 1967, 1969; Masuda and Pilet, 1983). The galactose content of the walls decreases in this accelerating region (Harris and Northcote, 1970). In the decelerating phase of cell enlargement (where cell walls are becoming less extensible), the glucose and xylose contents of the cell wall increase whereas galactose and mannose decrease

(Dever *et al.*, 1968). On the other hand, radio-labelled galactose was taken up and incorporated into the pectin and hemicellulose fractions at a maximum rate as the cell elongation rate decreased (Roberts and Butt, 1969, 1967). Thus, increasing turnover of cell wall galactose, presumably in the pectin and hemicellulose fractions, is associated with regions where the wall is becoming less extensible. Interestingly, treatment of growing root tissue with galactose reduced root extension by tightening the cell wall (Pritchard and Tomos, 1995). The autofluorescence of cell walls due to phenolics was unchanged along the growing zone of unstressed maize roots or following stress-induced wall hardening (Pritchard, 1994) so that there was little correlation of total wall phenolics with the changes in wall properties. Following reduction of growth by NaCl, which may also be due to a tightening of a cell walls, the growing zone of cotton roots had a slight increase in uranic acid and a decrease in the cellulose content of the wall (Zhong and Lauchli, 1993b). Despite these various observed changes in the gross chemistry of the cell wall, it is generally considered that they are not responsible for controlling the process of cell enlargement (Pritchard, 1994). If this is the case, the observed changes in cell wall properties may be the result of changes in the degree of cross-linkage between the components. Cross-linkages are not easy to measure directly, but measurements of the activities of enzymes responsible for their formation or cleavage offer an alternative experimental approach. Wall extensibility could be the result of an enzyme-catalysed breaking of load-bearing bonds within the wall. An increase in its activity during the acceleration phase would loosen the wall allowing faster extension for a given increase in turgor pressure (Cleland, 1967). Likewise a decrease in its activity would be associated with the deceleration phase of the cell elongation. Thus, there is a distinct possibility that the variable growth rate that we have observed in cotton is due to differences in the changes and reformation of cell wall cross

linkages during cell elongation.

It is clear from above discussion that the plant cell wall is a metabolically active compartment of the cell and that it must contain numerous enzymes (Lamport, 1970). From several sources of evidence, it is suggested that specific enzymes are involved in loosening and tightening the wall by changing the structure of the polysaccharides (Cassab and Varner, 1988). These cell wall enzymes are capable of degrading and resynthesising the cross links between the polymers, but their precise role in cell wall extension remain uncertain. Several enzymes which may be involved in wall metabolism during cell elongation have been discussed in Chapter 1. They include peroxidase, ascorbate oxidase, xyloglucan endotransglycosylase (XET), pectinesterase, galactosidases, arabinosidases, xylosidases, glucosidases and glucanases (Fry, 1982; Smith and Fry, 1991; Goldberg, 1984; Lamport, 1965; Labrador and Nicolas, 1985; Dopico *et al.*, 1989; Masuda *et al.*, 1985; Wong and MacLachlan, 1980; Hayashi *et al.*, 1984).

In the present study, it was decided to look at peroxidase. This was chosen because of its possible importance in cell wall metabolism (Masuda and Pilet, 1983) and for its experimental convenience. Although some of the other cell wall enzymes might be more important than peroxidase, they are often more difficult to assay. Also, the necessary substrates for these other enzymes were not easily available and shortage of time restricted our study to one enzyme.

In our first experiment, it was observed that both the soluble and the insoluble (cell wall) peroxidase activities were higher in the fast-growing roots than in the slow-

growing roots. This is the opposite from that which was expected. It has been reported by several workers that peroxidases can catalyse phenolic cross links between macromolecules such as lignin (Gross, 1977), protein (Labella *et al.*, 1968), hemicellulose (Whitmore, 1976), and ferulic acid (Fry, 1983). They are therefore believed to be involved in the cross linking of extensin monomers and feruloylated polysaccharides (Lamport, 1986). Peroxidase also catalyses the formation of covalent cross links between cell wall components, such as the dityrosine link between tyrosine sub-units of extensin (Fry, 1987). Similar diferulate cross links are formed as intramolecular bonds between pectin molecules (Wallace and Fry, 1993). Peroxidase may also cross link proteins by oxidative deamination of lysine (Stahmann and Spencer, 1977). Its activity might, therefore, be expected to correlate with wall tightening. Thus, slow-growing roots might be expected to grow slowly because they have higher peroxidase activity giving rise to tighter cell walls. Conversely, fast-growing roots would have lower peroxidase levels.

In another experiment, conducted more carefully by selecting eight quintiles according to root length, the soluble peroxidase activity on a per root basis did not vary much according to root length. The cell wall peroxidase activity on a per root basis was greater in short than in long roots however. This is more in keeping with the idea that short roots have tighter cell walls because they have higher cell wall peroxidase activities. However, both soluble and cell wall peroxidase activities on a per gram fresh weight basis decreased progressively with decreasing root lengths. This is again the opposite from what might be expected. There is another possible explanation for this however. This decreasing pattern is dictated by the changes in the fresh weight of the root tip, because the fresh weight of the root tips increased markedly as the length of

the root decreased. It must be concluded that the present experiments to relate peroxidase activity to root growth rate present an unclear picture.

The present experiments to study the distribution of soluble and insoluble (cell wall) peroxidase activities along the length of the root produced a more clear picture. In both long and short roots, there was always a high level of enzyme activity in the root tip compared with the part just behind the tip. It is difficult to come any firm conclusion, but one might speculate that, since enzyme activity is high at the tip of the root, the enzyme is associated with the cell elongation and/or cell division which takes place there. The second region of high activity in the more proximal part of the root may similarly be associated with hypocotyl elongation and/or the cell division or elongation which occurs as a part of lateral root initiation.

Peroxidase studies by other workers have also failed to produce firm evidence for a correlation between the enzyme's activity and cell wall tightening. Peroxidase activity in the cell wall of *Phleum pratense* roots increased as cell elongation rate decelerated (Avers and Grimm, 1959), but this may have been due to initialization of root hair formation rather than wall tightening. Total peroxidases activity was unchanged in accelerating and decelerating portions of the growing zone of maize roots (Grison and Pilet, 1978).

As already stated, the effects of the various hormones on cotton root growth were very much as would be expected from our knowledge of their effects in other species. The effects of gibberellic acid and abscisic acid were small and indole acetic acid had more pronounced effects. High concentrations of the latter hormone inhibited root growth,

while low concentrations stimulated growth. Similar results have been reported for *Medicago polymorpha* by Yahalom *et al.* (1991). They showed that low concentrations of IAA stimulated root elongation, while higher concentrations inhibited root elongation. It was also observed in our results that the high (inhibitory) concentration of IAA caused a large increase in the fresh weight compared with the control. This increase in root fresh was associated with root thickening.

Although the effect of GA_3 was small, it was nevertheless noted that 10^{-5} to 10^{-7}M GA_3 stimulated root growth. These results are similar to those of Srivastava *et al.* (1971), who reported that treatment of excised lettuce hypocotyls with GA_3 causes them to elongate and this enhancement of elongation is accompanied by an acceleration of wall synthesis. Barlow (1992) reported that cultured root apices of tomato bearing the Gib-1 mutation, which reduces the level of endogenous gibberellins, grew slower and were thicker than the roots of the wild type. All these effects could be reproduced in wild type roots by the addition of 0.1 μM paclobutrazol to the culture medium (Hedden and Graebe, 1985; Burden *et al.*, 1987). Paclobutrazol inhibits GA biosynthesis causing thickening of maize root apices (Baluska *et al.* 1993). Butcher *et al.* (1990) reported that the effects of both the Gib-1 mutation and paclobutrazol could be normalized by the application of GA_3.

It was observed in our study that ABA slightly inhibited root growth. This confirms the results of El-Antably and Larsen (1974), who reported that ABA inhibited root elongation in *Vicia faba* seedlings. Khan (1969) found that the ABA inhibition of root elongation in barley seedlings could be slightly reversed by treatment with GA_3. Effects on cell wall metabolism are also seen in other plant tissues. Kutschera and Schopfer

(1986a,b) revealed that IAA promoted and ABA inhibited maize coleoptile wall loosening both *in vivo* and *in vitro*. It is clear from these reports that gibberellins and abscisic acid may have a small but significant role to play in the regulation of root cell wall metabolism and growth in cotton.

It was noted in the present study that the soluble peroxidase activity was reduced by high concentrations of IAA during the first 24 hours of incubation and then stimulated between 24 and 48 hours. Insoluble (cell wall) peroxidase activity was reduced all the way to 48 hours. At the low concentration, the hormone had no effect on soluble peroxidase activity, but the insoluble (cell wall) peroxidase activity was stimulated. These results were opposite from that which was expected. The low concentration should reduce cell wall peroxidase activity because it stimulates root growth. High concentrations inhibited root growth and therefore peroxidase should be increased.

San-Jose et al. (1992) reported that, in oak shoots, peroxidase activity was similar in auxin-treated and untreated shoots during the first 4 days after treatment, but subsequently increased in treated shoots and decreased in untreated shoots. Dencheva and Klisurska (1982) and Klisurska and Dencheva (1983) presented evidence that the peroxidase molecule also possess IAA oxidase activity. Denchava and Klisurska (1986) also showed that this IAA oxidase activity of peroxidase increases as extension and differentiation of root cells proceed. This is one of the possible mechanisms through which peroxidase may participate in the regulation of growth and differentiation of primary root cells of maize.

The effects of IAA are not restricted to that on peroxidase activity. It has been proposed

that IAA can stimulate growth by increasing stress-relaxation in the wall (Cosgrove, 1985). Hoson *et al.* (1993) reported that breakdown of xyloglucans in the epidermal cell wall plays an essential role in auxin-induced elongation in dicotyledons. Hoson and Masuda (1992) concluded that the synthesis of matrix polysaccharides may contribute to IAA-induced cell elongation by fixing or extending polymers already present in the cell wall. Edelmann and Kohler (1995) suggested that IAA-induced wall loosening in rye coleoptile may be primarily mediated by cell wall changes other than cleavage of covalent, load-bearing bonds as hypothesized in various wall loosening models. They did not propose an alternative mechanism. Seara et al. (1988) reported, however, that a factor (IAA) that induces growth of epicotyls of *Cicer arietisum*, increases the autolytic capacity of the cell wall by 50%, suggesting that autolysis is related to the process of cell wall loosening that accompany growth. They further added that IAA promotes an increase in the specific activities of the enzymes involved in autolysis, mainly ß-galactosidase. Nardi et al. (1994) reported that humic matter, IAA and IAA-inhibitors stimulated peroxidase activity in *N. plumbaginifolia*, but their effects on cell elongation were not noted.

The present work has identified a number of related areas for future research. The differences in root growth are an expression of some aspects of variable vigour. Slow-growing seedlings are less vigorous than the fast-growing seedlings even though they are not placed under stress. If they were placed under a stress, then there would probably be even greater difference between them. Seed quality with respect to vigour is important for the farmer, because he really needs to have high vigour seeds. At the moment in case of cotton in Pakistan, he is getting seeds with mixed quality with regard to vigour. It is therefore important to direct research towards improving the quality.

It is important to know whether this variability in vigour is due to a physiological/biochemical or a genetic basis. Sheikh (1994) and Sethar (1993), both working on cotton roots, have studied quite a number of physiological and biochemical parameters, which indicate that there is nothing that is absolutely outstanding that might suggest that the problem is physiological. The slow-growing roots just seem to under perform in terms of all studied parameters, eg. leakiness of membranes and respiration. This does not disprove anything, but also it does not give us a lot of valuable information.

New physiological and biochemical approaches have been established by scientists, such as turgor pressure, osmotic pressure, water potential, solute potential measurements, etc. In future studies, it would be important to study changes especially in turgor pressure and water potential between slow-growing and fast-growing cotton seedling roots. This is because both turgor pressure and water potential are important factors in the cell enlargement process that is initiated by the yielding of the wall (Lockhart, 1965 and Ray *et al.*, 1972). Abnormalities in either parameter might have a negative effect on the cell enlargement process and therefore on root growth.

In addition to turgor pressure and water potential, cell wall enzymes also play an important role in wall metabolism (wall loosening and tightening) during cell enlargement. These enzymes include peroxidase, ascorbate oxidase, xyloglucan endotrans-glycosylase (XET), ß-galactosidase, pectinesterase, arabinosidases, xylosidases, glucosidases and glucanases. It would be interesting in future to study the differences, especially in XET and ß-galactosidase activities, between slow-growing and fast-growing cotton seedling roots, because they both are believed to be ideal candidates

for wall loosening (Smith and Fry, 1991; Seara *et al.*, 1988).

If the variability in seed vigour in cotton is genetically based, then it should be possible take the best seeds (those which are fast-growing) and use them as breeding stock. This could be done by growing the plants in the field, collecting the seed from them, collect the best seedling from them and so on over a number of generations. This should progressively improve the quality of the seed. On the evidence from Pakistan cotton breeders, it does not work, however, because the problem is not genetically based. In other words there is something about cotton which is heterogenous. It does not appear to give pure genetic lines very easily even from well-managed breeding programmes.

Another method of selecting good quality seed could be used. One method has been used for other species of the crops such as cereals. This involves the practice of growing the seed producing plants under optimum environmental conditions, particularly temperature and humidity (Schoolrel, 1960; Perry, 1969). It is understood by cotton scientists in Pakitan that seed coming from Punjab is better than seed produced in Sindh, due to better environmental conditions in the Punjab. To extend this knowledge, it would be necessary in Pakistan to search around within the good areas, down to the best farm, and even down to the best field on that farm. Variations in seed quality can be expected from field to field within the farm and also variations in the skill of the farmer will probably be important. For cereal seed production in the west, seed producers select certain farmers who are known to be good farmers and they pay them premium prices to produce high quality seed. The same practice could be adopted for cotton seed production in Pakistan.

It is also known that cotton seeds are harvested in Pakistan at a time to suit to use of the product for fibre production. The quality of seed is secondary, although they are using the same seed for planting in the next season. It may be necessary to choose the harvest time to suit seed production in some cases on selected farms to provide the best quality seed for sowing. In those cases the fibre production becomes secondary. It has also been demonstrated that the quality of cotton seed varies from different parts of plant, i.e. the quality of seeds from higher shoots is different from the seeds from lower shoots (Sethar, personal communication). Differences in seed quality were also noted between proximal and distal parts of the same shoot. If there are such differences, that suggests that it is probably physiological. The physiological experiments in Bangor have not enabled us to identify a single cause for this variable performance however.

Good quality (high vigour) seed could be established by the techniques used in plant biotechnology. Tissue culture and molecular biology are two important and interesting components of plant biotechnology, which may be used to transfer genes between species and propagate selected lines. The successful development of these new techniques have suggested that it is possible to generate improved cotton plants in this way (Mirza and Shaikh, 1983). The advantage of tissue culture is that it is much faster, producing large numbers of plants very quickly from one piece of tissue from a single seed or seedling. It provides a huge advantages in multiplying plants much more quickly than traditional methods. Tissue culture could be used for cotton by using high vigour seeds as the stock tissue for multiplication, but it might have the same disadvantage as using the best seed for reproduction through normal growth cycles in the field. We may still end up with the same range of seedling performance, because the variability may not be genetic in nature.

Molecular biology techniques can be used in two ways. Firstly, the development of recombinant DNA technology and its application to plants has provided opportunities for the investigation and understanding of the molecular basis of plant development and for the identification and cloning of desirable plant genes. The successful routine use of the Ti plasmid of *Agrobacterium tumefaciens* to introduce foreign genes stably into plant cells has created opportunities for the molecular genetic manipulation of dicotyledonous plants. In the case of cotton, several workers, for example Firoozababy *et al.* (1987), have reported that cotyledon tissues can be efficiently transformed and plants regenerated that express the newly incorporated genes. For improving cotton seed quality we are unable to do this at the moment, because the specific genes have not been identified. It could be done after identification of genes which are involved in seed vigour.

Finally, molecular biology techniques could be used to look for differences between slow-growing and fast-growing roots with respect to gene products (proteins). This could be done by gel electrophoresis (particularly two dimensional) to compare long and short roots to look for any specific differences in the proteins produced. For example, proteins which are consistently either missing or present at low levels in seeds that are growing slowly as compared with fast-growing seeds. After that, it would be possible to start the application of molecular biology to improve the seeds by identifying the genes which are not being expressed properly, i.e. the one producing abnormal low levels of their product proteins. The other possibility is that using DNA marker technology. This technology has provided a means for plant breeders to select desirable plants directly on the basis of genotype instead of phenotype. DNA markers, developed by RFLP (restriction fragment length polymorphism) and RAPD (random amplified

polymorphic DNA) techniques, provide a means for the transformation of QTL (quantitative traits loci) into medelian entities that can be manipulated more readily in classical breeding programmes. These techniques may also help to find and clone genes for genetic engineering (introduce foreign genes) purposes (Beckmann and Soller, 1986; Paran and Michelmore, 1993).

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