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Tor-Henning Iversen

THE ROLES OF STATOLITHS, AUXIN TRANSPORT, AND AUXIN METABOLISM IN ROOT GEOTROPISM

TRONDHEIM 1974

K. norske Vidensk. Selsk. Mus. Miscellanea 15 - 1974

THE ROLES OF STATOLITHS, AUXIN TRANSPORT, AND AUXIN METABOLISM IN ROOT GEOTROPISM

by

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ISBN 82-7126-041-3

ABSTRACT

Iversen, Tor-Henning. 1974. The roles of statoliths, auxin transport, and auxin metabolism in root geotropism. *K. norske Vidensk. Selsk. Mus. Miscellanea 15*: 1-216.

The behaviour of amyloplasts in geotropically sensitive organs and metabolic processes involving auxin in higher plants are discussed in this study. The validity of the starch statolith hypothesis is examined and statolith function is correlated with the mechanism of differential growth found in geotropical responses.

The development of geotropic curvature has been followed by light microscopy and scanning electron microscopy. The ultrastructure of root tip cells is described in detail in order to obtain information as to which organelles may act as gravity receptors. By suitably chosen manipulations of cress seedlings the amyloplasts can be directed to certain regions of the cell either before or after stimulation. Observations on the ensuing geotropic response and comparison with the observed movements of the amyloplasts makes the amyloplasts the most likely candidate for the statolith function. These results are supported by studies on the dependence of geotropic sensitivity on the presence of amyloplast starch. Roots and coleoptiles made starch-free by treatment with gibberellic acid and kinetin are unable to respond to gravity. Attempts have been made to study the role of auxin transport and metabolism in geotropism in plant roots. In translocation experiments with radioactive labelled indole-acetic acid (IAA) an acropetal flux of IAA was found both through root segments and intact seedlings.

Biochemical and cytochemical studies have also been performed to clarify whether IAA-oxidase and peroxidase are involved in the distribution of endogenous IAA upon geotropic stimulation of plant roots. Specific methods for ultrastructural localization of these enzymes have also been described.

On the basis of these experimental observations attempts have been made towards establishing the causal relationships in plant geotropism.

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PREFACE

In order to obtain a more complete and unified picture of the geotropical problems in higher plants the author in addition to including new results, has preferred to reconsider and summarize in this thesis the main results from the following previously published papers. It is to be noted that most of these papers have not been given in the Reference section. The magnification of the electron micrographs is indicated by the length of the horizontal line which is 1 μm unless otherwise stated.

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Chapter 1

EARLIER STUDIES ON THE STARCH STATOLITH HYPOTHESIS AND GEOTROPISM

A. General introduction

It has been known for a long time in the history of scientific botany that several plant organs are able to orientate themselves in relation to their environment. This orientation is expressed as a movement of the plant. It has also been recognized for several years that these movements could be brought forth by a number of external and internal factors. There is no doubt that one of the most important of these factors, when expressed in the most general way, is exposure under certain angles to a field of mass acceleration. Gravity is the natural representative of stimuli of this type and it plays an important role for the plant where the main roots grow downward, main stems upward and branches and leaves develop at relatively constant angles to the plumb line. These movements are called geotropic movements and the term geotropism, which was introduced by Frank (1868), describes the general phenomenon of the influence of gravity on plant organs.

The geotropic movements are the result of a directional stimulation by gravity. In this way the plant organs approach their normal orientation relative to the direction of gravity. The movements can either be due to differential cell elongation and/or embryonic growth ("growth movements") or to differential changes in the turgor pressure within certain cells ("turgor movements"). The geotropic growth movements are the best studied and can be induced by centrifugal forces as well as by gravity.

The means by which a plant organ perceives its orientation in a gravitational field is a very intriguing problem. From a physiological point of view, the force of gravity is practically uniform and constant on the earth. Gravity acts equally on the entire plant and also upon each cell of the organ. The question is then whether the gravity is stimulatory on the entire plant or only on certain parts of a organ, on certain cells or on one or more of the structural components of the cells. It seems as if the most likely way for an organ to perceive its orientation, is by movement of certain constituents of the cells. Therefore, attention has for a long time been focussed on the occurrence in some plant cells of amyloplasts ("statoliths") which are starch-

containing bodies capable of movement under the influence of a gravitational field. Several authors have attempted to elucidate the mechanism of graviperception in plants by referring to these particles.

In the first chapter a summary of the studies on the starch statolith hypothesis will be given following an introduction of the terminology used.

B. Terminology

Many terms have been used to describe different aspects of geotropism and the meaning of these terms has varied from one paper to another. A brief summary of the general terms used in this paper will therefore be given here.

It is generally accepted that a geotropical movement is a result of a reaction chain, similar to that applicable to a number of other physiological processes.

At least a theoretical distinction between various phases can be made (see e.g. Larsen 1962):

Physical phase	Physiological phases			
Susception	Perception (Reception)	Transmission	Reaction	After effects
	← Stimulation →			

This chain can be divided in at least two phases; a. A physical phase which may be called susception, b. Several physiological phases.

The term stimulation is commonly used to comprise the physical phase and the first physiological phase which may be called perception or reception. In geotropism the physical phase consists in the direct action of gravity, e.g. on the statoliths. The mechanical effect performed by the statoliths is perceived in the first physiological phase, which creates a certain "excitation." In the phase of transmission this "excitation" is forwarded to the site of the reaction where the reaction proper, the geotropic curvature, takes place. This reaction may be followed by after-effects.

The geotropic stimulation period can also be divided as follows. After a plant organ, e.g. a root, has been placed in a horizontal position, it takes a certain time before a curvature can be observed. This period is called the reaction time (or latent time) and it depends on the method

of observation. The presentation time is a term used to designate the duration of stimulation (by a given acceleration) required to produce a minimum response after the necessary reaction time (Larsen 1962). The difference between the reaction time and the presentation time was called the latent period by Rufelt (1954, 1957).

The direction in which the axis of a geotropically reactive plant organ can be maintained for prolonged periods of time whithout the organ carrying out geotropic reactions, is called the normal or liminal direction. The direction of the plumb line is usually taken as reference and if we choose the downward direction of the plumb line as zero (Fig. 1), the liminal direction of main roots is close to 0°, and for main shoots and coleoptiles it is close to 180°. The term orthotropic designates these organs, while organs which grow at an angle to the direction of the plumb line are called plagiotropic. The geotropic curvature is usually formed as a result of a preferential cell elongation of certain structural elements on one side of the organ. When orthotropic, subterranean organs such as main roots are geotropically stimulated in the horizontal position, the curvature is a consequence of a preferential elongation of the upper side which becomes convex. The result of this is a bending in the same direction as the force of gravity: a positive geotropic reaction. When orthotropic, aerial plant organs (e.g. main shoots, coleoptiles) are geotropically stimulated, their lower side becomes convex, and they carry out a negative geotropic reaction. The term non-geotropic (or ageotropic) is used to describe plant organs which do not carry out geotropic reactions as a consequence of changing the direction of their axis with reference to the plumb line.

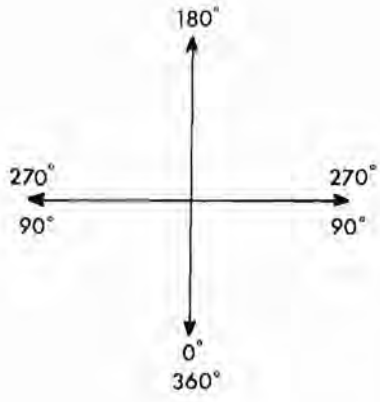


Fig. 1. Conventions for expressing the orientation of the axis of plant organs. The angles indicate the direction in which the tip of the organ is pointing. 135° thus means: pointing obliquely upward; 45°: pointing obliquely downward.

C. The statolith hypothesis

The term statoliths has for a long time been used to describe movable masses of high specific weight occurring in the cyst organs in Crustacea. The cyst organs serve as equilibrium organs and are therefore called statocysts. The function of the statocysts and the statoliths as gravity receptors is well known. In the decapod Crustacea the cyst is located in the basal segment of the two first antennae and the statoliths are attached to the sensory hairs which protrude from the walls of the cyst cavity. When the animal changes the position this causes a shift of the statolith mass. This displacement seems to be the first link of the chain which transforms mechanical energy into nervous activity ultimately resulting in a reaction serving to restore the normal position of the animal relative to gravity.

The occurrence in some plant cells of starch grains capable of movement under the influence of a gravitational field was first noted by Naegeli in 1858. The possible function of these organelles in graviperception was suggested by Berthold (1886) and Noll (1892, 1900). Noll, working with *Nitella* sp., ascribed the movable nucleoplasm the statolith-function in these plants.

In an attempt to elucidate the mechanism of graviperception in plants Haberlandt (1900) and Němec (1900), independently, reported experiments and observations that showed a close correlation between the occurrence and motility of starch grains on the one hand, and the presence of geotropic sensitivity on the other. Based on these observations they put forward the starch statolith hypothesis which in its classical form assumes that geotropic stimulation is brought about by the pressure of amyloplasts with starch grains on the cytoplasm lining the outer tangential walls of the sensitive cells.

The amyloplasts often occur in parenchyma cells as large freely-falling organelles having a density and surface : volume ratio such that they settle under gravity in a geosensitive cell in a few minutes. The distribution of such cells in higher plants shows such a close correlation, both in time and space, with the geosensitive regions that these cells have generally been regarded as statocytes containing starch statoliths.

Even if the amyloplasts are accepted as the most likely statoliths in green plant cells, it has, however, been suggested that other particels might function as statoliths (see e.g. Audus 1962). There are

a number of particles in the cell having a density considerably different from that of the surrounding cytoplasm. It has for instance been suggested that nucleus, mitochondria, microsomes, and protein molecules which all have a density exceeding that of the cytoplasm, might function as statoliths. These particles when influenced by gravity, tend to sink to the lowest point in the cell. The operation of statoliths lighter than the surrounding cytoplasm has also been suggested. These include oil droplets and lipid-rich granules which tend to float upwards in the cell.

Giesenhagen (1901) set forth the idea that the "shining bodies" ("Glanzkörper") present in the unicellular rhizoids of *Chara*, functioned as statoliths. Later Cholodny (1922) envisaged microsomes as graviperceptors and the idea that particles of microsomal size might act as statoliths has been favoured e.g. by Hertz and Grahm (1958).

Applying Stoke's law, Audus (1962) calculated the rates at which the various cellular organelles would move under the influence of gravity. The cytoplasmic medium is assumed to have a density of 1 and a viscosity between 5 and 20 centipoises while the density of starch grains is about 1.5 and that of mitochondria and microsomes about 1.2. As the movement of the particles also depends on the surface : volume ratio, Audus (1962) and Gordon (1963) concluded that microsomes of about 30 nm diameter and spherical protein molecules of 5 nm diameter, have a sedimentation rate too low for them to be considered as possible statolith particles. Particles of this size would also be disturbed by thermal agitation (Brownian movements) and the streaming of the cytoplasm (cyclosis). These factors eliminate the directional movements and the orientation of such small particles under the influence of gravity. As will be demonstrated in detail in Chapter IV the cell organelles which can be realistically considered as possible statolith particles in higher plants are starch grains and mitochondria.

D. The starch grains as gravity receptors

The starch statolith hypothesis has been the object of extensive experimentation and speculation and a number of studies have provided strong evidence that starch grains do act as statoliths and form an integral part of the gravi-perception system in higher plants. Both qualitative correlation between the presence of starch statoliths and geosensitive organs and the experimental approaches to the problem give support to the hypothesis:

1. Correlation between the presence of starch grains and geosensitivity in plant organs

The correlation between the occurrence of movable starch and the presence of geotropic sensitivity has been demonstrated by several investigators. This correlation holds even in a few species of plants which do not manufacture starch in their chloroplasts and do not have starch as the storage form of carbohydrate. These plants (e.g. *Iris* and *Allium*) do, however, posess movable starch grains in their geosensitive organs such as the root cap and the cotyledons, and in that way they therefore strongly support the starch statolith hypothesis.

There are, however, a few plants where the abovementioned correlation does not hold. As discussed by Wilkins (1966) these plants can be divided into two groups: 1. Plants containing movable starch grains but which lack geosensitivity and 2. plants that exhibit geosensitivity but have no movable starch grains.

There are a few examples of plant organs which are geotropically insensitive but posess movable starch grains. Haberlandt (1903) places the secondary roots of *Myosotis palustris* L. and *Oxalis acetocella* L. in this category, but these cases alone do not disprove the starch statolith hypothesis. It is possible that one or more links in the normal reaction chain may be missing in these species.

Audus (1962) cites two examples of geotropic sensitivity in starch-free organs of higher plants; the perianth of *Clivia nobilis* Lindl. and the aerial roots of the cattleya-like orchid *Laelia anceps* Lindl. These cases may be explained by assuming that other particles function as statoliths.

The literature also contains an example of a lower plant which falls in the first category. In the marine algae *Caulerpa* Haberlandt (1906) has shown that there exists mobile starch grains in the stems while the organ is geotropically insensitive.

As a whole, the correlation between the presence of starch grains which show rapid sedimentation under the influence of gravity and the occurrence of geotropic sensitivity is suggestive of the statolith function of the movable starch.

2. Correlation between the experimental manipulation of starch grains and geosensitivity

Several investigators have attempted to correlate geotropic responses of seedlings with assumed or observed movements of statolith starch. By suitably chosen manipulations of the seedlings it is possible to direct the amyloplasts to certain regions of the cell before or after stimulation, or both. By comparing the ensuing geotropic responses with the expected or observed movements of the starch grains, the results can be evaluated in relation to the starch statolith hypothesis. The results of the older experiments and the conclusions drawn from these have been variously interpreted for or against the starch statolith hypothesis by various reviewers.

Several authors have claimed that the time required for starch grains to move from their normal position to the lower side of the cell after the seedling has been placed horizontal, is approximately the same as the minimum duration of stimulus (presentation time) required to induce a detectable geotropic curvature. These presentation times vary from 1 min to about 20 min. In most cases, descriptions of the methods used to determine the speed of amyloplast movement are insufficient or entirely lacking. Hawker (1932, 1933), however, has given a detailed description of the methods. She found a close correlation between the presentation time and the rate of amyloplast movements at various temperatures (varying from 10° to 40°C) in the epicotyls of *Lathyrus odoratus* L. seedlings. An optimum at 30°C, where the rate of movement is highest and the presentation time shortest, was found.

The conclusions drawn from the experiments performed by Zimmermann (1924, 1927) and von Ubisch (1928) remained controversial for many years. The experiments made by Zimmermann (1927) are well known (e.g. Larsen 1971) but even so a short review of the experimental procedure and the results will be given here. Zimmermann determined the minimum geotropic stimulation time with 1 g for cress roots. After the stimulation, the seedlings were placed for 5 min in a vertical position, either normal or inverted. To compensate unilateral gravitational stimulation during the development of the geotropic curvatures, the seedlings were thereafter rotated paralell to the horizontal axis of a klinostat for 45 min. The response was recorded by counting the number of reacting roots. Zimmermann found that inversion of the seedlings enhanced the geotropic response and he interpreted his results in favour of the

starch statolith hypothesis. Both Zimmermann (1927) and Haberlandt (1928) regarded the sliding of the starch grains along the peripheral cytoplasm as stimulatory.

Von Ubisch (1928) carried out a similar type of experiment, but with a modification; the seedlings were first inverted for 20 min sufficient for the amyloplasts to settle in the "ceiling" end of the cell as opposed to the normal location ("floor end"). The pre-inverted seedlings were then stimulated in the horizontal position for a certain time and thereafter turned to a vertical position, either normal or inverted again. In the latter case the amyloplasts would slide only the short distance back to the ceiling and the stimulus, and therefore the response, should be less than that produced by turning the seedling to the normal position. It would have been anticipated that this manipulation should yield a result opposite to what Zimmermann observed. However, von Ubisch claimed that this was not the case and that inversion after the horizontal stimulation produced the largest curvatures and reduced the minimum stimulation time.

On the basis of her report von Ubisch claimed to have performed what she called the experimentum crucis against the starch statolith hypothesis. Her results represent a serious objection to the validity of the statolith theory. She has been criticized for insufficient documentation as she presented no quantitative data in her paper (Audus 1962) but her predictions and results have been confirmed by Larsen (1965). He repeated the experiments described by Zimmermann (1927) and von Ubisch (1928) with improved methods and quantitative recording of the degrees of curvature and fully confirmed their results. However, as discussed by Larsen (1965, 1971) a critical examination of the design of the experiments by Zimmermann and von Ubisch, shows that the effects of the manipulations of the plants by these two authors may have no bearing on the starch statolith hypothesis. In its original form the hypothesis assumed that geotropic stimulation is brought about by the pressure of amyloplasts on the sensitive cytoplasm lining the outer tangential walls of the sensitive cells. In accordance with this, the sliding of amyloplasts along these walls in a vertical direction should not involve any stimulation. On this background, only during the horizontal stimulation would there thus have been a susception of gravity, and Zimmermann and von Ubisch results must be regarded as tonic, i.e. interferring with the reactivity and sensitivity, rather than tropistic.

Larsen (1969) has redesigned the experiments on the correlation between starch grain manipulation and geotropic sensitivity. By letting

the amyloplasts slide along the cell walls at specified angles to the plumb line Larsen found a clear relationship between the amyloplast movements and the geotropic response and his results support the statolith hypothesis.

3. Correlation between the experimental removal of statolith starch and the elimination of geotropic responsiveness

Attempts to deprive responsive plants of their movable starch grains have been made in order to study the dependence of geotropic sensitivity on the presence of such starch. Although the statolith starch is most persistent and remains intact in the tissue after all storage starch has been removed, several investigators have succeeded in making statolith starch-free organs. Effective means have been chilling, starvation in darkness, and treatment with various chemicals. As stressed, however, by several authors (e.g. Wilkins 1966) great care must be exercised in interpreting the results of this type of experiment. The experiments should be carried out without permanent damage to the plant material and the organs should be capable of cell elongation after destarching. Unfortunately, the results of the older experiments on these lines have not been universally accepted as evidence either for or against the starch statolith hypothesis. The reason being that in several cases although it was concluded that no permanent damage had occurred to the cell (the organs reformed starch when returned to more favourable conditions), information on other related physiological processes was lacking. Measurements of growth rate and tests for geotropic reactivity should preferably be carried out simultaneously on the same plant material; this is of important relevance to the validity of the causal relationship between the disappearance of starch grains and geosensitivity.

One of the earliest experiments made to deprive plant organs of statolith starch was performed by Haberlandt (1902). Exposure of stems to low temperatures resulted in starch-free stems which had lost their geotropic sensitivity. Němec (1902) obtained similar results by embedding roots of peas and broad beans in plaster of paris. Similar results have been obtained by treatment with chemicals such as potassium alum. Using this treatment, Went (1909), working together with Pekelharing, succeeded in partially depleting *Lepidium* roots of their starch,

but some of the roots were still able to respond to gravity.

In the abovementioned experiments the reactions were not uniform in the entire plant material, and quantitative data on growth rates and geotropic responses are often scarce. Zollikofer (1918), however, presented a well-documented report on the correlation between starch-depleted plants and geosensitivity. By 3 to 4 days starvation in darkness, seedlings of *Tagetes*, *Dimorphoteca*, and *Helianthus* became starch-free, whereby their geosensitivity decreased markedly. Zollikofer also reported that in the same plants the growth rate and phototropic responses were unaffected. When the seedlings were illuminated, the starch was reformed after 2 to 4 days and the sensitivity to gravity restored.

There are also several reports of seed plant material which has been experimentally destarched and still retains geotropic sensitivity. Syre (1938) reported that treatment with sulphur dioxide results in complete destarching of the plants while the geosensitivity is unaffected. Similar results were obtained by von Bismarck (1959) with shoots of *Sphagnum*. By exposure of the stems to low temperature the cells were depleted of starch but the organs were after the chilling, still able to respond to gravity.

A detailed discussion of more recent reports on the correlation between starch-depleted plants and geosensitivity will be given in Chapter VI.

E. Purpose and outline of the present study

The aim of the present study is to elucidate, firstly, the validity of the statolith hypothesis as related to higher plants, and, secondly, to correlate statolith function and the mechanism of differential growth in geotropic responses.

In Chapter II a study of the structure of the plant root both at the cellular and sub-cellular level is presented. The effect of gravity on the development of geotropic curvatures is described in Chapter III. It will be shown that the geotropic curvature develops in two phases; the initial stage appears as an asymmetry in the root cap region, the disappearance of which is followed by a second phase with differential growth of cells in the elongation zone. The movement of amyloplasts and various other cell organelles which may function as graviprecipitable bodies in the statocyte cells, is demonstrated in Chapter IV. The results

from this chapter and the next (Chapter V) where the relationship between amyloplast movements and geotropic responses is studied, are discussed in relation to the classical statolith hypothesis. The importance of the statolith starch for the geotropic sensitivity will be demonstrated in Chapter VI. The movement of auxin and the relationship between auxin transport and the geotropic response of roots are dealt with in Chapter VII. In Chapter VIII is presented a detailed biochemical and electronmicroscopical cytochemical study of the auxin destroying enzymes and their relation to the gravity receptors in root cells. A general discussion of the result and a physiological interpretation of the graviperception mechanism will be presented in Chapter IX.

Chapter II

THE MORPHOLOGY OF THE ROOT TIP AND THE ULTRASTRUCTURE OF THE ROOT TIP CELLS

A. Introduction

In the search for gravity receptors in plants, most investigations up to the present time have concentrated on the study of organelles which, because of their dimensions, are identifiable in the light microscope. The information, however, which this instrument can give is limited because of the resolution. More detailed information on the ultrastructure of the gravity receptors and their interrelationships with other cell components can be obtained by the use of the electron microscope. Pioneer work in this field of gravity studies was made by Audus (1962) and Griffiths & Audus (1964). Since then the use of the electron microscope as a tool in geotropical studies has been greatly extended.

The value of the electron microscope in gravitational studies has been clearly demonstrated by Sievers (1965, 1967 a, b, c). In several ultrastructural studies he has demonstrated and analyzed the statolith function in the positive geotropic reaction of the rhizoids of *Chara*. Based on detailed observations of the ultrastructure of the organelles of the rhizoid tip, Sievers was able to show that, in this plant, the geotropic curvature can be explained by a rather direct action of statoliths, where the Golgi-apparatus and the socalled "Glanzkörper" (statoliths) act together as a self-regulating cellular system. A similar possible linkage between the Golgi-apparatus and the geotropic reaction in higher plants has been suggested by Shen-Miller & Miller (1972 a). They examined the distribution of dictyosomes and the vesicle production from these organelles in the avascular tip cells of oat coleoptile upon geostimulation. In a closely related study Shen-Miller & Miller (1972 b) observed the intracellular distribution of mitochondria after geotropic stimulation of the oat coleoptile. In this report they presented results which indicated that the redistribution of mitochondria upon geostimulation is dependent on the fixation procedure used for the electron microscopical work.

The usefulness of the electron microscope in other studies related to geotropical problems has also been demonstrated by Pickard & Thimann (1966) in their study of the geotropic response of starch-depleted wheat coleoptiles. A study on similar lines was performed by

Nougarède & Pilet (1971) who studied the activity of gibberellic acid on the ultrastructure of the amyloplasts in roots of *Lens*. The electron microscope has also been used by Juniper & French (1970) who examined the starch grain ultrastructure and the fine structure of cells that perceive gravity in root tips of maize.

In an ultrastructural study of the root cap of *Lepidium sativum* L., Sievers & Volkmann (1972) pointed to the importance of the asymmetry of the peripheral columella cell. They suggested, on basis of their observations on the localization of the endoplasmic reticulum membrane complex, that geoperception in roots may be a function of pressure exerted differentially by amyloplasts on this membrane system.

The electron microscope has been used extensively in the present study as a necessity for further progress in the search for the linkage between the gravity receptors and the mechanism which ultimately results in the geotropic curvature. In the present chapter detailed ultrastructural studies of different cell organelles in several plant species will be dealt with and their possible connection to the geotropical developmental pattern in higher plants will be discussed.

B. Materials and Methods

Plant material and cultivation

Seeds of the garden cress (*Lepidium sativum* L.), maize (*Zea mays* L.), dwarf wax bean (*Phaseolus vulgaris* L. cv. "Gullhorn"), cabbage (*Brassica oleracea* L. cv. Ditmarsker), and sunflower (*Helianthus annuus* L.) were sterilized with a 3% calcium hypochlorite solution for 15 min, rinsed with sterile water, and germinated on moist filter paper in petri dishes. The seedlings were kept in the dark at 21°C until they had attained a root length of 2.5 to 4 cm. They were then ready for the fixation procedure.

Light microscopy

The roots were cut with razor blades in segments of 1 cm and immediately thereafter fixed in Nawaschin II solution for 12 h. After fixation the roots were carried through the embedding process to paraffin, sectioned to 9 to 15 µm thickness and stained with toluidine blue,

Feulgen or periodic acid and Schiff's reagent (PAS), using the procedure described by Jensen (1962). By this last method, the starch grains stain intensively red, whereas the cellulose walls, cytoplasm, and nuclei are less intensively stained.

Occasionally, the roots were prefixed in 3% glutaraldehyde and postfixed in 2% OsO_4, dehydrated in ethanol, and embedded in Epon. From these semi-thin sections (0.5 to 3.0 µm) were obtained, and they were stained with the PAS-reagents after gentle heating of the sections.

Transmission electron micsoscopy (TEM)

Root segments (1-2 mm) were cut and in accordance with the purpose of the experimental procedure fixed in one of the following fixatives:

1) Two hours at 20°C in 3% glutaraldehyde in 0.1 M sodium phosphate buffer at pH 7.2. The segments were rinsed for 2 h at room temperature in four changes of the same buffer and postfixed for 2 h at 4°C (occasionally at 21°C for special purposes) in 0.1 M phosphate buffered 2% osmium tetroxide (OsO_4), pH 7.2 ("double-fixation").

2) Two hours at 20°C in 3% glutaraldehyde and rinsing as above but postfixed for 1 h at 4°C in 2% potassium permanganate ($KMnO_4$) in veronal-acetate buffer (Luft 1956).

3) One hour at 4°C in 2% $KMnO_4$ in veronal-acetate buffer containing 1.5% sucrose and 0.014% calcium chloride.

4) Two hours at 4°C in 2% OsO_4 in veronal-acetate buffer (Caulfield 1957) containing 0.014% calcium chloride.

Dehydration was carried out in ethanol of increasing concentration at room temperature and finally in two baths of propylene oxide. The material was embedded in Epon for 48 h at 60°C. Ultrathin sections were cut from near the median plane of the roots with glass or diamond knives. The sections were mounted on copper grids and stained for 15 min in 1% uranyl-acetate diluted in ethanol or water and thereafter for about the same time with lead citrate (Reynolds 1963). The sections were examined in a Siemens Elmiskop 101, and JEOL 100B electron microscope.

Scanning electron microscopy (SEM)

For the study of the roots in SEM, roots were fixed for 2 h at $4^{\circ}C$ in 2% OsO_4 in 0.1 M sodium phosphate buffer at pH 7.0. After thorough rinsing in the same buffer, the roots were dehydrated through a graded ethanol series of increasing concentration, and transferred from the 100% solution to xylene. The roots were then infiltrated in paraffin and excess paraffin was removed with xylene. A thin film of gold palladium alloy was applied to the roots under vacuum and the roots were examined in a Cambridge Scanning Electron Microscope.

C. Results

The root anatomy

The general anatomy of the cress root tip is illustrated in Figs. 2 and 3. The root cap occupies the first 200 to 250 µm of the tip when analyzed from the sections. The natural length of the root cap is approximately 500 µm but due to the fixation procedure the mature cells in the extreme root cap region will disappear. Between 250 and 300 µm from the tip is the meristematic region, which usually consists of the quiescent centre and the apical initials, from which the protoderm and cortex differentiate. Behind this region the first cell identifiable as a xylem cell (a metaxylem vessel element) is found. Shortly behind this the xylem has differentiated and provascular cells can be identified. The vascular region becomes well defined at 500 to 600 µm while the first phloem cells appear even further behind. There is little if any increase in diameter of the root past 500 µm, where the elongation zone starts.

The cell development in the root occurs in two directions, apical and basal to the apical initials. The cells of the root cap and the remainder of the root are the results of the apical and basal development, respectively. The cells of the root cap develop rather quickly into functionally mature cells. The region behind the apical initials is normally transversally divided into the central cylinder, pericycle, cortex, protoderm, and epidermis (Fig. 3).

The epidermis arises from the root cap by periclinal divisions. The term protoderm, is usually applied to the outer layer of the young

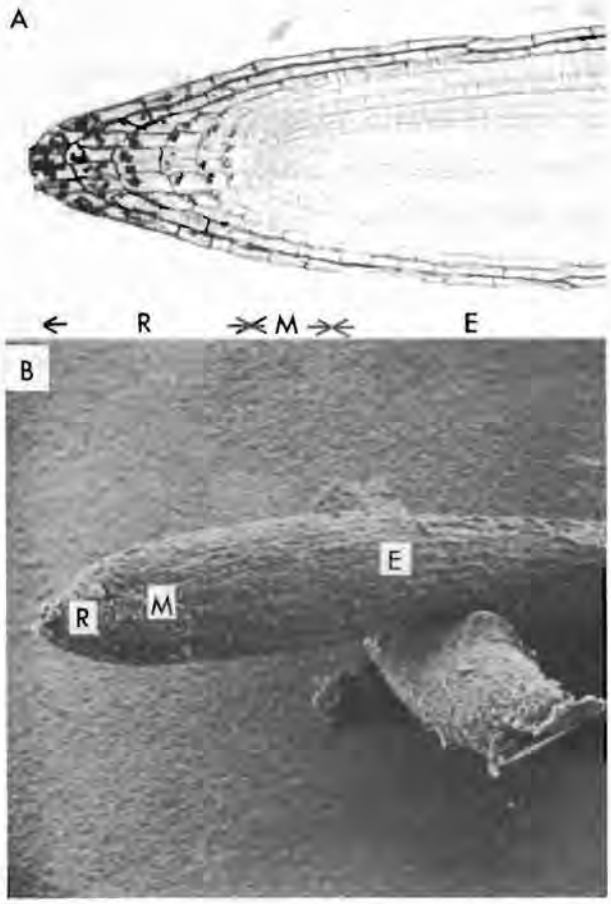


Fig. 2. *The anatomy of the cress root tip demonstrated in the light microscope (A) and the scanning electron microscope (B).* The root cap (R), meristematic region (M), and the apical parts of the elongation zone (E) are indicated.

root. The root protoderm has a common origin with either the cortex or the root cap.

In cress roots the central region of the root cap is distinct from the peripheral part as it has few or no longitudinal divisions. This region which forms a center core in the area between the apical initials and the extreme root tip, is very conspicuous (Figs. 2 and 3) and is usually referred to as the columella. The tip cells are located at the acropetal portion of the columella cells and are probably derived from them.

The central part of the root cap of cress (Fig. 3) contains 7 (occasionally 8) storeys, marked I to VII, of developing (I), functional (II-V), or degenerate (VI, VII) statocytes. Amyloplasts are present in

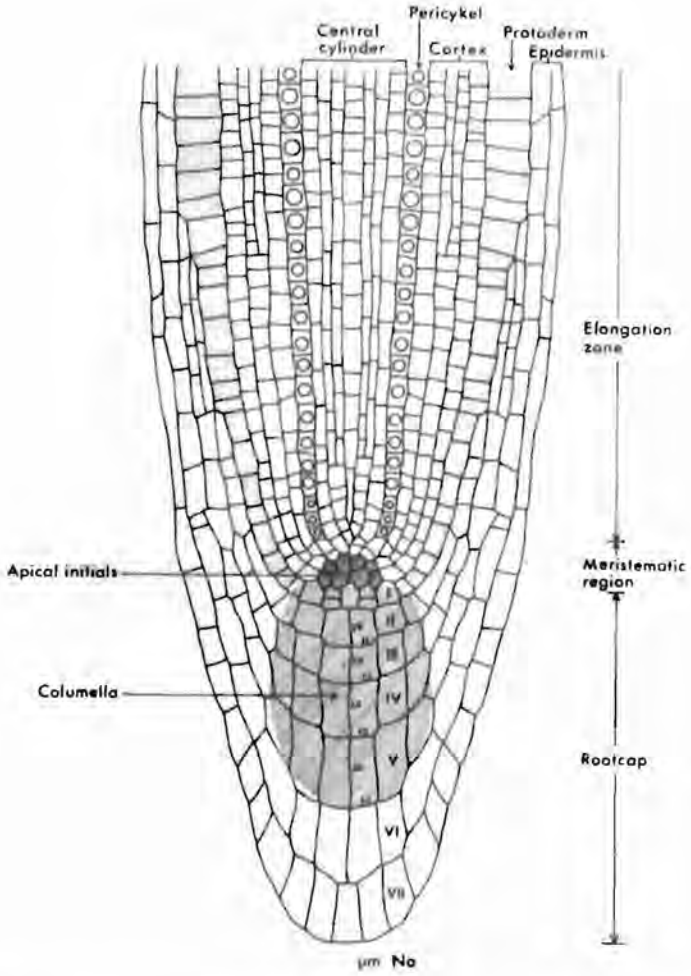


Fig. 3. *Semi-schematic map of the different parts of the root tip of cress.* Numbers I to VII indicate the storeys of starch-containing cells, and the average lengths are indicated in µm.

Storeys II through VI, usually also in No VII (Fig. 3). The average length and width in µm of the central statocytes in each storey are given in Fig. 3.

The ultrastructure of the root tip cells

The appearance of the groundplasm (hyaloplasm) in the root tip cells is dependent on the fixative used. The groundplasm contains granules varying in size from 10 nm in diameter downwards to the limit of resolution. When fixed in permanganate as the only fixative the matrix of the groundplasm is finely and uniformly granular and e.g. ribosomes are absent (Fig. 4 A). Permanganate produces excellent fixation

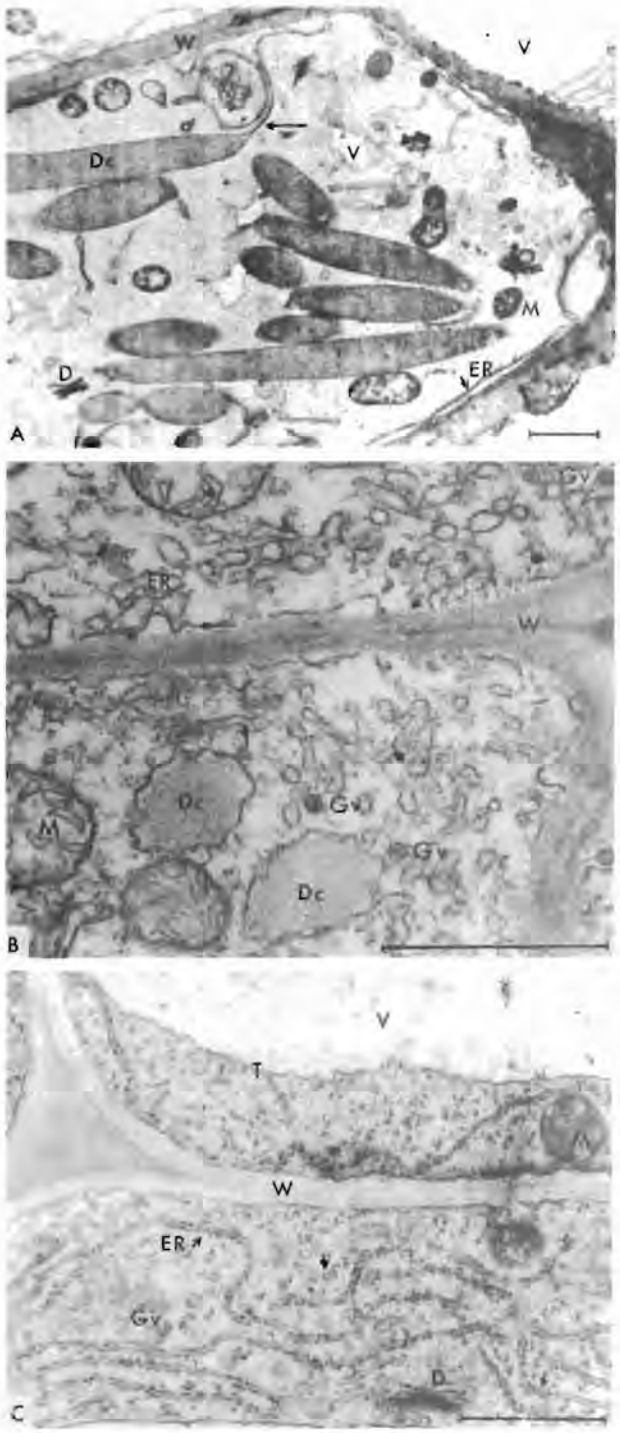


Fig. 4. *Appearance of root tip cells fixed in different fixatives.*

A. Potassium permangante. Membranes of the endoplasmic reticulum (ER, arrows) are clearly seen, dilated cisternae of the ER (Dc) are present, the groundplasm is uniformly granular, and ribosomes are missing. Root of cress.

B. Osmium tetroxide. The ER-membrane system (ER, arrows) is not as well preserved as in A. Dilated cisternae of the ER (Dc) can be seen in cross sections. Ribosomes are present. Root of cress.

C. Glutaraldehyde/osmium tetroxide (double fixation). Membranes are well preserved and both ribosomes and polyribosomes can be seen attached to the ER or free in the cytoplasm. Root of dwarf wax bean.

In addition the following organelles are indicated: V = vacuoles, M = mitochondrion, Gv = Golgi vesicles, T = tonoplast, D = dictyosomes, W = cell walls, arrows = polyribosomes.

and staining of the membranes in the root tip cells, but fails to fix and may even destroy nucleic acids and proteins. In the present study permanganate has been used either along or in combinations with glutaraldehyde, only for special purposes e.g. for the preservation of the endoplasmic reticulum membranes of the root tip cells.

Osmium tetroxide alone, as a staining fixative also has its limitations. It is a much less specific stain than permanganate and it may sometimes fail to preserve endoplasmic reticulum membranes. The

groundplasm exhibits a higher degree of granularity after osmium fixation (Fig. 4 B) than after permanganate fixation. In conjugation, however, with glutaraldehyde (double-fixation) osmium tetroxide reveals a high proportion of the cell contents in a well-fixed and stained condition. The extensive preservation which this double-fixation gives for plant root cells is extremely valuable, particulary where fine detail is required (Fig. 4 C). In the present study the double-fixation procedure has been used as a general fixative.

Nucleus

The nuclei of the columella cells (Fig. 5 A) and the epidermal root cap cells (Fig. 5 B) are irregular in outline compared to the nuclei of meristematic cells (Fig. 5 C). The interphase nucleus as shown in Fig. 5 C, occupies up to one half of the volume of the cell whereas in differentiated cells (Fig. 5 B) it is much smaller relative to the cell.

The nucleus is surrounded by the nuclear envelope which is a double membrane (Fig. 5 B, arrow) with membrane pores, and the outer membrane is continuous with the granular endoplasmic reticulum (Fig. 6). Nucleoli are present in most of the cells. Nucleoli which contain large amounts of RNA and protein, differ in size in meristematic and other root tip cells. As shown in Fig. 5 the rapidly dividing regions (Fig. 5 C) have nucleoli about three times the size of those in the basal part of the columella region (Fig. 5 A).

In the columella region the nucleus is always to be found near the ceiling of the cell. In the extreme root cap cells, where the vacuole occupies most of the cell, the nucleus is usually located in the groundplasm lining the longitudinal cell walls.

Endoplasmic reticulum (ER)

The endoplasmic reticulum appears in sections as lines of double unit membranes permeating the groundplasm. On the basis of serial sectioning of cress root tips, sections from bean root tip cells (Fig. 7), and freeze-etched preparations made by Branton & Moor (1964), a schematically three-dimensional picture of the ER-membrane system in and between two root cap cells, has been made (Fig. 6).

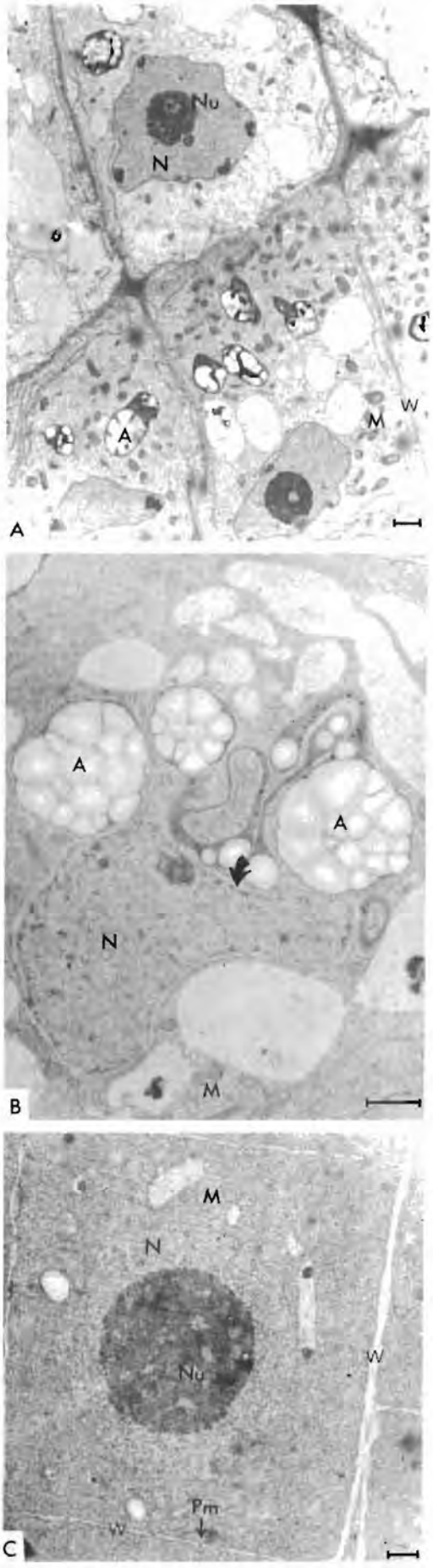


Fig. 5. *The appearance of nuclei in cells from different regions of the root tip.* Nuclei (N) with nuclueoli (Nu) in cells from the columella region in cress (A), in the epidermal cells of cabbage (B), and in the meristematic cells of cress (C). Cell walls (W), mitochondrion (M), plasmodesmata (Pm, arrow), amyloplasts (A), and the nuclear envelope (arrow, B) are also indicated.

Fig. 6. *Three-dimensional representation of parts of two root tip cells.* This reconstruction is based on serial sectioning (compare Fig. 7) and freeze-etched preparations of root tip cells. The endoplasmic reticulum (S-ER, G-ER) forms branched, fenestrated sheets throughout the cells and passes from one cell to another via the plasmodesmata (Pm). Both surface and transverse views of mitochondrion (M), dictysome (D), and nucleus (N) are shown. Cell wall (W), vacuole (V), and polyribosomes (P) are also indicated.

The drawing is slightly modified from the original picture (Fig. 7) in order to demonstrate that the ER forms branched, fenestrated sheets throughout the cells and passes from one cell to another via the plasmodesmata.

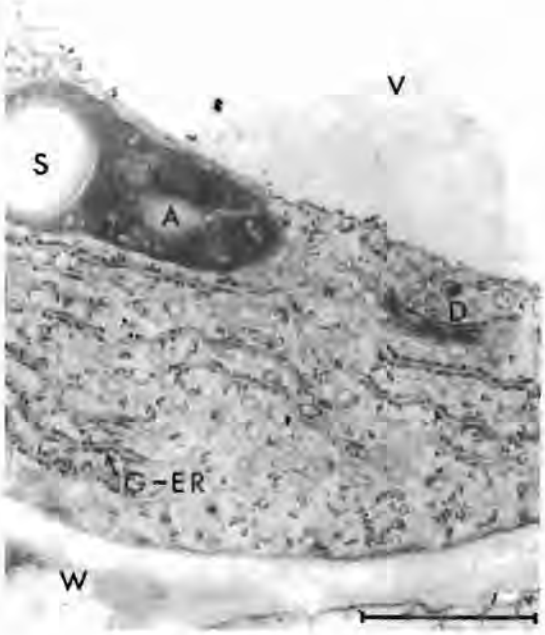


Fig. 7. *Ultrastructure of a part of root tip cell from dwarf wax bean.* This figure has been the basis for the upper part of the artistic interpretation presented in Fig. 6. The following organelles are indicated; amyloplast (A) with starch (S), dictyosome (D), granular endoplasmic reticulum (G-ER, arrow), and vacuole (V).

As can be seen from the figures, ER is quite extensive in the root cap cells. ER is often connected to the nuclear membrane, frequently surrounds it and may be generated from it. In the root tip cells ER appears sparse in the cells of the meristematic region but well developed in the expanding cells both of the elongation zone and the root cap. It is assumed that the area of ER increases more or less proportionally with the volume of the cell in the root cap. A striking feature of the ER in the columella cells (Storey II, III, and IV) is that the membranes are often concentrated in the distal part of the cells and apparently without conjunction with the nuclear membrane. The peripheral membranes tend to be paralell to the cell wall. The ER profiles paralell to the cell walls can also be seen in the more vacuolated apical and peripheral columella cells. In the cells on the periphery of the root cap the ER membranes form irregular rings and loops.

When seen on thin sections the ER appears as two dark lines 8-10 nm thick separated by a less electron dense line 15-20 nm in thickness. Each of the dark lines represents a unit membrane. The space

between the two dark lines, has been called enchylema (Frey-Wyssling 1964): This space can be widened and form dilated cisternae of ER (Fig. 4 A; Iversen & Flood 1969, Iversen 1970).

As demonstrated on Fig. 6 the ER membrane system is hollow and forms the so-called cisternae. It is assumed that the internal spaces are continuous throughout the cell. The lumen of the nuclear envelope is in open conjunction with the ER cisternal matrix (enchylema).

Some parts of the ER are covered by ribosomes and these ribosomes are always located on the outer surfaces of the membranes (Fig. 7). The ER coated with ribosomes is termed "rough-surfaced" or "granular" ER while ER without ribosomes is called "smooth-surfaced" or "agranular". In the root tips examined in the present study both types have been observed (Figs. 7 and 4 C).

It has been assumed (Buvat 1963) that most of the ER near the cell wall is rough whereas that in the rest of the cytoplasm is smooth. This idea has not been confirmed as the observed species of plants used in the present study often contain both types of ER-membranes in conjunction with each other (Fig. 6). The only difference is that although the rough ER is invariably sheet-like, the smooth ER can be either sheet-like or tubular.

Ribosomes

Ribosomes are present in all types of root tip cells which have been studies, but the number per unit volume varies. The particles appear in the cells as osmiophilic particles of about 15-25 nm in diameter. The ribosomes have been observed both free in the cytoplasm and attached to the ER (e.g. Fig. 4 C). In the meristematic and undifferentiated cells the ribosomes are mainly free in the groundplasm and not on the ER, while the attachment to the ER is more common in differentiated root cells. It has been suggested that ribosomes attached to ER are more active in protein synthesis than free ribosomes, but the significance of the attachment to ER is not known.

Frequently ribosomes appear to be grouped into clusters (polyribosomes) with the appearance of spirals or circles. These clusters can be observed free in the groundplasm but they also occur attached to the ER (figs. 4 C, 6, and 7). The number of ribosomes in each polyribosome chain is of the same order in cress and cabbage root tip cells; 10-15 particles. It is possible that polyribosomes are groups of ribosomes

attached to a thread of messenger RNA. At high magnification a fibre about 1 to 2 nm in diameter has been observed connecting the particles of the chain. In a few investigations it has been reported that such fibres can be destroyed by treatment with ribonuclease.

Mitochondria

Numerous mitochondria are present in all root tip cells of the different species studied. They showed the following ultrastructural features typical of mitochondria (Fig. 4 B); a surrounding double membrane, cristae, matrix and occasional dense granules. The majority of them in meristematic cells are circular or slightly ellipsoid in cross section, but of more varying form and size in the differentiated cells of the root cap. They are commonly about 0.3 µm in diameter if cylindrical, but if spherical or elongated they are up to 3 µm long.

The number of mitochondria in root cap cells of cress has been estimated, based on serial sectioning, to be from about 200 to 2000. The meristematic cells and Storey I in the root cap region have the smallest amount and the number of mitochondria increases through the apical end of the root cap.

Golgi apparatus

In the present study the term Golgi apparatus will be restricted to describe a collection of dictyosomes which are interassociated. As the plant cells may contain several Golgi apparatus each consisting of many dictyosomes, the term apparatus is used to denote both singular and plural. The dictyosome in a plant root cell can be demonstrated e.g. on Fig. 4 C. The dictyosome consists of several cisternae (usually four to seven in each dictyosome) and depending on the plant material the diameter of the cisterna is on the average 1-3 µm and the stack is about 0.5 µm high.

As can be shown on Figs. 4 C, 6, and 8 the cisternae has a vesiculated or dilated periphery. This is clearly seen in the outer root cap cells of maize where hypertrophied dictyocomes produce large vesicles which move through the plasmalemma and may displace it from the cell wall (Fig. 8). The function of the Golgi apparatus in these cells is assumed to be in the formation of cell walls and extracellular structures.

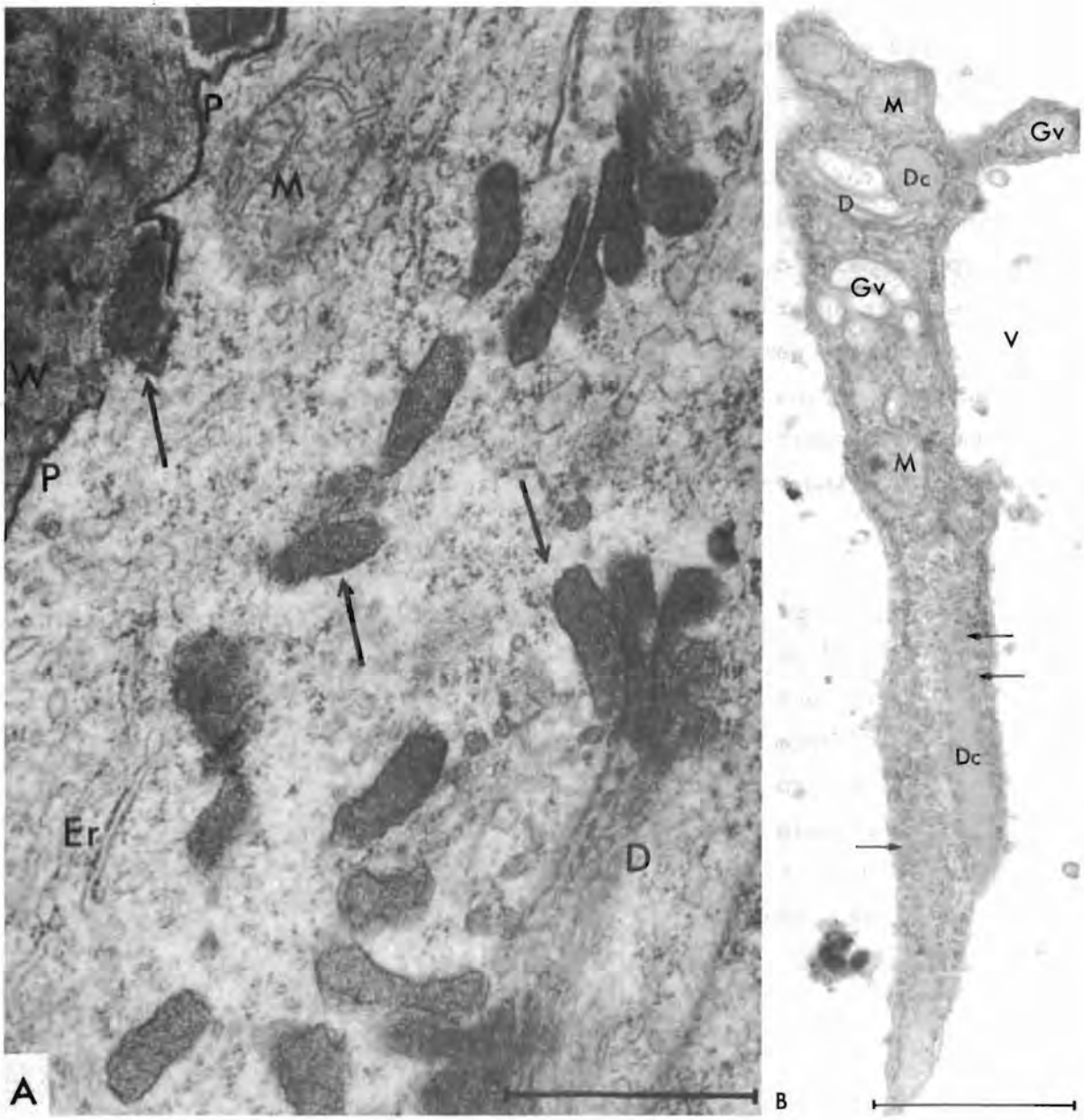


Fig. 8. *Dictyosomes and Golgi vesicles in epidermal root tip cells.*

A. Golgi vesicles (arrows) moving from the dictyosome (D) in cytoplasm to the cell wall (W) in maize.

B. Dictyosome (D) and Golgi vesicles (Gv) in vacuolated cells of cress. Polyribosomes are present (arrows).

Note the difference in electron density of the Golgi vesicles (OsO_4-fixation). The following cell organelles are indicated: plasmalemma (P), mitochondrion (M), endoplasmic reticulum (ER), dilated cisternae of the ER (Dc), and the vacuole (V).

There is especially in maize but also in the other plant species studied an apparent difference in the secretary activity of the dictyosomes. A conversion from small vesicle production to large vesicle production occurs in the cells when moving from the meristematic region through the columella region to the apical root cap cells (Fig. 9). Also the number of vesicles increases in the same direction. Due to the hypertrophied condition of the dictyosomes in the apical root cap cells, the Golgi apparatus become very evident.

As demonstrated on Fig. 8 the displacement of plasmalemma results from passage of the dictyosome derived vesicles to the plasmalemma and the deposition of the vesicle content outside the membrane. At the same time the vesicle membrane is incorporated in the plasma membrane.

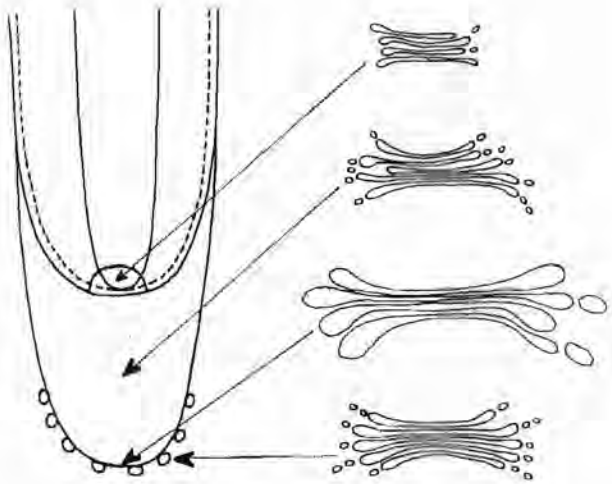


Fig. 9. *The appearance of the dictyosomes in different regions of a root tip of maize.* The production and size of Golgi vesicles are indicated (drawn to the same scale).

As more vesicle content is deposited between the cell wall and plasmalemma, the displacement becomes more severe. In maize the vesicle product accumulation moves to the exterior of the root tip to form a droplet on the root cap which has been characterized as a polysaccharide (Morré et al. 1967). In cress, bean, and cabbage a similar droplet on the root cap has not been observed and the hypertrophication is less severe.

The dictyosome vesicle product accumulation has not been seen as distinctly in cress, bean, and cabbage as in maize. There has, however, been demonstrated on electron micrographs of these species an accumulation of material between the cell wall and plasma membrane. The electron density of this accumulated material corresponds with the electron density of the cytoplasmic dictyosome vesicles of the same plant

species. This correlation between the electron density of the vesicle content and the cell wall accumulated material is most marked in the epidermal and cortical cells of the root tip.

The number of dictyosomes in each cell depends on the plant species. The number of dictyosomes in each cell section has been estimated for the different species, and in all cases dictyosomes have been shown on the average to be almost as numerous as mitochondria. In columella cells of cress the number of dictyosomes per cell has been estimated to be in the region of 200 to 300. In the more peripheral root cap cells the number increases two to three times the number in the columella cells. In maize root tip cells a number of the same order has been found.

Amyloplasts

The amyloplasts which are plastids filled with starch, appear in different forms and with different starch content depending on the plant species and the root zone examined. The membrane around each starch grain is assumed to have arisen from invagination of the inner membrane of the amyloplast envelope. These cisterna-like projections of the inner membrane can most clearly be demonstrated in starch-depleted amyloplasts after permanganate fixation (cf. Fig. 42 D).

As mentioned the number of starch grains within an amyloplast varies with the species and the tissue. Mature amyloplasts with starch grains have not been observed in meristematic root cells. In this region, however, proplastids can be identified at a certain stage in their development before they have developed starch grains. At an intermedate stage of development the proplastids are very similar to mitochondria in appearance. It only becomes possible to distinguish proplastids with certainty after the inner layer of the double outer membrane invaginates and form vesicles.

The maturing of the amyloplasts starts in the basal cell regions of the columella. In cress root cap cells of this region (e.g. Storey I; Fig. 5 A) each amyloplast contains one to a few starch grains. The amyloplasts here are scattered all over the cell but in the storeys II-IV they are located at the floor end of the cell when the roots are growing in the normal position (Fig. 10).

The number of amyloplasts in these cells is on the average about twenty and about the same number is found in the more apical and epidermal cells of the root cap. In these cells the amyloplasts are

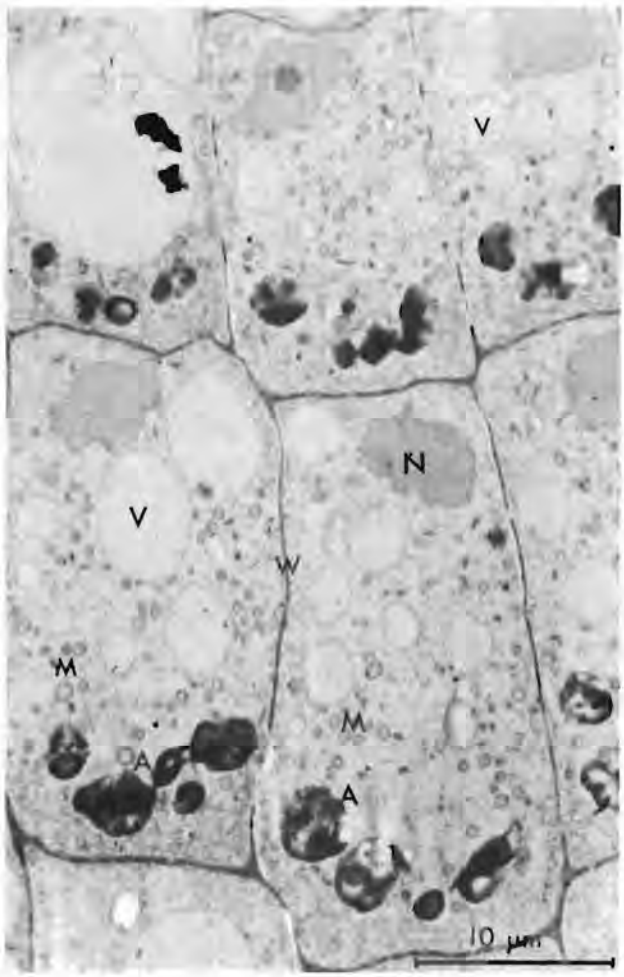


Fig. 10. *Part of the root cap region in cress roots.* Storeys II and III are shcwn and the amyloplasts (A), nuclei (N), vacuoles (V), mitochondra (M), and cell wall (W) are indicated.

confined to the cytoplasm lining the cell walls but they can occasionally be observed in cytoplasmic trabeculae through the vacuole.

Based on series of ultra-thin sections the size, form, and interrelationships of the amyloplasts can be depicted. Fig. 11 consists of selected electron-micrographs from a series of 30 sections (each of 700 Å thickness) taken from Storey VI in the root cap of cress. As shown the dimensions and form of the amyloplasts can be estimated; e.g. no 1 (Fig. 11, A to E) is roughly spherical and has the dimensions of 1.2 µm for the least diameter and 2.5 µm for the greatest diameter. From this serial sectioning can also be shown that the cells in this region do not contain one regular, ovoid vacuole but consist of several more or less irregular small vacuoles which coalesce at a few points (Fig. 11, D to G).

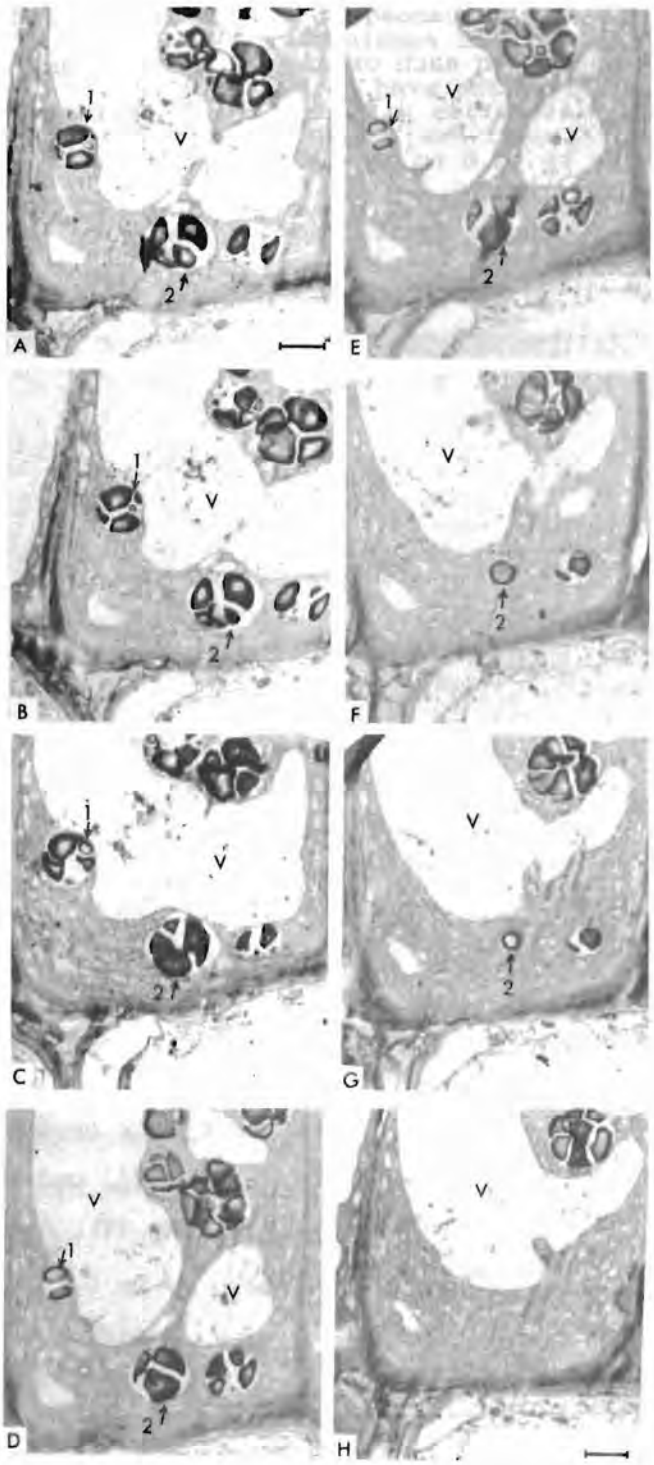


Fig. 11. *Serial sectioning of peripheral root cap cells of cress.* The outlines of the amyloplasts can be demonstrated (Nos 1 and 2, arrows) and their dimensions determined. Vacuoles (V) are indicated.

The most marked difference between amyloplasts in different species of plants involves the number of starch grains found in each amyloplast. In cress the amyloplasts in the root cap contain 1-10 starch

conjunction with others, has the morphological features necessary for statolith function? Which cell organelles are functionally involved in the linkage between detection of gravitational stimulus and its physiological implementation and to what extent are they involved? Finally, are specific cell organelles implicated and if so, do they function in the development of the differential growth which ultimately leads to a geotropic curvature? These questions cannot be answered at the present time but ultrastructural studies may contribute to the solution of these problems.

Griffiths & Audus (1964) stressed that there was no close and constant associations of amyloplasts with any other organelles in the root cap cells of *Vicia faba* L. They observed that in a few specimens ER entwined the amyloplasts at the distal end of the cell but they assumed that this was a fortuitous observation and without physiological significance. The present study has confirmed that there generally do not exist associations between amyloplasts and other cell organelles. The observation, however, that the ER-membrane system forms extensive fenestrated sheets in the root cap cells (Fig. 6) makes it necessary to take this into account when discussing the movements of amyloplasts in geotropically stimulated plant roots. It may be assumed from this that the amyloplasts or other cell organelles do not move freely in the cells. In determinations of the velocity of fall of various organelles, the values for the viscosity of cytoplasm have their limitations. The viscosity is poorly defined on account of the inhomogeneous composition of the cytoplasm and the velocity of fall of organelles through the cytoplasm is also influenced by the sheets of the ER-membranes. Both the falling velocity of amyloplasts upon geotropic stimulation and their final location are influenced by the ER-complex. Griffiths & Audus (1964) concluded that the tangled mass of ER, normally located at the apical end of the cell, was displaced as a result of the amyloplast movement, into the upper half of the cell during geotropic stimulation. They assumed that this was merely a question of mutual exclusion as the large amyloplasts occupying space in the lower sectors made it impossible for them to contain aggregations of ER. The matter of ER-displacement will be discussed in detail in Chapter IV but it seems necessary at the present time to stress that the connections between ER-membranes from one cell to the next through plasmodesmata (Fig. 6) make the ER-complex fairly rigid and stationary, and therefore displacements of this system seem unrealistic.

In sections the ER-membranes constitute only a limited area of the cell. From serial sectioning the impression of an extensive membrane-complex is confirmed (Fig. 6) and the area of ER increases more or less proportionally with the volume of the cell in the root tip. Clowes & Juniper (1964) and Juniper & Clowes (1965) have estimated there to be about 400 μm^2 of ER in dividing cells and up to 10.000 μm^2 in fully differentiated cells of the root cap.

In a detailed study Sievers & Volkmann (1972) pointed to the structural differences in the peripheral columella cells of cress. They reported that the ER-complexes have a parabolic shape and the surface areas of the peripheral ER-complexes form an acute angle with the plant organ axis. They related the positions of the amyloplasts before or after geotropic stimulation to the location of the ER-membrane system and suggested that geoperception in roots may be a function of pressure exerted differentially by the amyloplasts on the ER-complex.

The distribution and location of the ER-membrane complex in columella cells of cress has previously been reported (Iversen 1968), but the structural differences between the central and peripheral columella cells were not stressed. In the present study this has been taken into consideration and this matter will be discussed in Chapter IV.

The participation of the Golgi apparatus in the geotropical reaction chain has been suggested by several authors. Both the Golgi apparatus and the endoplasmic reticulum, and the vesicles produced by both, are closely associated with wall increment. Several studies have indicated that in specific plant cells, the cisternae and vesicles contributing to the plasmalemma and cell walls, are derived from the Golgi apparatus. The production of large vesicles from the hypertrophied dictyosomes and the accumulation of these vesicles on the cell wall in the outer root cap of maize (Fig. 8), has previously been observed by several authors (Mollenhauer et al. 1961, Mollenhauer & Whaley 1963). Using autoradiographical methods Northcote & Pickett-Heaps (1966) demonstrated how tritium-labelled cell wall precursors were transported in the Golgi vesicles to the cell wall in the root cap cells of *Triticum*. These results were taken as conclusive evidence for the role of the Golgi apparatus in cell wall increment. Similar experiments have been performed both on lower and higher plants (e.g. Barton 1968, Brown et al. 1969, Morré 1970) and the participation of the Golgi apparatus in the formation of the cell plate as well as the primary and secondary cell wall, is generally accepted.

The close correlation between the displacement of Golgi vesicles and the geotropic curvature has been clearly demonstrated in the *Chara* rhizoid (Sievers 1965, 1967, a, b, c, 1971; Sievers & Schröter 1971). In the normally oriented rhizoid the dictyosomes can be observed above the statoliths ("shining bodies" ; see Chapter I). When the rhizoid in the normal position grows as a result of the incorporation of Golgi vesicles in the cell wall, this growth is uniformly distributed over the entire growing tip region. In a horizontally positioned rhizoid the statoliths sink to the lower part of the tip and thereby they block the lower passageway of the Golgi vesicles and widen the upper path. As a result, a differential growth occurs on the upper and lower sides; the growth of the first becomes intensified, the latter stops. This causes the tip to develop a positively geotropic curvature downward.

Complications arise for various reasons when this theory based on a comparatively simple system, is applied to higher plants. This has been done by Shen-Miller & Miller (1972 a) who examined the distribution and activation of the Golgi apparatus in the tip cells of the oat coleoptile. They found a differential distribution of dictyosomes in the avascular tip cells upon geostimulation and they also observed a differential activation of the dictyosomes with respect to gravity in the tip cells of the lower tissue. Similar differences in the distribution and activations occurred also in other regions of the coleoptile. Based on these observations the authors suggested that the Golgi apparatus participates in the differential elongation of cells after geostimulation of coleoptiles.

The ultrastructural study of the occurrence of dictyosomes in the different regions of the root tip presented in this chapter, indicate that these organelles, at least in maize, contribute to the growth of the cell wall. The occurrence of the quiescent and active forms of dictyosomes can also be closely correlated with the respective regions of cell growth and enlargement. It is, however, at the present time difficult to decide at which stage in the geotropic reaction the Golgi vesicles are involved; do they act as geosensors or do they contribute to the secondary physiological phases preceding geotropic curvature, or both?

A possible role for microtubules in geotropic reaction patterns has, up to the present time, not been considered. It is wellknown from animal cells that microtubules are implicated in a great variety of subcellular structures and functions. They are found in the sperm manchette, in elongated animal cells including neurons, and as the marginal

band in erythrocytes. They also compose the fibres of mitotic spindles, the tubular structures in centrioles and basal bodies, and the 9 + 2 array of axonemal filaments in cilia and flagella. Microtubules are assumed to function in a variety of ways; e.g. they participate in a motile system in cilia and flagella. They are also believed to perform a cytoskeletal role in the production and maintenance of cell asymmetries and they may be involved in the transport of cytoplasmic materials.

The occurrence of microtubules as components of plant cells was reported by Ledbetter & Porter (1963). Studies on the function of microtubules in plants have been concentrated on their involvement in the mitotic processes and their control of cell wall microfibrils. It is now generally accepted that they play a directive role in morphogenesis, either by controlling the movement and positioning of other cellular substructures, or by serving as a cytoskeleton. One aspect, which has not yet gained considerable attention, is their ability to be polymerized and depolymerized rapidly at different intracellular loci as conditions change during growth and differentiation. In this connection the paper presented by Atema (1973) is of special interest. It is there proposed that ciliary microtubules are actively involved in the reception and transduction of sensory information from the distal portion of the dendrite to the basal body area of the receptor cell in specific sensory systems. The receptor cells of these sensory systems are ciliary in nature and each cilium contains the 9 + 2 arrangement of microtubules characteristic for motile cilia and flagella. It is assumed that environmental stimuli may cause stimulus-specific conformational changes in the microtubule protein (tubulin). These changes may be propagated along the tubulin polymer filaments and once this coded informations has reached the cell body a classical transduction mechanism most likely takes over.

The fact that microtubules also exist in all types of root tip cells introduces the fascinating possibility that a similar mechanism to the above could account for the initial physiological phases (perception and transmission) in geotropic reactions. However, too little is known at the present time about the properties of the protein composition in plant microtubules to say whether or not these organelles are relevant to the problem of elucidating the graviperception mechanism in plants.

E. Summary

The anatomy of the root tip and the ultrastructure of root tip cells have been examined by light and electron microscopy. Special attention has been given to the anatomy of the root cap of cress which was found to contain 7 (or occasionally 8) storeys of starch-containing cells.

Examination of the ultrastructure of root tip cells from several plant species was performed after different fixation procedures. Organelles which might possibly be involved in one or more stages of the geotropical reaction chain, were examined in detail and their morphological interrelationships if any, are described. It is concluded that there do not exist associations between starch-containing amyloplasts and other cell organelles in the root cap. It was also observed that the connections between ER-membranes from one cell to the next through plasmodesmata, give the ER-complex a mechanical rigid and stationary form which makes displacements of this membrane system upon geostimulation unlikely. Details on the occurrence and morphology of the Golgi apparatus in different regions of the root tip have been presented and the function of this organelle in the differential growth in the geotropic curvature has been discussed. The possible function of microtubules in geotropism of plants has also been considered.

Chapter III

THE DEVELOPMENT OF GEOTROPIC CURVATURES IN ROOTS

A. Introduction

The typical orthogeotropic reaction in roots is the positive geotropic curvature which has been assumed to be produced by actively growing cells located in the subapical regions of the organ. The curvature appears as a response to a geotropic stimulation, e.g. after the root has been placed in a horizontal position for a certain period of time. The root responds with a geotropic curvature both to finite quantities of stimulus and to prolonged stimulation. In the first case this can be demonstrated by interrupting the gravitational stimulation before any curvature has become visible. The nullification of the unilateral stimulation is performed by returning the root to the normal position or by rotating the plant parallel to the horizontal axis of a klinostat. After a certain time (the reaction time) in the new position the curvature can be observed.

The development of the geotropic curvature of a *Vicia faba* L. root which was placed in the horizontal position in moist soil and exposed for prolonged stimulation, has been described by Sachs (1874). The apical part of the root was marked with India ink and the development of the different zones (2 mm intervals) was followed for several hours. The increase in distance between the marks was interpreted as a measure of the elongation rate in the different zones.

As demonstrated in the modified illustration of Sachs' original illustration (see Larsen 1962; Fig. 1) a slight curvature can be observed over the entire growing region after 2 hours. After 7 and 23 hours the strongest curvature is found in the zones 1-3 and 2-3, respectively. After 23 hours, the apical zones which have reached a nearly vertical direction, are straight.

The geotropic curvature of the *Vicia* roots was established by Sachs (1882) to be due to the lower half growing more slowly than the upper half. The decrease in the growth rate of the former has been confirmed by Audus & Brownbridge (1957). This differential growth has previously usually been explained by the Cholodny-Went theory, which for many years has been accepted as a basic principle in geotropism. The theory which will be discussed in detail in Chapter IX, postulates that auxin produced or liberated in the root tip moves basipetally into the elongation zone. By gravitational stimulation, the auxin accumulates

in the lower half of the root. Optimal or supraoptimal quantites of auxin in the elongation zone of the roots and the possibility of lateral auxin transport are prerequisites to the application of the theory to root geotropism. After stimulation, the deflection of auxin to the lower side of the root produces a retardation of growth there and a lessening of inhibition in the upper half. The present understanding of the mechanism of differential growth in the geotropic process is, however, very poor. Several investigators (Audus & Brownbridge 1957, Audus & Lahiri 1961, Wilkins 1971) have accepted that the release or synthesis of a growth-inhibiting substance may be involved.

In Sachs' (1874) classical illustration of the curvature of a root stimulated in the horizontal position, the extreme tip was given a symmetrical shape and Sachs accepted that the anatomy of the tip region was unaltered and reached its normal, vertical position by the curvature of the more basal parts. The shape of the tip in the original illustration was later modified by Rawitscher (1932) who assumed that the curvature very soon after the stimulation became markedly asymmetric and only later resumed its normal, domelike shape. The asymmetry in the root tip has also been noticed by several other investigators (e.g. Riss 1914) and Larsen (1962 p. 153) points out that the geotropic curvature initially begins at the extreme root tip.

In a few reports results have been presented where light appears to affect the geotropic development of roots. Lake & Slack (1961) who worked with several higher plants, suggested that exposure to light was a prerequisite for geotropism in some roots. *Vanilla* roots, which are diageotropic when grown in the dark, become positively geotropic after exposure to blue light (Irvine & Freyre 1961). In a recent article Tepfer & Bonnett (1972) have shown that the geotropic behaviour in radicles of root cultures of *Convolvolus arvensis* L. is influenced by light. Most of the experiments in the presented work have been performed in the dark but as handling of the cress seedlings for the geotropical experiments has been done under weak, green light it was felt necessary to examine if there were light and geotropic interactions also in the cress roots.

In the present chapter a detailed study of the geotropic development of cress roots in total darkness and under the influence of green "safelight", is presented.

B. Materials and Methods

Cultivation

Seedlings of garden cress (*Lepidium sativum* L.) were raised from seeds sterilized with a 3% hypochlorite solution for 15 min. The seedlings were germinated on moist filter paper in petri dishes kept in the vertical position in the dark. In some experiments the seedlings, 24 or 30 h after soaking, were transferred to a plant chamber, where they were kept for 24 h. Here the seedlings were positioned on a glass plate (115 x 78 x 0.8 mm) so that the roots grew between two 1 mm thick slices of 1.25% agar. The slices were protected by a plastic cover kept in place by two rubber bands. When the seedlings had attained a root length of 2.5 to 3.5 cm they were ready for the experiments.

Geotropic experiments

Handling of the seedlings for the geotropical experiments was done under weak, green light unless otherwise stated. The light was in the spectral range 500 to 550 nm (Fig. 14), produced by a 20 Watt Osram FL 20 Green fluorescent tube and a gelatin filter made according to Withrow & Price (1957). The spectral intensity of the light was measured with a Model SR Spectroradiometer (ISCO, U.S.A.) placed at a distance

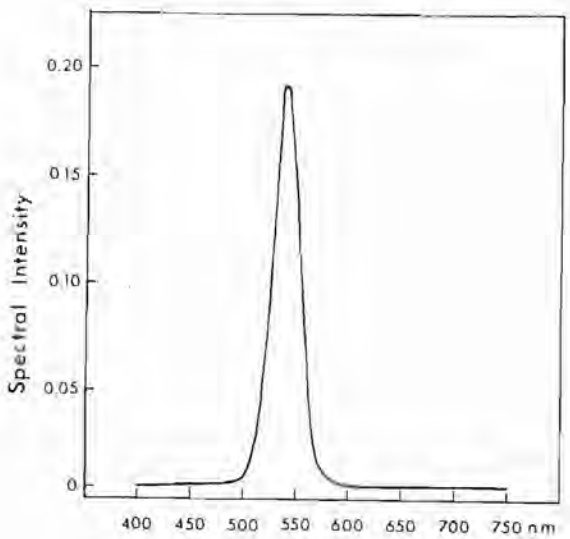


Fig. 14. *The spectral intensity of the green "safelight."* The spectral intensity is expressed as $\mu W \cdot cm^{-2} \cdot nm^{-1}$.

of 15 cm from the light cource.

In the continuous stimulation experiments the plant chamber with the seedlings was placed on a specially constructed klinostat. It consisted of a revolving frame (Fig. 15) equipped with a camera, an electronic flash assembly, and a holder arrangement for the plant chamber. The klinostat axis proper goes through the plane of the plant chamber which is in the central position of the frame. The camera ("Robot Royal", Berning und Co., Düsseldorf) and the electronic flash (Braun F 270) were attached to the frame on each side of the plant chamber on an axis perpendicular to the plane of the plant chamber. Distances from plant chamber to camera and to flash were adjustable. For routine work the film plane was 16 cm from the plants. The camera loaded with Kodak Microfile panchromatic film, was focused by extension rings. A green filter (Ilford Spectrum Green filter No. 604) transmitting in the range of 500-550 nm was placed in front of the flash window. After camera and flash distances were set, the klinostat was balanced by means of magnet weights.

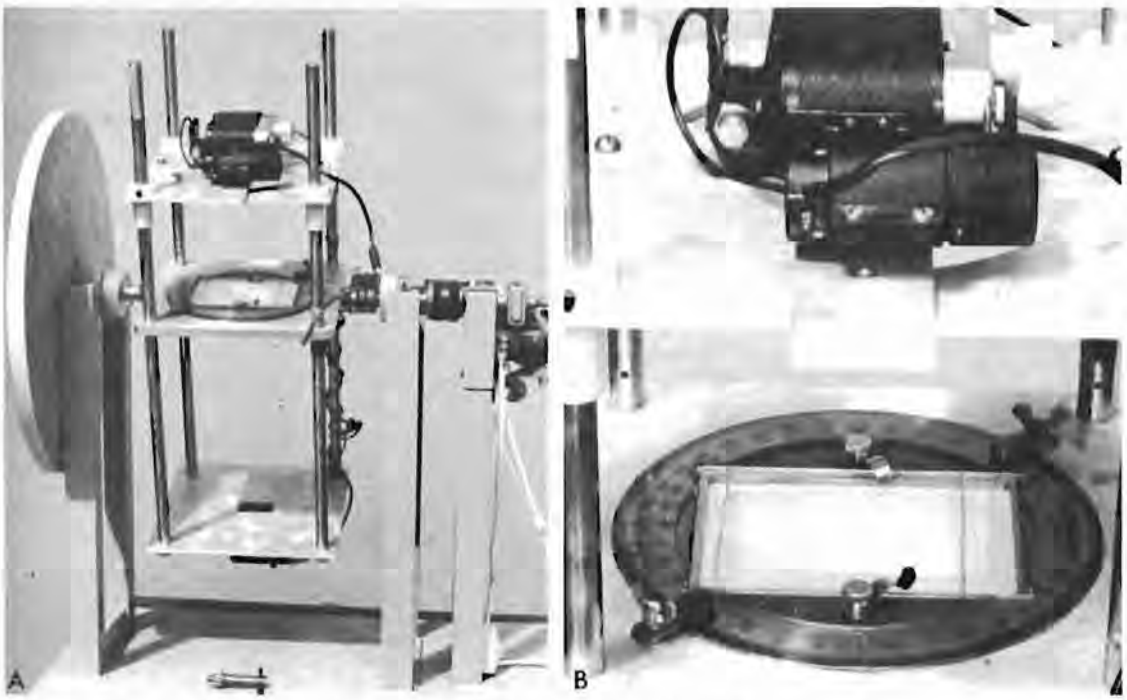


Fig. 15. *Klinostat for geotropical experiments.*

A: The main parts of the klinostat can be seen (from left): rotation regularity control disk, revolving frame with magnet weights, camera, plant chamber, electronic flash assembly, and the motor.

B: Close up view of the plant chamber on the revolution frame. The plant chamber is positioned in the gap between the two parts of the klinostat axis proper which are attached to the frame. Notethe scale of degrees.

The klinostat was driven by a Parvalux SD 10 capasitor-induction constant speed motor furnished with a double reduction gear box giving 2 rpm. To control the regularity of rotation a circular disk (diam. 50 cm) was attached to the main axis. The rim of the disk was divided into 1600 equidistant lines. When the rim was illuminated with a stroboscope a slight irregularity was observed. This was eliminated with a mechanical brake on the main axis.

The klinostat was connected to a timer and a Heat Servo Recorder. By means of the timer an exposure was made every 10 min. The number of exposures and time intervals were controlled by the recorder.

In order to permit the angle of stimulation to be varied, the plant chamber was made rotatable in its own plane. The angle of stimulation was read in degrees on a scale connected to the plant chamber (Fig. 15 B). If stimulated at angles deviating from the horizontal position, the plants were returned to the latter and kept in this position during rotation parallel to the klinostat axis.

As described by Larsen (1957, 1965, 1969) a closed cuvette, two-thirds filled with ethanol, was photographed together with the plants before rotation. Angles of curvatures were measured on images of the roots projected from the negatives onto a scale of degrees by means of a Letz Model SM microscope equipped with a straight monotube and drawing prism. The meniscus of the ethanol in the cuvette served as a base line for determining the horizontal and vertical directions on the negatives and for measuring the magnitude of the curvature of the roots.

The angle between the base line and the median line in a length of a section corresponding to about 500 µm on the living root (Fig. 16) represents the magnitude of curvature. A comparison has been made of two methods for measurement of the response (Fig. 17).

When the angles of curvature are measured on the photographic film they are found to be slightly but significantly smaller than when determined on photo-micrographs of sections through the median plane of the roots. The difference between the results of the two measurement methods might be explained by a mechanical alterations of the curvature in the latter case, due to osmotic reactions in the roots during the preservation procedure.

With the aid of the first technique very small curvatures could be detected and it was found that a certain number of roots exhibit small curvatures, even when kept in the normal vertical position. If these initial curvatures exceeded $\pm 5^{\circ}$, the respective roots were disregarded.

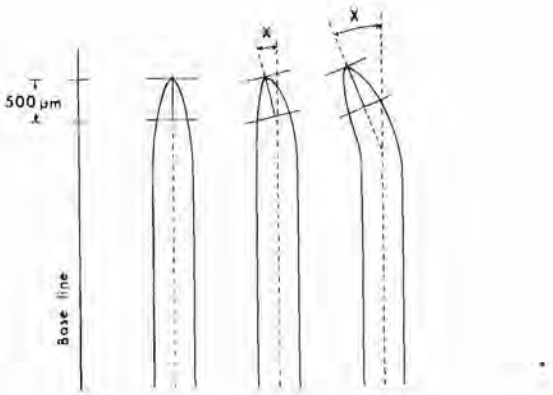


Fig. 16. *Measurement of angles of curvature.* The magnitude of curvature is determined on the basis of the angle which the median line in the apical 500 µm of the root tip makes with the base line (Larsen 1957, 1969).

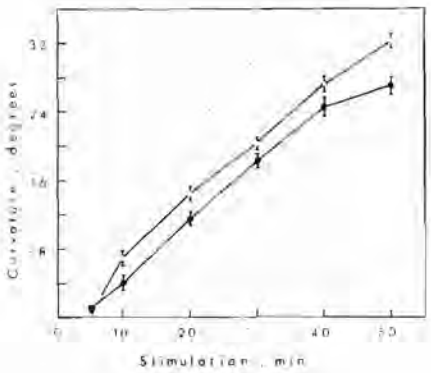


Fig. 17. *Geotropic curvatures of cress roots after continuous stimulation in the horizontal position.* The curvatures were measured both on enlarged images of the roots on film (●) and on light micrographs taken from the median plane of sectioned roots (o). The vertical bars indicate the mean errors.

Root elongation was determined by projecting the negatives onto a wall and measuring the length of the enlarged root images by means of a chartometer. The measurements of the roots were made from the root hypocotyl junction.

Light microscopy

Following the geotropic experiments, the roots were fixed in Nawaschin II solution for 12 h, carried through the embedding process to paraffin, sectioned for 9 µm thickness, and stained with PAS-reagents. The fixative has been shown to stop physiological processes in the root tips in less than 45 sec after they are placed in the solution. As a routine precaution, if the roots had been geotropically stimulated, they were kept oriented in the same position as in the experiment for the first 2 min in the fixative. By lightly squeezing the proximal part of the root with a pair of forceps, a mark was made which after the embedding would serve to identify the orientation of the root during stimulation.

Scanning electron microscopy

The scanning microscope has been used in the study of the curvature of the roots. The major problem in preserving the roots' surface topology adequately for the scanning electron microscope is the removal of water from the roots with a minimum of tissue damage. After the geotropic experiments, the roots were fixed for 2 h at 4°C in 2% OsO_4 in 0.1 M sodium phosphate buffer at pH 7.0. The roots were thoroughly washed free of osmium tetroxide, dehydrated through a graded ethanol series of increasing concentrations, and transferred from the 100% solution to xylene. Taken from xylene, the roots were infiltrated in paraffin and excess paraffin was removed with xylene. A thin film of gold-palladium alloy was applied to the roots under vacuum and the roots were examined in a Cambridge Scanning Microscope.

C. Results

1. Course of geotropic reactions in response to continuous stimulation

The course of the geotropic curvature developed in cress seedlings which have been growing with their roots accomodated between two agar plates and stimulated continuously in the horizontal position is shown in Fig. 18. After 3 hours the curvature is distributed over the

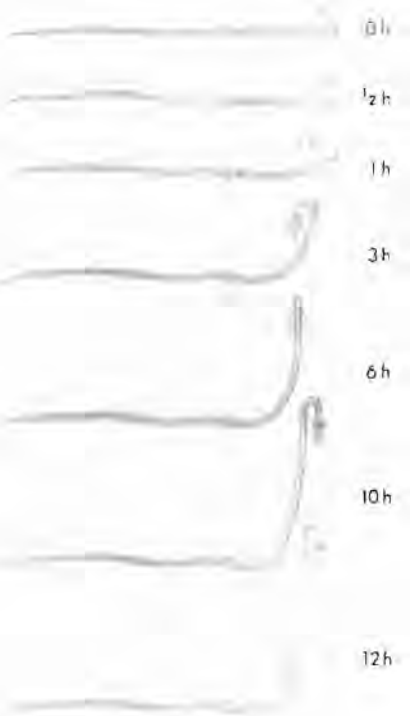


Fig. 18. *The successive stages in the geotropic curvature during horizontal stimulation.* The illustration is based on photographs taken at different time intervals after the beginning of stimulation. The cress roots have been kept continuously in the horizontal position between agar slices.

entire growing region. The development of the curvature is almost the same whether the roots are growing on moist filter paper in petri dishes or when growing between agar slices. Twelve hours are required after the start of the stimulation to reach the end position, in which the roots continue to grow at a constant angle (20° to 30°) to the vertical.

Table 1. *The average length of the epidermal root cap and cortical cells in the root tip of cress after horizontal stimulation.* The values are based on measurements of light micrographs of sections. The mean errors (e) are indicated.

Cell type	Zone no.	Distance from root tip, µm	Stimulation time and cell length, µm ± e					
			0 min			10 min		
			Upper	Lower	Diff.	Upper	Lower	Diff.
Epidermal ..	1	0– 300	55.0 ± 0.71	54.1 ± 0.68	0.9	55.5 ± 0.68	54.1 ± 0.65	1.4
Cortical	2	300– 600	7.9 ± 0.43	7.8 ± 0.41	0.1	8.1 ± 0.30	8.0 ± 0.32	0.1
.. ..	3	600– 900	8.2 ± 0.38	8.2 ± 0.39	0.0	8.7 ± 0.36	8.6 ± 0.33	0.1
.. ..	4	900–1200	11.1 ± 0.53	10.5 ± 0.50	0.6	11.1 ± 0.50	10.4 ± 0.51	0.7
.. ..	5	1200–1500	20.5 ± 0.47	20.1 ± 0.46	0.4	20.7 ± 0.43	20.1 ± 0.44	0.6
.. ..	6	1500–1800	36.0 ± 0.62	36.0 ± 0.59	0.0	36.0 ± 0.55	36.0 ± 0.56	0.0

Cell type	Zone no.	Distance from root tip, µm	Stimulation time and cell length, µm ± e					
			30 min			50 min		
			Upper	Lower	Diff.	Upper	Lower	Diff.
Epidermal ..	1	0– 300	65.5 ± 0.64	60.4 ± 0.62	5.1	60.5 ± 0.58	57.5 ± 0.59	3.0
Cortical	2	300– 600	9.9 ± 0.28	8.0 ± 0.30	1.9	8.3 ± 0.29	7.4 ± 0.31	0.9
.. ..	3	600– 900	9.2 ± 0.34	8.6 ± 0.36	0.6	7.9 ± 0.27	6.8 ± 0.23	1.1
.. ..	4	900–1200	13.7 ± 0.42	12.1 ± 0.40	1.6	12.4 ± 9.46	10.0 ± 0.47	2.4
.. ..	5	1200–1500	18.8 ± 0.41	18.7 ± 0.40	0.1	25.0 ± 0.48	20.0 ± 0.46	5.0
.. ..	6	1500–1800	36.1 ± 0.52	36.0 ± 0.51	0.1	46.1 ± 0.52	40.0 ± 0.55	6.1

The curvature which starts at the extreme root tip produces an asymmetry in the tip after a very short stimulation period. These stages during the first 50 min in the development of the geotropic curvature have been examined in detail and the results are presented in Fig. 19 and in Table 1. In the first 10 min after the start of the horizontal stimulation a small curvature appears in the root tips (Fig. 17). After 30 min of stimulation (Fig. 19) a downward asymmetry is clearly visible in the root cap region. In the same time, the root elongation after different pretreatments is on the average 400 µm (Fig. 20) and the curvature in the root tip region is 19.5° (Fig. 17). Even if the asymmetry is not apparent after 10 min stimulation on the light-micrograph of the root section presented in Fig. 19, it can, however, be observed after 10 to 20 min of horizontal stimulation (Table 1 and Fig. 22). The asymmetry which is a result of the difference in the elongation rate of the epidermal cells in the root cap and clearly

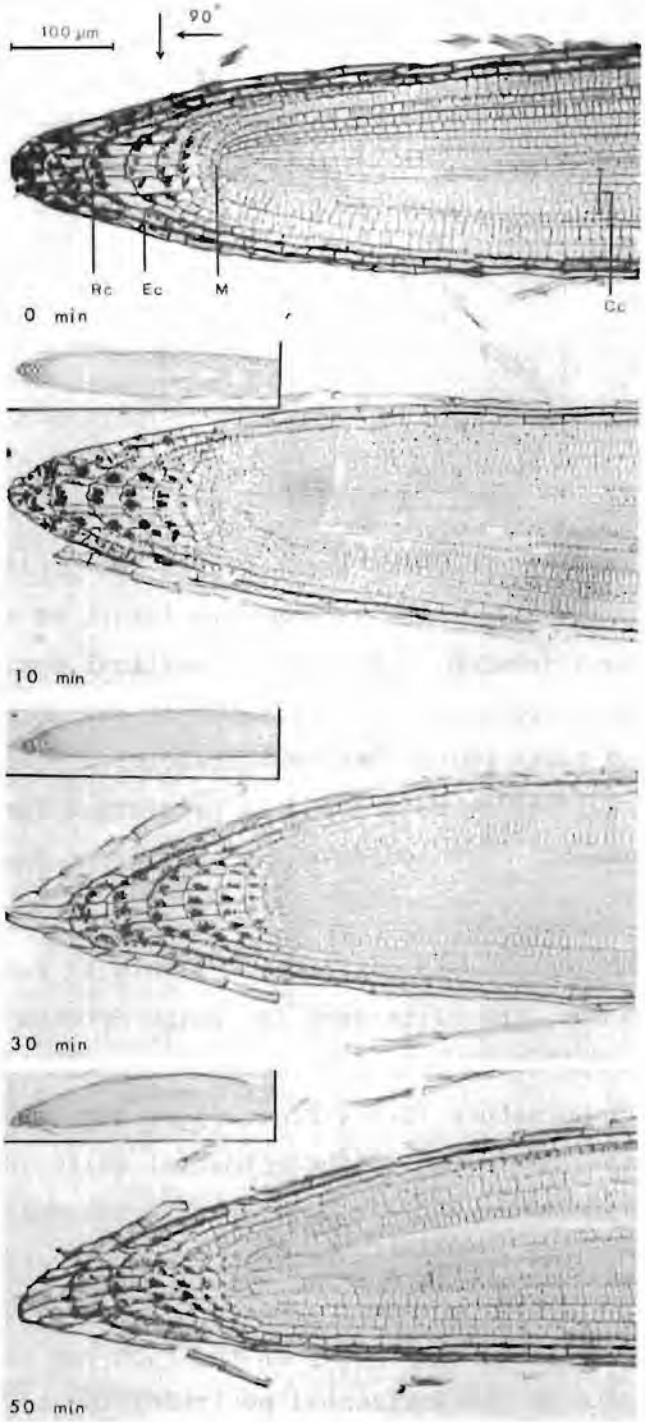


Fig. 19. *The development of the response in the initial stages of the geotropic curvature.* The asymmetric extreme cress root tip is shown after 30 and 50 min horizontal stimulation. The various cell types and regions are indicated: Rc: root cap, Ec: epidermal cells, M: meristematic region, Cc: cortical cells in the apical part of the elongation zone.

visible after 30 min of stimulation has decreased after 50 min (Fig. 19 and Table 1). The larger curvature obtained in the same time period is the result of the increased growth of the upper compared to the lower

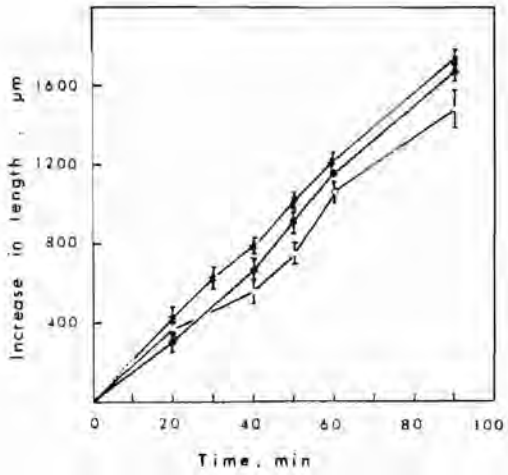


Fig. 20. *Straight growth of unstimulated and geotropically stimulated cress roots.* The increase in length of the median line of roots growing in the normal vertical position (●), unstimulated roots which are rotated in the horizontal position (o), and roots which have been stimulated horizontally for 10 min (...) before rotation (★), is plotted against time. The vertical bars indicate the mean error (means of 30 plants).

cortical cells in the elongation zone (Table 1, zones 4 to 6). During the first 10 min, the difference in length between the upper and lower cells in the epidermal root cap regions is predominantly responsible for the small curvature (6.9°; Fig. 17) as demonstrated in Table 1. The differences in length of the epidermal cells increase during the next 20 min. The decrease in asymmetry in the root cap is followed by an increase in the difference between the upper and lower cortical cells in the elongation zone.

Although the asymmetry in the root cap is not clearly visible after 10 min in the horizontal position, the movement of the amyloplasts in the statocyte cells has started. The amyloplasts move as close as possible to the lower longitudinal cell wall after 10 to 15 min (se Chapter IV) and the cluster of amyloplasts is spread along the lower cell wall after 30 min (Fig. 19). As the curvature of the root increases and the angle between the gravitational force and the root cells decreases, the location of the amyloplasts changes. After 50 min of horizontal stimulation the clusters of amyloplasts have slid along the lowermost cell walls towards the floor of the cell (Fig. 19). This movement is supposed to continue until the roots have attained their final position.

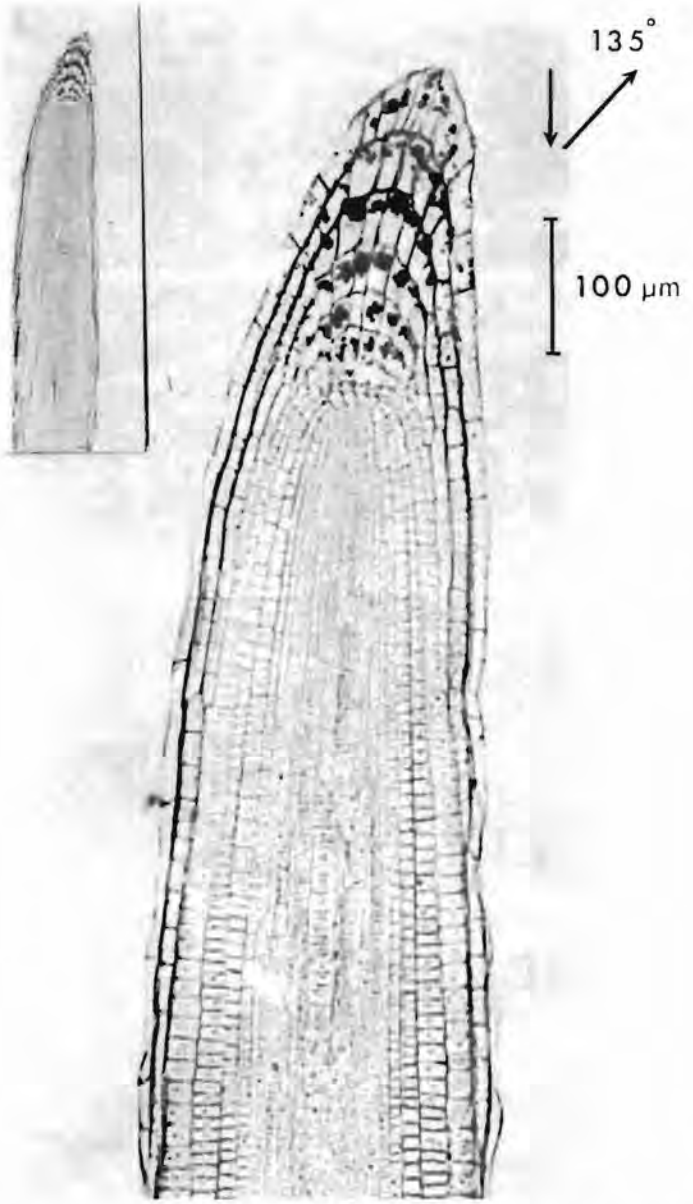


Fig. 21. *The asymmetry in the extreme part of a geotropically stimulated root tip.* The cress root has been taken from the normal position and stimulated at 135° for 10 min.

When the roots are inclined at an angle of 135° from their normal position, i.e. the root tips are pointing obliquely upwards, the asymmetry in the root cap can be clearly demonstrated after 10 min (Fig. 21). The resulting geotropic curvature which increases with time will eventually be larger than after stimulation for the same period of time in the horizontal position (Fig. 38). When stimulated at 135° the falling amyloplasts move a maximum of 7 µm during the 10 min stimulation period (Storey III; Fig. 32).

Fig. 22. *Scanning electron microscopy of the geotropical response.* The cress roots have been stimulated continuously in the horizontal position for B: 10 min, C: 30 min, D: 50 min, A: unstimulated root. The asymmetric region in the extreme tip is indicated by the arrow (B).

The development of the geotropic curvature during continuous stimulation has also been demonstrated by scanning electron microscopy. After 10 min of stimulation, a slight asymmetry can be observed in the extreme root tip (Fig. 22 B). The course of the geotropical curvature which appears concurrently with the elongation of the root is demonstrated in the micrographs taken 30 min after the start of the stimulation and thereafter (Fig. 22 C and D).

2. The influence of gravity on root elongation

Measurements of the elongation of opposite sides of the apical part of geotropically reacting root tips during continuous stimulation have been made. As shown in Table 2, the difference between the convex (upper) and concave (lower) sides increases with time. The measurements are based on enlarged light micrographs of the root cap, meristematic region, and apical part of the elongation zone. In the first 50 min after the beginning of the horizontal stimulation, the elongation rate of the apical upper side is, on the average, 1.5 µm per min (Table 2), while the elongation of the lower side seems to be inhibited. This difference in elongation between the two sides is responsible for the asymmetry in the root tip.

Table 2. *Length (µm) of the convex and concave sides of the apical part (0-900 µm) of geotropically curved cress root tips.*

The values are means of measurements of 3 to 5 roots and based on light micrographs of sections taken from and near the median plane of the roots. The roots have been stimulated continuously in the horizontal position

Stimulation time, min	Convex side	Concave side	Difference convex/concave side
0	900	900	0
5	903	903	0
10	917	904	13
20	930	900	30
30	951	903	48
40	960	894	66
50	976	896	80

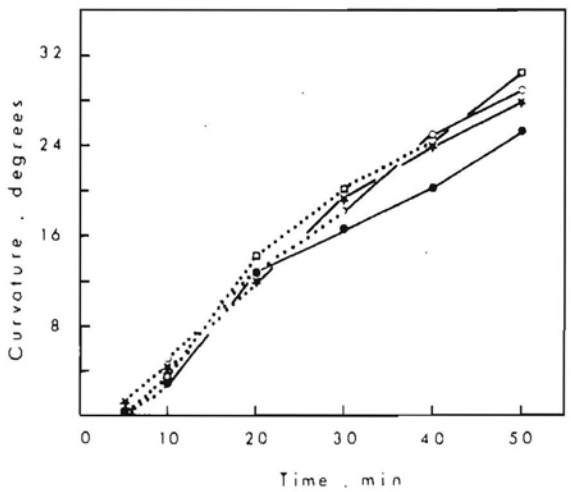
The average total elongation of the entire root in the same 50 min period is 820 µm (Fig. 20), which gives an elongation rate of 16,2 µm per min and a curvature of 32.7° after 50 min.

The influence of gravity on the over-all elongation of the intact roots growing in various positions has also been investigated. Unstimulated roots rotated at 2 rpm parallel to the horizontal axis of the klinostat (Fig. 20) elongate slightly slower than the control roots growing in the normal vertical position. If the roots are stimulated in the horizontal position for 10 min and then rotated, they elongate slightly faster than the two other groups (Fig. 20).

3. The influence of various quantities of stimulus on the geotropic response

The reaction time has been found to be about 5 min for cress (Larsen 1965). In the present study it was found that when the simulation time exceeded 5 min the ultimate curvature at the end of a 5 min period seems to be independent of the stimulation time. In Fig. 23 are shown the results from experiments in which cress roots have been stimulated in the horizontal position for 10, 20, 30, and 40 min before rotation on the klinostat. The total curvatures recorded after 50 min are not influenced by differences in the stimulation period when this is extended beyond 10 min.

Fig. 23. *Geotropic curvatures of cress roots stimulated for various lengths of time prior to rotation.* Before klinostat rotation at 2 rpm, the roots were kept in the horizontal position for 10 min (●), 20 min (★), 30 min (○), and 40 min (□) as indicated by the dotted parts of the respective curves.



4. The effect of green light on the geotropic curvature

The few reports of interaction between light and gravity in the geotropic development of roots made it necessary to find out if the green "safelight" used in the experiments in this study influenced the

geotropic curvature of normal, untreated cress roots. Handling of the seedlings for routine geotropic experiments was done under weak diffuse green light in the spectral range 500-550 nm (Fig. 14). As shown in the figure the intensity of the light was very weak with a peak at 540 nm. For routine work the plants were handled at an average distance of 15 cm from the green light source. The spectral intensity at this place was at the maximum 0.186 μWatt$\cdot$cm$^{-2}\cdot$ nm^{-1}. An electronic flash, with a green filter transmitting in the same range, was used to record the curvatures.

In order to test the influence of this light on the roots, seedlings were raised from sterilized seeds growing between agar plates in total darkness (dark-grown roots). Roots were also obtained from seedlings handled during the growing period, in the diffuse green light (light grown roots). Geotropic stimulation of the latter group was performed in a weak, green-illuminated darkroom.

In the experiments using dark-grown roots, precautions were taken to avoid even the slightest light exposure to the soaked seeds after the sterilization period. Exposure to the gleam of light (duration: 1/1000 sec) from the electronic flash, was avoided. Therefore, several dark-grown control groups had to be photographed at zero time, in the horizontal position to give the average initial curvature for the test groups which could not be photographed. It was not possible to follow the development of the curvature of the test roots at specific time intervals for the same reason. First and final exposure of the test roots was taken either 30 or 60 min after the start of the geotropic experiments.

When the stimulation period is only 30 min, there is no difference between the development of geotropic curvatures in cress roots from sterilized seeds germinated between agar slices from the beginning and roots from seeds germinated first on filter paper and then transferred to agar (Table 3). When the stimulation time is extended to 60 min, however, the seedlings which have been cultivated from seeds in petri dishes and later transferred to agar seem to develop a slightly larger curvature (28,4°) than the roots germinated in agar (26,8°). The slight difference may, however, be explained by the difference in root length (cf. Table 4).

The influence of the root length on the geotropic curvature has also been examined. For the dark-grown roots the curvature is clearly dependent on the root length of the seedlings (Table 4 A). When,

Table 3. *The effect of growing conditions on the development of geotropic curvature of cress roots.* The roots have been pretreated in the following manner:

A. The sterilized seeds were germinated in petri dishes for 24 hours and then transferred to agar plates for an additional 24 hours.

B. The sterilized seeds were placed directly on agar slices and the seedlings kept there for 48 hours.

The handling of both groups of seedlings was performed under green safelight. The mean error (e) and the stimulation period (S) are indicated.

Pre-treatment	Root-length (mm)	Number of roots (n)	Curvature, degrees ± e	
			30 Min (S)	60 Min (S)
A	31.0	29	14.3 ± 0.6	28.4 ± 0.7
B	26.0	33	13.9 ± 0.9	26.8 ± 0.5

however, the roots have attained a certain length (> 25 mm) the curvature seems to be independent of the root length whether the stimulation period is 30 or 60 min (Table 4 B).

Table 4. *The correlation between cress root length and geotropic curvature.*

A: The roots have developed from seeds between agar plates in total darkness (dark-grown roots).

B: The roots have been handled in weak, green light when transferred from petri dishes to agar plates (light-grown roots).
The mean errors (e) are indicated.

Stimulation Period (min)	Root length, mm ± e	Curvature, degrees ± e	Total numbers of experiments	plants
60	18.2 ± 0.4	16.5 ± 0.9	5	39
60	32.5 ± 0.6	25.7 ± 0.6	6	56
60	37.7 + 0.8	26.1 + 0.9	4	28
B				
30	26.1 ± 0.8	14.4 ± 0.9	3	16
30	32.0 ± 0.4	12.8 ± 0.6	8	72
60	26.1 ± 0.8	29.1 ± 0.7	3	16
60	33.0 ± 0.5	28.4 ± 0.6	3	29

As can be seen from Table 4 the green safelight in the dark-room influences only to a slight degree the development of geotropic curvature. The roots which have been stimulated for 60 min in total darkness curve on the average 25.9° (Table 4 A) which is slightly less than the corresponding group of light-grown roots at the same root length (28.7°; Table 4 B). The increase in curvature after the light-treatment has also been demonstrated in another set of experiments where both groups of seedlings have been cultivated from seeds on agar plates (Table 5). Stimulation, for 60 min, of the roots kept in total darkness and the light-influenced roots give the average curvatures 25.7° and 27.8°, respectively. Although the average root lengths are different in the two groups, it is not expected that an increase in root length of the light-grown roots will influence the final curvature to any great extent (cf. Table 3).

Table 5. *Geotropic curvatures in dark- and light-grown roots of cress.* Both groups of seedlings have been cultivated from sterilized seeds on agar plates (e = mean error)

Pre-treatment	Stimulation period (min)	Root length, mm ± e	Curvature, degrees ± e	Total numbers of experiments	plants
Dark-grown	60	32.5 ± 0.6	25.7 ± 0.6	6	56
Light-grown	60	26.0 ± 0.6	27.8 ± 0.6	5	45

D. Discussion

The experiments indicate that the initial stages in the development of the geotropic curvature of cress roots appear to develop in two phases:

a. an initial curvature in the root cap and meristematic region which results in an asymmetry in the extreme root tip, and b. the geotropic curvature in the basal parts of the root tip.

In the first phase the asymmetry is caused by a difference in the rate of elongation of the epidermal cells on the upper and lower side of the root cap region. After the initial stage the actual curvature progresses towards the elongation zone, and this second phase continues until the root tip reaches its final direction, 20-30° from

the vertical. Whether this curvature is a result of differential cell elongation or of changes in the rate of embryonic growth or both, is still a matter of dispute. Wagner (1936) found a temporary increase in the number of mitoses in geotropically stimulated root tips of *Allium*, perhaps with a predominance in the upper half, but contrary to these results Younis (1954) did not find any difference in the number of cells in the upper and lower halves of stimulated root tips of *Sinapis* and *Vicia*.

According to Jensen (1955) four major stages of cellular development occur in the apical three millimeters of *Vicia faba* L. root tips. They are the stages of 1) cell division 2) radial enlargement 3) beginning elongation and 4) active elongation. The mature root cap cells are in this connection excluded. In the last period of the first 50 min of horizontal stimulation of cress roots (Fig. 19), the curvature is assumed to be caused by beginning elongation of cells in the upper half of the procortical and cortical region approximately 1200 to 1800 µm from the tip. In the present study, this differential elongation has been expressed as the length of the cortical cells on the upper and lower sides (Table 1) but it should be remembered that the increase in length is only a simplified expression of the complicated metabolic processes which occur in the procortical cells. The basipetal extension of the curving region after 2 to 3 hours of stimulation is the result of differential growth of cells in the elongation zone, which is localized 2 mm from the tip and backwards.

The rate of curvature of cress roots does not seem to be dependent on the stimulation time when the roots have been stimulated for 10 min or more prior to rotation at 2 rpm on the klinostat (Fig. 23). These results are in agreement with the results presented by Larsen (1957) working with *Artemisia* roots. This independence of the rate of curvature from stimulation time seems to be peculiar to roots. In contrast the rate of curvature of hypocotyls of *Helianthus* has been shown to be dependent on the duration of the stimulation (Andersen & Johnsson 1972).

As shown in Fig. 17, cress roots growing between agar platelets do not reach the vertical position within a 12-hour stimulation period. The curvature pattern in various growth media has been extensively studied by Holman (1916) on leguminous plants. He observed that in compressed sawdust the root tip of *Vicia faba* L. reached the vertical position after 9 to 18 hours. Although the initial rate of curvature is much higher in roots growing in air a counter reaction prevents

the attainment of a vertical position, and the roots continue to grow at an oblique angle to the gravitational force. This was confirmed by Bennet-Clark et al. (1959) who found that roots of *Vicia faba* L. grown in soil or air attain a maximum curvature of 85° and 41°, respectively, after stimulation for 12 hours. In a recent work, Zinke (1968) confirmed Holman's results with *Vicia* roots. He also observed that roots of *Pisum arvense* L. and *Zea mays* L. curved 85° and 41°, respectively, in air when the stimulation time was 24 hours. The pattern of curvature was unaffected for both plants when growing in sand. Zinke (1968) concluded that the course of the geotropic curvature is the result of a combined action of positive and negative reactions, the intensity and duration of which differ in roots of various species.

A comparison between cress roots growing in agar and on moistened filter paper has shown that the curvature patterns are similar and independent of the external conditions. In both cases the roots attain the final angle which is 20° to 30° with the vertical after 12 hours (Fig. 18), and they continue to grow at this angle in a plagiotropic manner.

This failure to obtain the vertical position within a reasonable period of time may be correlated with the movements of amyloplasts. As the curvature increases and the angle between the vertical line and the root tip decreases, the amyloplasts are assumed to move slowly from the position near the lower longitudinal wall (Chapter IV) and towards the original normal position at the "floor" (Larsen 1965) of the root cap cell.

The appearance of the asymmetry in the root tip of *Lupinus albus* L. was used by Riss (1914) as a measure for a proper geotropic response. After 2.5 min in the horizontal position, the roots were returned to the normal vertical position and kept there for 80 min. During this period a slight asymmetry ("leichte Asymmetrie") had appeared in the extreme root tip. The asymmetry was identified as a slight concave lower side. In experiments with *Vicia faba* L. and *Lupinus albus* L., Overbeck (1926) studied the initial stages in the geotropic reactions in roots. He observed that the early stages in the curvature appeared as a result of a different turgor pressure on the opposite sides of a stimulated root. Although these curvatures could be straightened by plasmolysis and therefore should be regarded as turgor movements, they are nevertheless geotropic responses (Larsen 1962).

The results of the present experiments may indicate that the

initial stages in the geotropic curvature are turgor movements. The observation that the time-limited elongation of the upper cells of some zones of the root tip, which appears between 10 and 30 min, is reversed after 50 min (Table 1) supports this interpretation. A correlation to turgor movements is also indicated by the location of the increased elongation to the epidermal cells, which are the most highly vacuolated cells and therefore the most sensitive to changes in the osmotic conditions in the root tip. The possible connection between such movements and the statolith function is difficult to see.

The question is now whether the asymmetry which appears in the initial stages of the geotropic curvature constitutes one phase in the development of the curvature and the subsequent curvature in the elongation zone another. If these are two separate processes they might be initiated by two different starting signals. Alternatively, the geotropic curvature might be initiated as an asymmetry caused by differential elongation of the epidermal cells of the root cap, followed by a flow of information from the root cap to the elongation zone. Irrespective of whether the curvature develops in one or two phases it is important to direct attention to the fact that the initial geotropic reaction as a response to the amyloplast movement starts in the root cap. This must be taken into account when discussing the subsequent stages in the geotropic response.

If we accept the Cholodny-Went theory and the existence of two phases, it can only be applied to the curvature which appears in the elongation zone, i.e. the second phase. At the present time the geotropic reaction is generally accepted to be an auxin-regulated growth process. Auxin is assumed to promote the synthesis of cell wall material and to participate in the active extension of the cell wall by intussusception. The asymmetric distribution of amyloplasts in each root cap cell may lead to a biochemical polarization of the cell. In an unexplained manner the entire columella region becomes transversally polarized, and the signal which is emitted basipetally results in the asymmetric distribution of auxin in the elongation zone. The same explanation can not be applied directly to the first phase. It is highly probable that the movements of the amyloplasts are also responsible for the reaction pattern in the first phase. A transversal polarity must be initiated in the root cap region, but since the geotropic response occurs in the epidermal zone in this region, this phase is independent of a longitudinal transport and therefore it is unlikely that auxin participates in the first phase.

In their detailed study on the interaction of gravity and light on the geotropic behaviour of roots of *Convolvulus arvensis* L., Tepfer & Bonnett (1972) observed that in darkness the roots grew horizontally, while exposure to light induced a positive orthogeotropic response. Red light elicited the response and it was reversed by a far-red-light treatment following the red exposure. They suggested that phytochrome mediated the light response and that the pigment was located in the root cap or the meristem.

Other studies of the light and gravity interaction controlling the geotropic development in roots have been made by several investigators (Lake & Slack 1961, Irvine & Freyre 1961, Scott & Wilkins 1969). In all these reports it is demonstrated that irradiation with blue and red light, or both, affects the geotropic response.

In the present chapter it has been demonstrated that cress roots develop a normal curvature in total darkness. Although a detailed study with interference filters and selective irradiation of the root apex was not performed, it has been shown that the diffuse weak, green "safelight" in the darkroom only slightly increases the final curvature after stimulation of the roots in the horizontal position for 30 and 60 min. This result indicates that although red light is highly unsuitable for use as a "safelight" in a physiological darkroom, the green light in the region between 500 and 550 nm appears to have the least physiological effect on the geotropical experiments.

E. Summary

Roots of cress growing between two agar slices develop an asymmetry in the extreme root tip region after 10 to 20 min of horizontal stimulation. After prolonged stimulation (exceeding 50 min) the asymmetry disappears and after 3 hours the curvature is distributed over the entire growing region. The course of the initial stages in the geotropic curvature has been followed by light microscopy and scanning electron microscopy. - When stimulated at an angle of 135° with the gravitational force, the asymmetry in the root tip is clearly visible after 10 min of stimulation.

The asymmetry in the root cap can be explained by a difference in the elongation rate of the epidermal cells on the upper and lower sides of the stimulated root. The disappearance of the asymmetry is followed by a second phase in which there is a differential growth of

the cortical cells on the two sides of the elongation zone. The average elongation rate of cells in the upper half of the apical region during the first 50 min of continuous stimulation is 1.5 µm per min, while the elongation rate of the entire root is 16.2 µm per min. Only small modifications in the elongation rates were observed when stimulated and unstimulated roots were rotated parallel to the horizontal axis of a klinostat at 2 rpm.

The ultimate curvature developed after 50 min is unaffected by stimulation times exceeding the reaction time which for cress roots has been found to be about 5 min.

The geotropic curvature of cress roots is only slightly influenced by weak, green light in the spectral range 500 to 550 nm. In total darkness the roots curve approximately at the same rate as in green "safelight" in the darkroom.

Chapter IV

MOVEMENTS OF AMYLOPLASTS AND OTHER CELL ORGANELLES IN ROOT CAP CELLS OF GEOTROPICALLY SENSITIVE ROOTS

A. Introduction

Numerous reports indicate a close correlation between the occurrence of movable amyloplasts in various plant organs on the one hand and the presence of geotropic sensitivity on the other. Measurements of the actual time it takes for the amyloplasts to move a certain distance in a cell are much less numerous. Various authors claim to have found good agreement between presentation times (minimum stimulation times) and observable amyloplast movements. Information on the speed of amyloplast movements is of importance in the sort of experiment where it is desired to direct the amyloplasts towards specified regions of the cell before or after the geotropic stimulation. Descriptions of the methods employed for determining the speed of the movements are, however, often lacking or insufficient in the previously presented papers.

Detailed observations on amyloplast movements in the transversal direction in statocytes of manipulated plants have been made by Hawker (1933) who studied the endodermis cells in epicotyls of *Lathyrus odoratus* L. Larsen (1965) attempted to determine the time required for the amyloplasts to move in the longitudinal direction from the "floor" end of the root cap cells to the "ceiling" end in inverted roots of *Lepidium sativum* L.

As described in Chapter II the root cap of *Lepidium* has 7 (or occasionally 8) storeys of stach-containing cells, but only in 3 of these do the amyloplast move appreciably within the few minutes usually allowed for stimulation. The so-called columella (cf. Figs. 2 and 3) is generally characterized by its content of movable amyloplasts. In each storey these are found only in a limited number of cells, usually 6 in a median longitudinal section, distributed symmetrically about the root cap axis. In the preliminary microscopic study of Larsen (1965) on fixed root tip sections the starch grains appeared to be packed on the ceiling of the cell after 6 min of inversion. On basis of these results, inversion for 8 min was considered sufficient to secure a complete transfer of the amyloplasts to the opposite end of the most reactive cells.

For various reasons it was found important to study the time relationships of amyloplast movements more closely. In the following sections results based on light microscopical examinations of the wan-

derings of the amyloplasts will be presented.

More detailed information, obtained using electron microscopy, on their exact location, particularly in relation to other organelles and the cell wall, together with a preliminary report on the movement of the mitochondria, will also be included in the present chapter.

B. Materials and Methods

Cultivation

Seeds of the garden cress (*Lepidium sativum* L.) were sterilized with a 3% calcium hypochlorite solution for 15 min, rinsed with sterile water, and germinated on moist filter paper in petri dishes (diam. 9 cm) kept vertical in the dark at 21°C. When the seedlings, about 66 h after soaking, had attained a root length of 2.5 to 4.0 cm, they were ready for experiments. By rotating the petri dishes, the seedlings were turned to the desired position in relation to gravity. Some seedlings were kept in the normal position and served as controls.

Light microscopy

At the specified time after stimulation in the desired position, representative plants were lifted out and their roots fixed in Nawaschin II solution. After 12 h in the fixative, the roots were carried through the embedding process to paraffin, sectioned to 9 µm thickness and stained with periodic acid and Schiff's reagent (PAS).

Amyloplast movements as a response to gravity cease in less than 45 sec after placing the root in the fixative. This was established by taking some roots from the normal position and immediately fixing them in the inverted position. The location of the amyloplasts in sections of such roots was the same as in control sections (Fig. 24). As a routine precaution, after stimulation at a given angle a root was kept orientated as in the experiment during the first 2 min in the fixative. As described in Chapter III, by lightly squeezing the proximal part of the root with forceps, a mark was made which after the embedding would serve to identify the orientation of the root during the stimulation.

For confirmation of the results from 9 µm thick paraffin embedded sections, semi-thin plastic embedded sections (0.5 to 3.0 µm) were examined under the light microscope. The plastic embedding medium

was partly removed by weak heating and the sections were stained with the PAS-reagents before examination.

Electron microscopy

After stimulation, apical root segments, about 0.5 cm long were removed from the roots without change of orientation and fixed according to one of the following procedures:

1) Two hours at 4°C in 2% osmium tetroxide in veronal-acetate buffer (Caulfield 1957) containing 0.014% calcium chloride.

2) Two hours at 20°C in 3% glutaraldehyde in 0.05 M sodium phosphate buffer at pH 6.8. The segments were rinsed for 30 min in four changes of the same phosphate buffer and postfixed for 2 h at 4°C in 0.05 M phosphate buffered 2% osmium tetroxide, pH 6.8.

Dehydration was carried out in ethanol of increasing concentrations at 4°C followed by propylene oxide and embedding in Epon. Semithin and ultra-thin sections were cut from near the median plane of the root tip on an LKB Ultrotome I and a Reichert ultramicrotome. The ultrathin sections were mounted on copper grids and stained for 20 min with 1% aqueous uranyl acetate followed by lead citrate for 15 min. Sections were observed in a Siemens Elmiskop I, AEI EM 6B and JEOL 100 B electron microscope.

C. Results

Fig. 3 (Chapter II) may serve as a key for identifying the various storeys of the stach-containing cells. The storeys, marked I to VII, consist of developing (I), functional (II-V) or degenerate (VI, VII) statocytes.

Small amyloplasts are present in the youngest storey of the root cap cells (I), which otherwise appear distinctly embryonic. Occasionally two such storeys of embryonic cells are present. The older of these is regarded as No I in the numbering system. Larger amyloplasts are present in Storeys II through VI, and usually also in No VII, but only those in Storeys II, III, IV and (to a much smaller extent) V will sediment with gravity. The average dimensions (length and width in µm) of the central statocytes in each storey are also given in Fig. 3.

The location of the amyloplasts of the central cells in

Storeys II, III, IV, and V was estimated by inspection of photomicrographs of median sections made from each root. On basis of the micrographs, a scoring procedure was applied to usually 4 cells near the median line of the root cap. The scorers estimated the average distance from the respective cell walls to the position of the center of the cluster of amyloplasts expressed as percentages of the total length or width of the cell. In Storeys I and VII the score would usually be near 50% regardless of manipulation of the root. In Storey I, however, the amyloplasts were generally scattered over most of the cell volume, whereas in Storey VII, they sometimes tended to assemble around the center of the cell. Storeys I and VII will not be considered in the following treatment of the results.

Each micrograph was usually scored by at least two persons and a mean was calculated for each storey. The variability was determined

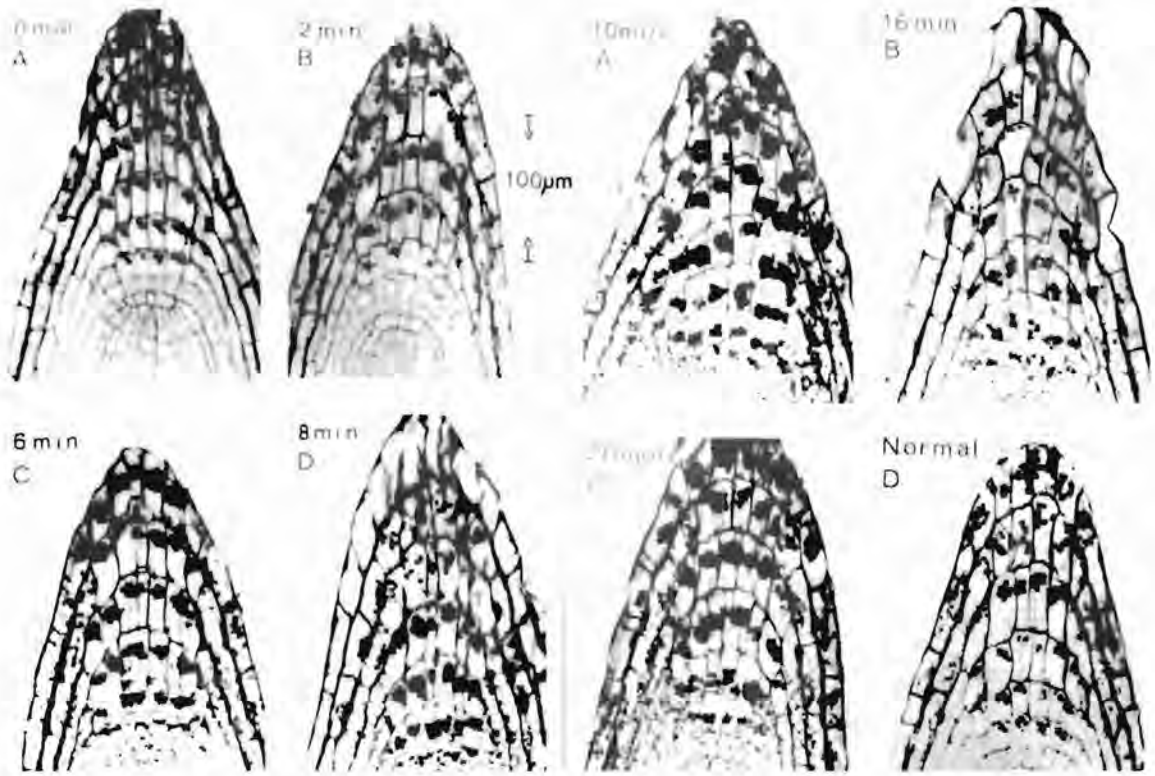


Fig. 24. *Position of amyloplasts after inversion of the cress root for 0 to 20 min as indicated.*

Upper left: Control, root not inverted, but the micrograph was rotated 180° for comparison. Lower right: Inverted for zero min, i.e. taken from the normal position and immediately fixed in the inverted position. On two of the sections, the starch-free, distal storey of cells (to be counted as No VIII) is retained (8 min) or has only been partly sloughed off (16 min). Total length of scale 100 µm.

on basis of the number of cells scored per storey. The mean percentages were converted to absolute units (µm) on basis of the average cell dimensions given in Fig. 3.

1. Movement of amyloplasts after inversion of the roots

For studying the movement of amyloplasts in the longitudinal direction in the statocytes the roots were kept in the inverted position for various periods of time (Fig. 24). In the scoring procedure the scorers estimated the distance from the floor (distal cell wall) to the center of the group of amyloplasts expressed as percentages of the total cell length. These data which have also been converted to absolute units are plotted against inversion time as shown in Fig. 25. The left-hand ordinate indicates the distance in µm of the amyloplasts from the floor of the cell (top); and the right-hand ordinate indicates the same distance in percentages of total cell length. The height of each frame indicates the average cell length near the median of the root cap.

In Storeys V and VI, the position of the amyloplasts did not show any clear dependency on the time of inversion. In Storeys II, III, and IV, on the other hand, the amyloplasts move quite regularly for 6 to 12 min, but thereafter they virtually come to a stop. As a result it takes 10 to 12 min before any of the amyloplasts comes approximately as close to the ceiling as they were to the floor before the inversion; and this is true only of Storeys II and III.

Considering the crudeness of the original percentage score the amyloplasts in Storey II, III, and IV (Fig. 25) can be said to move at approximately the same speed. This is about 6 µm within the first 5 min of relatively unimpeded movement and it gives 72 µm per hour at 21°C.

2. Movement of amyloplasts in statocytes of horizontal roots

The amyloplasts in roots which are placed in the horizontal position get close to the lower (longitudinal) cell wall in less than 15 min (Fig. 26). This applies as for the inverted roots, predominantly to Storeys II, III, and IV.

For studying in detail the movement of amyloplasts across the statocytes in horizontally placed roots, seedlings were kept inverted for 8 min in order to remove the amyloplasts from the mass of endoplasmic

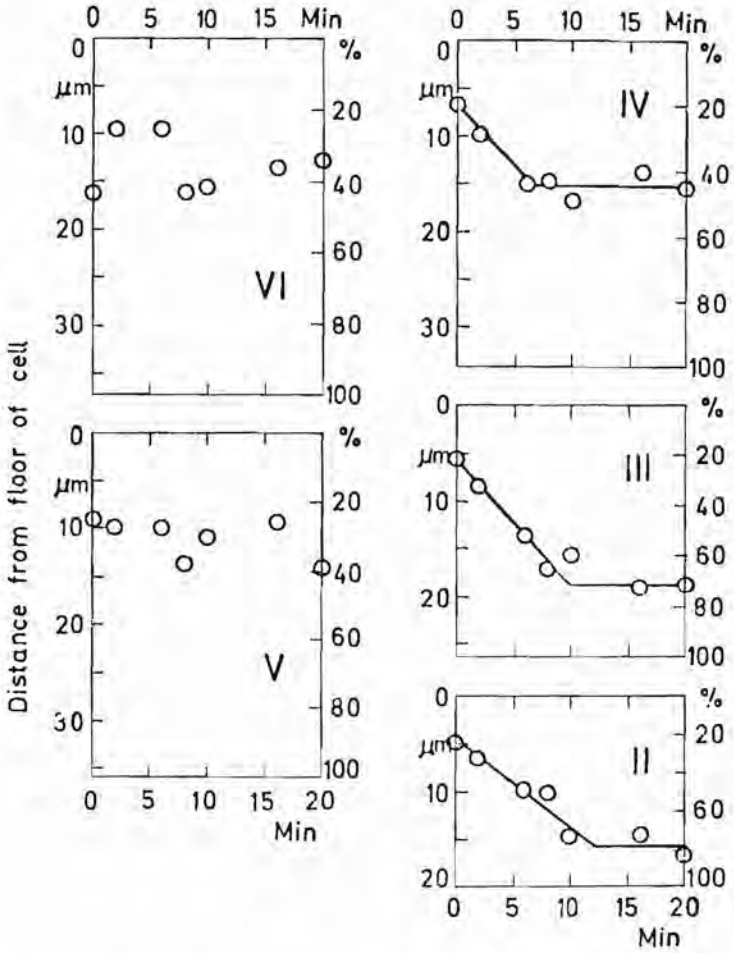


Fig. 25. *Positions of amyloplasts after various times of inversion of the cress root.*

Ordinates: Distance from floor of cell in µm (left) and in percentages of total cell length (right). Roman numerals indicate number of storey of starch-containing cells (cf. Fig. 3). The height of each frame indicates average cell length near the median of the root cap.

Fig. 26. *Longitudinal section of tip of cress kept horizontal for 15 min.*

reticulum (ER) on which they are resting when in the normal position. This ER can be seen on the various electron micrographs, for instance Fig. 27.

Next the roots were stimulated for 8 min in the horizontal position to get the amyloplasts as close as possible to one of the longitudinal cell walls. At the end of this stimulation, the seedlings were rotated 180° about their long axes, the formerly lower horizontal walls now being uppermost. The roots were kept in this position for various periods of time (up till 20 min) and then fixed in Nawaschin II solution, embedded and sectioned. Photomicrographs of the stained sections were used for estimating the location of the amyloplasts (Fig. 28).

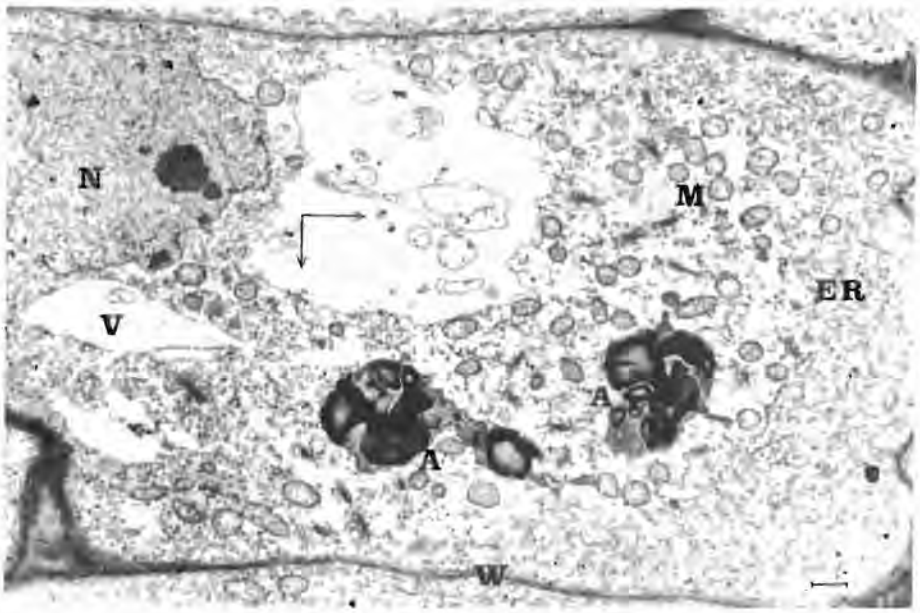


Fig. 27. *Electron micrograph of a statocyte in the columella* (Storey III) *of the root tip of cress.*

The root was turned from the normal to the horizontal position and stimulated for 20 min. Arrows indicate the direction to apex (right) and direction of gravity. Nucleus (N), vacuole (V), cell wall (W), endoplasmic reticulum (ER), mitochondria (M), and the amyloplasts (A) with starch grains are indicated.

In Fig. 28 B several of the amyloplasts seem to rest on the lower longitudinal cell walls. This is, however, a false impression caused by the thickness of the sections. In electron micrographs the amyloplasts were never seen to rest directly on the plasmalemma, but on

a layer of endoplasmic reticulum (ER) and other organelles, as exemplified in Fig. 27.

The location of the center of the clusters of amyloplasts at the various times is plotted in schematic maps of the cells drawn to mutually correct relative, average size in Fig. 29.

In this figure, the plus signs and the large open circles indicate, respectively, the position at the start of the experiment (the normal position) and the position after 8 min of inversion. The square with a circle inside indicates the location at the end of 8 min of stimulation in the first horizontal position, which equals the zero time in the second horizontal position (i.e. after rotation of the root 180° about its axis).

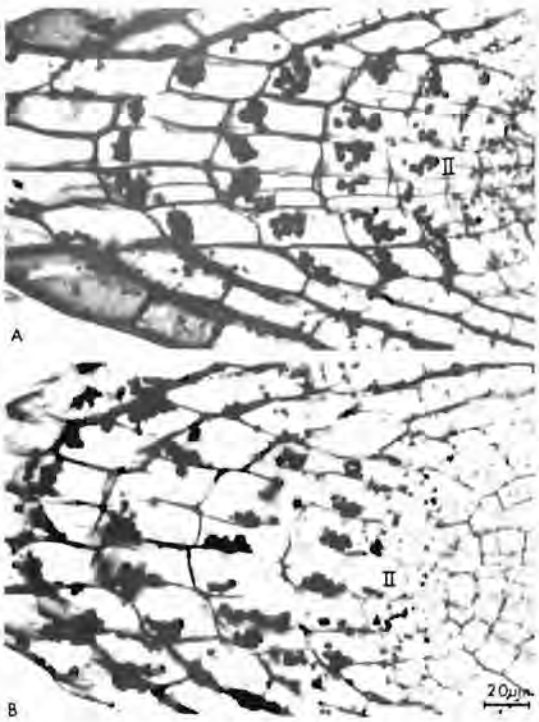


Fig. 28. *Longitudinal section of root tip of cress stimulated in the horizontal positon.*

The roots have successively been inverted for 8 min, stimulated horizontally for 8 min, and rotated 180° about their long axis. The formerly lower horizontal walls are uppermost in the pictures. Storey II is marked. The roots have been kept in the last position for (A) 2 min and (B) 16 min.

The arrow pointing from the open circle to the square indicates that the amyloplasts during stimulation in the first horizontal position not only approached the cell wall which was the lower at that

time, but also moved closer to the floor of the cell. The position observed at various times after the start of the second stimulation period are connected by lines, so that the time sequence of the observations can be followed, the filled circle in the square being 0 min and the subsequent points 2, 6, 8, 10, 16, and 20 min. It is unlikely that a single cluster of amyloplasts would follow such irregular courses as the ones shown in Storeys II and II, but a general pattern does emerge.

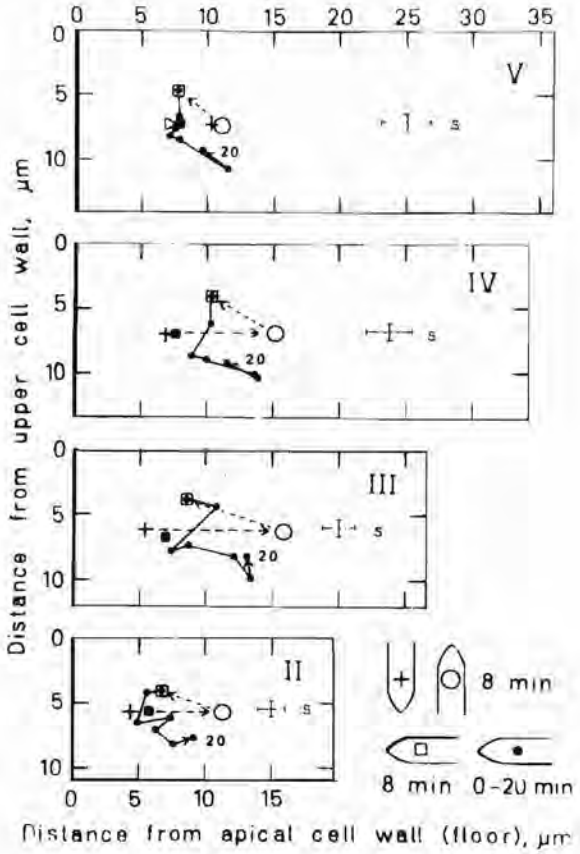


Fig. 29. *Location of amyloplasts during successive stages of movement.* The frames are schematic maps of the central root cap cells, drawn to mutually correct relative, average size. The apical cell wall ("floor") is marked as a heavy line. Roman numerals: storey numbers. - Plus signs: location at rest in the normal position cf. Fig. 25. - Triangle in Storey V: probable location at rest in the present series. - Open circles: location after 8 min inversion of the root. - Open squares: location at the end of 8 min stimulation in the first horizontal position, equal to the location at zero time in the second horizontal position (i.e. after rotation of the root 180° about its axis). - Filled circles: location at successively 0, 2, 6, 8, 10, 16, and 20 min in the second horizontal position. - s: standard deviation (the mean error is 0.33 to 0.2 s). - Filled squares: (for variability, see text): location after rotation for 15 min parallel to the horizontal axis of the klinostat.

At first the amyloplasts follow a mainly downward course. This phase is completed in 6 min (or perhaps less) in all storeys except Storey V, where it takes 8 min. Thereafter the amyloplasts move slowly away from the floor, still sinking somewhat until at about 16 min they have about the same distance from the lower wall as they had from the upper at the start. In Storeys II, III, and IV they do not move beyond the point they would have reached if the root had been inverted for the same or a longer time. As regards Storey V, the amyloplasts have a longer range of movement than observed previously (Fig. 25), namely from 7 to 11 µm from the floor. Their probable normal location at rest as estimated from these observations and those on roots rotated on a klinostat (Section 3) is indicated by a triangle in Storey V.

The travel of the amyloplasts in the downward direction during the first 6 min in Storeys II, III, IV, and V was 2.5, 3,9, 4.7, and 3.0 µm, respectively. This makes 0.42, 0.65, 0,78, and 0,50 µm (mean 0.6 µm) per min. This is only about one-half the average initial

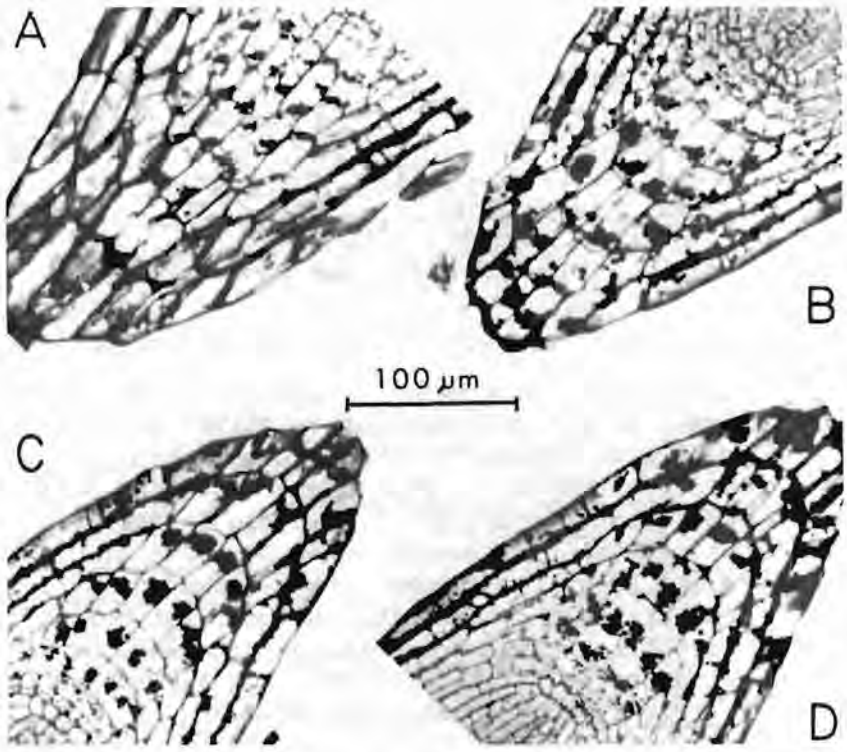


Fig. 30. *Longitudinal sections of tips of representative cress roots stimulated for 10 min at 45° and 135°.*
A and C: without prior inversion. - B and D: after pre-inversion for 16 min.

speed of movement in the longitudinal direction upon inversion of the roots: 1.2 µm per min. [In Hawker's experiments (1933) the amyloplasts moved across the cells of horizontal *Lathyrus* epicotyls at 0.9 µm per min at 20°C]. The speed of the amyloplasts in the longitudinal direction in the period from 6 to 16 min was only 0.3 µm per min in Storey II and from 0.4 to 0.8 µm per min in the other storeys. After 16 min the root tip is already inclined downward by about 17°, which may explain the tendency of the amyloplasts to begin moving back toward the floor end of the cell.

3. Movement of amyloplasts in statocytes of roots inclined at 45° and 135°

The response to stimulation at 45° and 135°, with and without previous inversion of the roots, has been used as an argument in support of the starch statolith hypothesis (see Chapter V). The actual location of the amyloplasts in the statocytes of roots stimulated for 10 min at those angles was determined on photomicrographs (Fig. 30) and plotted in a schematic map of the cells (Figs. 31 and 32). As discussed in Chapter V the amyloplasts were generally found at the expected locations; and treatments which allowed the amyloplasts to slide parallel to the longitudinal cell walls were those which gave rise to the largest geotropic curvatures (Fig. 40).

a. Stimulation at 45° without previous inversion (Figs. 30 A and 31 A)

When roots are turned 45° away from their normal, downward direction, the amyloplasts only move toward the nearest (lower) longitudinal wall and do not slide along the cytoplasm. They accumulate in the lowermost corner of the statocyte (Fig. 30 A). In Fig. 31 the location at the start (normal, downward direction of the root tip) marked by a plus sign, is taken from Fig. 25, but in accord with the results of experiments in Sections 2 and 4, the amyloplasts in resting statocytes in Storey V were evidently located somewhat closer to the floor in the roots used in the present series than in the ones used for Fig. 25. Their probable location at rest is indicated by a triangle in Storey V.

b. Stimulation at 45° after inversion for 16 min

(Figs. 30 B and 31 B)

When roots are kept inverted for 16 min, the amyloplasts move in the direction of the ceiling of the statocyte. The new position (from Fig. 25) is marked by an open circle in Fig. 31 B. If the roots are now turned to the 45° position, the amyloplasts start falling, and will thereby reach obstacles along the lower, longitudinal cell wall and slide downward along this, but separated from it by various organelles (see Figs. 33 and 34). The lengths of the path of this sliding parallel to the cell wall were found to be 9, 10, 4, and 4 µm, respectively, in Storeys II, III, IV, and V. - In this experiment, if the roots had been rotated parallel to the horizontal axis of the klinostat

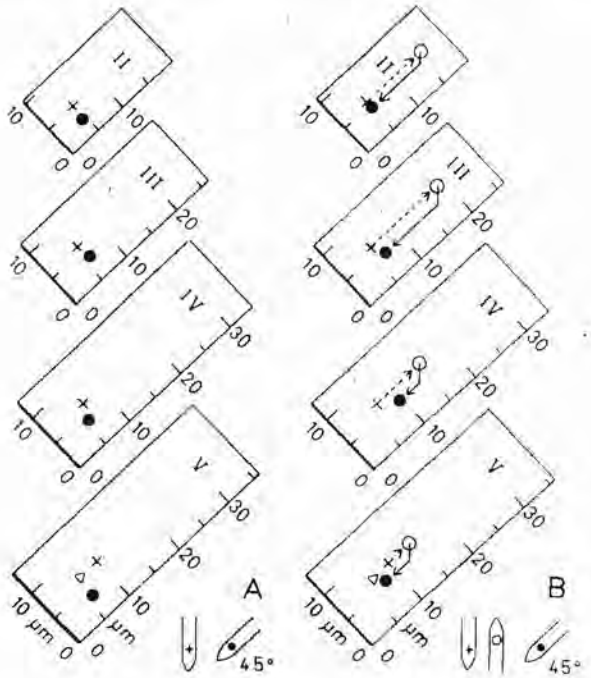


Fig. 31.

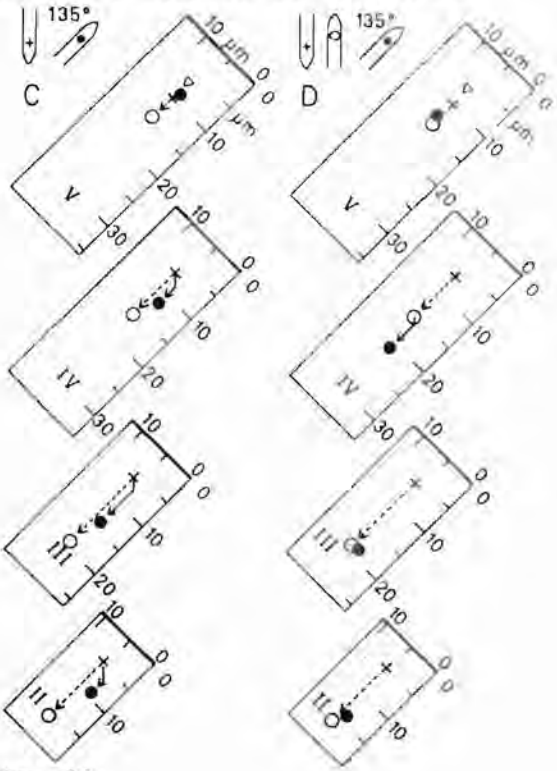


Fig. 32.

Fig. 31. *Location of amyloplasts before and after 10 min stimulation at 45°.* The frames are schematic maps of the central root cap cells, drawn to mutually correct relative, average size. The apical wall ("floor") is marked as a heavy line. - A: no inversion prior to stimulation. - B: inversion for 16 min prior to stimulation. - Roman numerals: storey numbers. - Plus signs: location in the normal position cf. Fig. 25. - Triangles in Storey V: probable location at rest in the present series. - Open circles: location after 16 min of inversion. - Filled circles: location after 10 min stimulation at 45°.

Fig. 32. *As Fig. 31 except that the roots were stimulated at 135°.*

for 20 to 30 min instead of being killed in the fixative, they would have produced considerably larger geotropic curvatures than in case A, where they had not been kept inverted first (see Fig. 38).

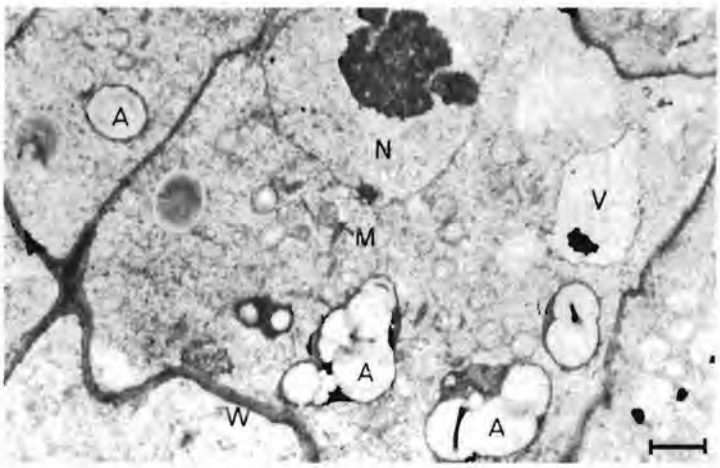


Fig. 33. *Electron micrograph of section of statocytes in Storey II of root cap of cress.*

Symbols as in Fig. 27. The root had been stimulated for 10 min at 45° without prior inversion (treatment A in Figs. 30 and 31). The amyloplasts (A) are present only in the lowermost corner of the cell. Apical end downward to the left.

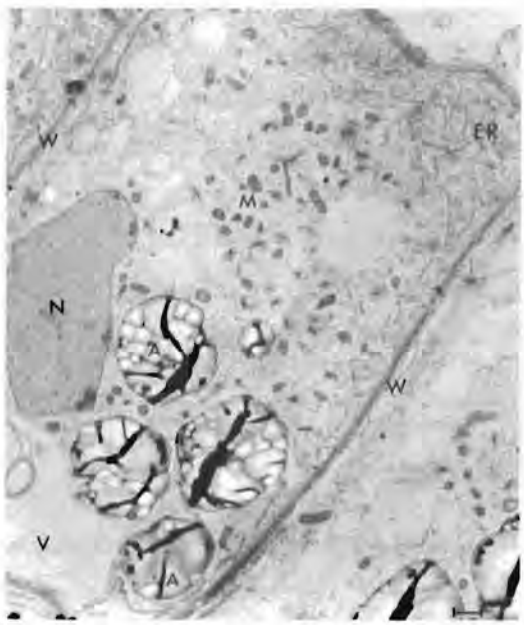


Fig. 34. *As Fig. 33, except that the root was stimulated at 135°.* Apical end upward to the right.

c. Stimulation at 135° without previous inversion
(Figs. 30 C and 32 C)

When roots are turned to a position 135° removed from their normal direction, the amyloplasts start falling and sliding as in case B, but in 10 min they do not get as far as if they had been inverted completely (the open circles in Fig. 32 C). In Storey V, they do not seem to move at all, but this is probably because their initial position was actually closer to the floor than indicated by the plus sign. In Storeys II, III, and IV, they slide parallel to the cell wall for distances of 2, 7, and 3 µm, respectively. Such roots would have produced larger geotropic curvatures than in case A (45°), where there was no sliding.

d. Stimulation at 135° after inversion for 16 min
(Figs. 30 D and 32 D)

In roots kept inverted for 16 min, the amyloplasts have presumably moved as far as they can toward the ceiling of the statocyte. If the roots are then turned to the 135° position, one would expect the amyloplasts to move only a short distance in direction of the lower, longitudinal cell wall, but not to slide along the wall. This was actually found to be the case in Storeys II, III, and V. In Storey IV, the amyloplasts seem to have slid about 4 µm parallel to the cell wall, but considering the consistent pattern in the other storeys, this result is probably due to experimental errors. At least it is evident that there is much less sliding along the cell wall in this type of experiment than in cases B and C; and if similarly treated roots had been allowed to carry out geotropic curvatures, these would have been smaller than in those cases, when recorded after 20 to 30 min (Fig. 38).

e. Electron micrographs

Figs. 33 and 34 are electron micrographs of statocytes from Storey II of root caps of roots that had been stimulated for 10 min at 45° and 135°, respectively. These figures show that the amyloplasts accumulate in the lowermost corner of the cell, but also that they are prevented from touching the plasmalemma by the presence of other organelles, particularly endoplasmic reticulum (ER). The nucleus is always

near the ceiling of the cell, and in many cases, but not in Fig. 34, this body together with the vacuoles will serve to keep the amyloplasts at a considerable distance from the ceiling after inversion of the root.

4. Location of amyloplasts in statocytes of roots rotated on a klinostat

By rotating orthogeotropic plant organs oriented parallel to the horizontal axis of a klinostat, it is possible to eliminate at least the directional component of the physiological effect of gravity on such organs. It would, therefore, be interesting to establish the location of the amyloplasts in the statocytes of klinostat rotated roots and compare their distribution with that of differently treated ones.

Fig. 35. *Micrograph of a median section of the root cap of a cress seedling after rotation for 15 min parallel to the horizontal axis of a klinostat at 2 rpm.*

Fig. 35 is a micrograph of a median section of the root cap of a cress seedling rotated parallel to the horizontal axis of a klinostat for 15 min at 2 rpm. At this rate of rotation, gravity has negligible influence on the direction of growth of the roots, whereas at slower rates, e.g. 0.03 rpm, the root tip will oscillate with the frequency of the rotation (Larsen 1957). The amyloplasts in the functional

statocytes are not clumped together as densely as in normal, inverted, or stimulated roots. The center of the region they occupy is, therefore, located somewhat further away from the floor of the cell than in the normal, vertical root. In many cells it is possible to distinguish individual amyloplasts (11 can be counted in one cell in a section 9 µm thick, i.e. 2 to 50 µm thinner than the cell width). The amyloplasts are, however, not scattered over the entire cell volume. After rotation for longer periods the amyloplasts in root and coleoptile statocytes undoubtedly spread out over larger cell volumes as seen from photomicrographs by various authors: von Ubisch (1928, *Lepidium* roots, 2 hours), Dedolph et al. (1967; *Avena* coleoptiles, 72 hours), and Grey & Edwards (1968; *Triticum* roots and coleoptiles, 45 hours). In the klinostat rotated *Triticum* roots, the distribution of the amyloplasts was similar to that in roots of plants which had been orbiting in Biosatellite II for the same length of time.

The location of the center of the region occupied by the amyloplasts was estimated in the usual manner and is plotted in the cell maps of Fig. 29 as filled squares. As expected, this center is located near the median of the statocyte. As a reference was chosen the longitudinal wall to the right when the micrographs was oriented in the normal position. As a mean of 4 rotated roots and a total of 16 cells per storey, the following distances from that wall were found (expressed as percentages of cell width): 50.4, 54.5, 50.9, and 50.0 ± 1.7 (mean error), respectively, in Storeys II to V. The standard deviation, 6.8, was based on 120 degrees of freedom, since the variances of the 8 groups of measurements could be pooled. The distances from the floor of the cell, expressed in µm, were 6.0 ± 0.34, 7.1 ± 0.45, 7.6 ± 0.59, and 7.6 ± 0.61. As regards Storey V, the figure 7.6 µm is another indication that the amyloplasts can get closer to the floor than found previously (10 µm; Fig. 25).

5. The effect of gravity on the location of movable cell organelles in the statocyte cells

In a preliminary electronmicroscopical study, measurements have been made on the distribution of amyloplasts and other cell organelles upon geotropic stimulation in the horizontal position. Roots of cress were stimulated for 20 min in this position and then immediately thereafter fixed in osmium tetroxide or glutaraldehyde followed by

osmium tetroxide. The roots were oriented, embedded and sectioned in the median plane of the root cap. Electron micrographs were taken of individual cells from the columella region and from the micrograph a drawing was made on paper of the outline of the cell and its amyloplasts and mitochondria. Each cell was divided into four quarters, the axes of division running parallel to the long axis of the root and the direction of gravity, respectively. As shown on Fig. 36 B, for convenience of recording and subsequent analysis the quarters have been designated 1 to 4, where the quarters 3 and 4 represent the lower part of each cell when oriented in the horizontal position.

The results from an analysis of the distribution of mitochondria and amyloplasts in the cell are presented in Fig. 36 A. The values are based on measurements of the columella cells from three different roots. As shown in diagram (a) the amyloplasts are evenly distributed in the quarters of the apical end of the cell when the root is kept in the normal vertical position. Upon geotropic stimulation the amyloplasts start moving and after 20 min in the horizontal position, they are redistributed along the distal part of the lower longitudinal cell wall. As can be seen from the diagram (b) the amyloplasts are displaced from quarter 1 to a final resting position in quarters 3 and 4.

The mitochondria are more evenly distributed in the statocyte cell when the roots are in the normal, vertical position, than the case was for the amyloplasts. A significant trend, however, towards a higher concentration of mitochondria in the distal regions of the cells (quarters 1 and 3) can be demonstrated when the sections are taken from roots growing in the normal position. The "normal" localization of the amyloplasts (diagram a) and the localization of mitochondria (diagram c) show the same pattern; the highest concentration is in the lower (distal) part of the root cap cell. One would expect that the concentration of mitochondria in a normal root tip cell would be the same for each quarter unless this organelle to a certain extent is movable. The uneven distribution could also be explained by the localization of the nucleus in the quarters 2 and 4 (Fig. 36 B). This gives a more restricted free space to occupy for the mitochondria in these quarters. When the roots have been stimulated in the horizontal position for 20 min a redistribution has occuried. A slight but significant accumulation of mitochondria can now be demonstrated in the two lower quarters of the cell (diagram d). The percentage of the total number of mitochondria in the lower and upper half is 58 and 42, respectively. Whether this difference

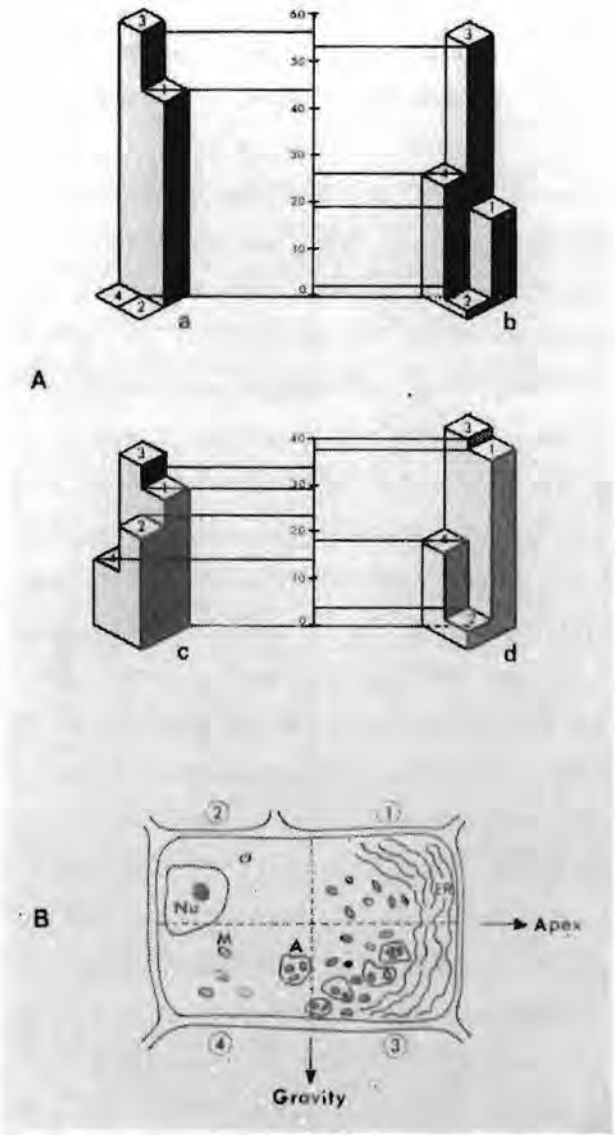


Fig. 36. *Diagram showing the distribution of amyloplasts and mitochondria between the four quarters of central columella cells of cress as a result of geotropic stimulation in the horizontal position for 20 min.*

A: The numbers of amyloplasts (a and b) and mitochondria (c and d) per quarter of cell sections given as percentages of the total number in each section. The diagrams show the distribution of amyloplasts in the normal (a and c) and horizontal (b and d) position, respectively.

B: Schematic drawing of a columella cell showing method of quartering and representative distribution of mitochondria (M), amyloplasts (A), endoplasmic reticulum (ER) upon horizontal stimulation for 20 min. The position of the nucleus (Nu) and the root apex is also indicated.

is real is difficult to say after this preliminary investigation where all the measurements presented in this section, are based on scoring of thin sections. A more reliable result can only be obtained by using

stereological and morphometrical methods on serial sections and by subjecting the results to a detailed statistical analysis.

D. Discussion

The main purpose of the present study was to get a picture of the movements of the amyloplasts under various manipulations of the roots based on observations on photo- and electron micrographs rather than assumptions. A number of attempts have been made to test the starch statolith hypothesis by comparing geotropic responses with presumed or observed movements of amyloplasts which the investigator had intended to direct to specified regions of the cells (Jost 1902, Fitting 1905, Buder 1908, Richter 1914, von Ubisch 1928, Larsen 1965, 1969, 1971). The present study was undertaken in connection with the kind of experiments presented in the next chapter (Chapter V).

After changing the orientation of the root, it was found that the amyloplasts perform the expected movements only in a limited number of the cells. The fact that the amyloplasts are accumulated on the floor of the majority of the root cap cells when the root has been growing in its normal direction for some time, does not mean that all these amyloplasts are movable in response to gravity. In the older cells, the amyloplasts have fallen to the distal end of the cell when the cell was younger, but are now more or less immobile.

The range of movement of the amyloplasts is restricted to a volume near the middle of the cell in Storeys II and III, in the apical half in Storey IV, and still closer to the floor in Storey V. Further movement is evidently prevented by other organelles, ER, vacuoles, and nucleus, the latter always to be found near the ceiling of the cell. As seen on the various electron micrographs (e.g. Figs. 27, 33, and 34; cf. also Fig. 6), the amyloplasts never touch the plasmalemma.

Estimations of the location of the amyloplasts in statocytes of roots stimulated at 45° and 135°, either directly or after inversion for a certain time, verified assumptions made previously (Larsen 1965, 1969, 1971; see also Chapter V): treatments which allow the amyloplasts to slide parallel to the longitudinal cell walls are those that give rise to the largest geotropic curvatures. This is true also in pre-inverted roots, in which the largest curvatures are produced by stimulation at the smaller of two supplementary angles. This effect, which may be called the pre-inversion effect, is observed only in a certain period after

the beginning of the stimulation. When roots are stimulated for 10 min at 45° and 135° and then rotated parallel to the horizontal klinostat axis, the pre-inversion effect is manifest in a period within the limits of 10 and 30 min on the klinostat. After 40 min on the klinostat, the roots stimulated at 135° invariably showed the largest curvatures, regardless of whether they had been pre-inverted or not (Chapter V). A similar pattern was observed in roots stimulated for 15 min at 15° and 165° (Larsen 1969). The suppression of the further development of the curvatures after stimulation at the smaller of the two supplementary angles was ascribed to tonic effects.

The once so popular hypothesis that the accumulation of statoliths on the physically lower cell walls was essential for the susception of gravity cannot be reconciled with the results of time studies on amyloplast movement and observed minimum stimulation times (presentation times). In cress roots 12 sec of horizontal exposure is enought to induce a curvature of 2°.

Another possibility is that the mere change of the direction of pressure of the amyloplasts on other organelles is essential for gravity susception. As pointed out by Sievers & Volkmann (1972) this is an instantaneous effect of the reorientation of a plant organ, which, if essential, would be in accord with the observed short minimum stimulation times. Sievers & Volkmann seem to be of opinion that it is the removal of the pressure exerted by the amyloplasts against the distal ER, particularly in the peripheral columella cells, that constitutes the susception. This concept might well explain the effect of stimulations of short duration in roots taken from their normal, vertical position and stimulated at various angles, but it does not explain why previously inverted roots respond to gravitational stimulation at all. At the beginning of the stimulation their amyloplasts were not in contact with the distal ER, which remains in place under the inversion. In roots stimulated at 90° for 3.75, 10, or 15 min, the responses of normal and pre-inverted roots are practically identical (Larsen 1969, Chapter V).

The differential response to stimulation at two supplementary angles (the pre-inversion effect) has been shown to be closely associated with the movement of amyloplasts. There is probably a certain lag time between the reorientation of a root and the beginning of amyloplast movements, but the results shown in Fig. 29 indicate that this lag period can be less than 2 min. In Storey IV the average downward movement of the amyloplasts in the first 2 min was 2.5 µm (from 6.1 to 3.6 µm,

the difference being statistically significant at the 5% level). Admittedly, the movement would probably have been slower if the amyloplasts had been located at their normal rest position on the distal ER, but the beginning of movement might well fall within the order of the minimum stimulation time. The assumption that the stimulation is due to the displacement of the amyloplasts and continues while they are moving would make it possible to apply the same principle to the susception in normal and in previously inverted roots. The pre-inversion effect, however, is only temporary and is followed, after a certain time of klinostat rotation, by a suppression of the development of the response to the smaller of two supplementary angles whether the roots have been pre-inverted or not.

According to the calculations of Audus (1962) and of Gordon (1963) the mitochondrion and the amyloplast are the smallest organelles that, after stimulation of the root, could settle in cells at rates compatible with the presentation time. The present results have given conclusive evidence that the amyloplasts sediment to the lower part of the cell within the chosen times (max. 20 min), a conclusion also reached by Griffiths & Audus (1964) in detailed, quantitative studies on root cap cells of *Vicia faba* L. The preliminary reports on the mitochondria redistribution presented in this chapter indicate a similar trend as for the movements of amyloplasts. The uncertainty, however, concerning the methodological problems makes these results on the movements of mitochondria less reliable. Griffiths & Audus (1964) observed a certain displacement of the mitochondria to the lower part of the root cells of *Vicia* but the difference was not statistically significant. Working with oat coleoptiles Shen-Miller & Miller (1972 b) found that mitochondria undergo a small degree of sedimentation upon geotropic stimulation. The number of mitochondria was greater in the bottom than in the top of cells of horizontally stimulated coleoptiles; in the avascular tip and outer epidermis of the subapical regions this difference was restricted to the lower tissues. The number of mitochondria and their distribution were reported to differ in different fixatives. These differences were suggested to be caused by distortion of the ultrastructure of the mitochondria as a result of permanganate fixation (Shen-Miller & Miller 1972 b) which they assumed rendered some of the mitochondria unrecognizable for scoring. This explanation seems unlikely as permanganate is recognized as a specially excellent fixative for preserving the membranes of the organelles including the inner and outer membranes of the mitochondria. They also suggest that the reason for

the mitochondrial response differences could be an effect of the rate of the fixation of the tissues. It is, however, difficult on basis of their reports (Shen-Miller & Miller 1972 a, b) to find out if they have kept the coleoptiles oriented in the horizontal position during the fixation procedure.

The idea that by sedimentation the amyloplasts must displace other cell constituents, has been favoured by some investigators. In their studies Griffiths & Audus (1964) found clear indications of shifts in the total quantities of mitochondria, dictyosomes and ER as an indirect result of amyloplast sedimentation. Perbal (1971) observed that mitochondria and ER in roots of *Lens* were displaced to the upper part of the cells as a result of amyloplast movements. A similar displacement of the ER was also observed in *Vicia* root cells (Griffiths & Audus 1964) and in *Zea mays* L. (Juniper & French 1973). These results are at variance with the observation of the behaviour of ER in cress roots reported by Sievers & Volkmann (1972). The present author had previously (Iversen 1968), like Sievers & Volkmann, noted that the mass of distal ER in the statocytes is shaped like a flat dish (cf. Fig. 6) on which the amyloplasts are resting when the root is in its normal position. Fig. 37 illustrates the distribution of amyloplasts in cress root cells kept at various positions in relation to gravity. As can be seen from the figure when the amyloplasts have come to rest in the new position of the root, the ER is still in the original position and has not been displaced, as also observed by Sievers & Volkmann (1972).

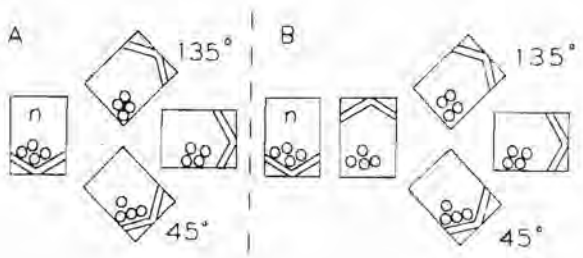


Fig. 37. *Schematic map of the location of amyloplasts (circles) in relation to the distal mass of endoplasmic reticulum (V-shaped structure) in statocytes of cress roots when the amyloplasts have come to rest after geotropic stimulation at 45, 90, or 135°.*

A: roots normal (n), B: roots inverted before stimulation.

The findings reported in this chapter will later be further discussed in relation to the statolith hypothesis (see General Discussion).

E. Summary

The movement of amyloplasts has been followed in the root cap of *Lepidium sativum* L. which contains 7 (or 8) storeys of starch-containing cells. In the youngest one (or two) of these storeys the amyloplasts are small and the cells appear embryonic. In the 6 non-embryonic storeys the amyloplasts are large. Upon inversion of the roots, the amyloplasts in the 3 youngest of the 6 non-embryonic storeys start falling toward the opposite end of the cell at about 72 µm per hour (at 21°C), but they maintain this speed for only 6 to 12 min, after which they virtually come to a stop. As a result, it takes 10 to 12 min before any of the amyloplasts get approximately as close to the ceiling as they were to the floor before the inversion; and this is true only of the 2 youngest of the non-embryonic storeys. The positions of the amyloplasts in the cells of the 2 oldest starch-containing storeys are erratic and show little, if any, dependency on the preceding time of inversion. As regards Storey No V, the amyloplasts in this have only a certain range of movement upon inversion.

Previously inverted cress roots were also placed in a horizontal position and the amyloplasts in the statocytes of the root cap allowed to fall through their entire range of movement across the cell. Under these conditions the amyloplasts first follow a mainly downward course for 6 to 8 min at a speed between 0.5 and 0.8 µm per min. For the next 10 min they move slightly more slowly in a direction away from the apical end of the cell, still sinking somewhat, but without reaching the plasmalemma along the lower wall.

Experiments presented in the next chapter have shown that conditions assumed to allow the amyloplasts to slide parallel to the longitudinal cell walls are those that give rise to the largest initial geotropic curvatures. Such conditions are for instance (1) stimulation at 135° (root tips pointing obliquely upward) and (2) inversion of roots for 16 min followed by stimulation at 45°. Treatments assumed not to permit extensive sliding of the amyloplasts produced smaller initial geotropic curvatures, namely (3) stimulation at 45° without pre-inversion and (4) inversion followed by stimulation at 135°. The location

of the amyloplasts after these four kinds of treatment has been determined on photomicrographs and the assumptions concerning the paths and extent of sliding of the amyloplasts confirmed.

Observations on electron micrographs showed that under all conditions the amyloplasts are separated from the plasmalemma by other organelles, such as ER, nucleus or vacuoles.

In roots rotated for 15 min parallel to the horizontal axis of the klinostat at 2 rpm, the amyloplasts are not clumped together as densely as in normal, inverted or stimulated roots, but they are not scattered over the entire cell volume.

In preliminary experiments the movement of organelles other than amyloplasts have been followed. The results from these experiments are discussed in view of other observations.

Chapter V

THE RELATIONSHIP BETWEEN AMYLOPLAST MOVEMENTS AND GEOTROPIC RESPONSE

A. Introduction

One of the methods applied to testing the validity of the starch statolith hypothesis consists of directing the amyloplasts to specified regions of the cell before or after stimulation and correlating the geotropic responses of the organs with the presumed or observed movements of the amyloplasts. Experiments on these lines have previously been carried out by several investigators and the results of some of these experiments were interpreted as not supporting the statolith hypothesis (Jost 1902, Fitting 1905, von Ubisch 1928); while others (Richter 1914, Buder 1908, Zimmermann 1927) were taken as supporting it.

Larsen (1965) repeated the experiments described by Zimmermann (1927) and von Ubisch (1928) with improved methods. In a later paper Larsen (1969) attempted to relate the optimum angle of geotropic stimulation to the movement of the amyloplasts. He observed that the optimum angle of supraminimal geotropic stimulation is larger than 90°. This agreed well with the sketched movements of amyloplasts in hypothetical model statocytes, assuming that sliding of the amyloplasts in the cytoplasm along the cell wall is a more efficient stimulus than a mere static pressure on a limited area (see Fig. 1 in Larsen 1969). On basis of such models, one would expect stimulation at 135° (root tips pointing obliquely upwards) to be more effective than stimulation at 45°, which is in agreement with observations. If the sliding of the statoliths along the cell wall is really more effective than their mere pressure on a limited portion of the wall, then one would expect stimulation at 45° to be more effective than stimulation at 135° if the statoliths were situated in the opposite end of the cell before the stimulation begins (Larsen 1965, 1969, 1971).

Accumulation of the amyloplasts in the opposite half of each of the functional statocytes can be achieved by keeping the seedlings in the inverted position for 8 to 20 min (Fig. 24). In experiments with seedlings which had been kept inverted for 8 min (Larsen 1969) and then stimulated for 3.75 min, at various angles, however, the

optimum was 90°, and not 40° to 60° as expected. The responses to 45° and 135° were nearly identical, and so were the responses to other pairs of supplementary angles. When the stimulation time was increased from 3.75 to 15 min, stimulation at 165° yielded curvatures many times larger than those obtained at 15°, when measured after 10, 20, and 30 min of klinostat rotation; so the response curves, that is curvatures plotted against angle of stimulation, were skewed to the right just as the curves for not inverted roots. A closer study, however, revealed that during the very first part of the geotropic curvature, i.e. in the second half of the 15 min stimulation period, before klinostat rotation, curvatures developed faster at 15° than at 165°. At 15°, they soon stopped or decreased, whereas at 165° they continued to increase for at least 30 min.

This experiment showed that conditions do exist under which the geotropic response curve is skewed to the left. In the present chapter experiments will be described in which such skewness can be demonstrated more clearly by choosing 16 min of pre-inversion and 10 min of geotropic stimulation.

B. Materials and Methods

Cultivation

Etiolated seedlings of the garden cress (*Lepidium sativum* L.) were raised from sterilized seeds, their roots growing in a plant chamber between two 1 x 35 x 75 mm slices of 1.25% agar. All the experiments were carried out at 21°C.

Experimental procedure

As shown in Chapter IV (Fig. 25) when seedlings had been kept inverted for 8 min, the amyloplasts in their root cap cells had moved more than halfway towards the opposite end of each cell. Relative to the total range of free movement in the cells, the distances traversed in 8 min were ca. 65%, 80%, and 100%, respectively, in cell storeys Nos II, III, and IV (Larsen 1969).

In the present study, the inversion time was extended to 16 min, thus securing that the amyloplasts had moved as far as they would

go in all cells. The longer inversion time, however, has the disadvantage of larger spontaneous movements than would occur in 8 min.

Stimulation in the various positions was fixed at 10 min. Since the reaction time for continuous stimulation at 90° with 1 g is about 5.5 min for *Lepidium* roots, a certain curvature was present, also at other angles, at the end of 10 min stimulation period.

Immediately after the end of the stimulation period, the plants were rotated at 2 rpm parallel to the horizontal axis of the klinostat described in Chapter III, and photographic exposures of the geotropic responses were made automatically during the rotation.

The geotropic curvatures were measured on the photographic negatives as described previously (Chapter III).

C. Results

1. Seedlings not inverted prior to stimulation

For the sake of comparison, a series of experiments were carried out with seedlings which had not been inverted prior to stimulation. When the responses to 10 min of stimulation were plotted against angles of stimulation (Fig. 38 A), the resulting series of curves showed an optimum well above 90°, getting more and more pronounced with increasing duration of klinostat rotation, here extended to 60 min.

2. Seedlings inverted prior to stimulation

Fig. 38 B shows the results of a similar series of experiments carried out with roots of seedlings which had been kept inverted for 16 min prior to 10 min of stimulation at the various angles. As in the previous case, the curvatures developing during the 10-minute stimulation period itself were small. At the end of the period (t = 0), the roots had been curving for only about 4 min. The curvature recorded at t = 0 after stimulation at 135°, however, is exceptionally low, only 0.2°, and differs significantly (at the 5% level) from curvatures recorded at the other stimulation angles, including 45° where the curvature at t = 0 is 1.8°. The low result at 135° is

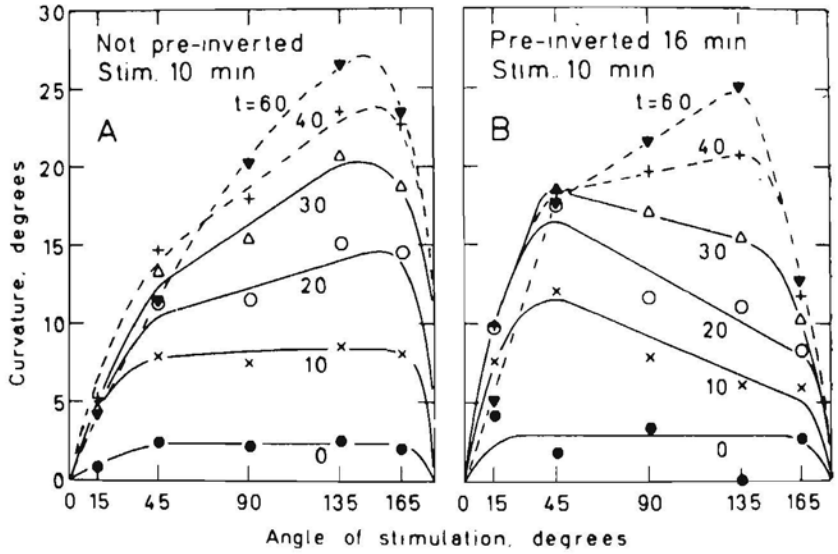


Fig. 38. *Geotropic curvatures of cress roots plotted against angles of stimulation.*

Zero degrees on the abscissa means root tips are pointing downward. Roots stimulated for 10 min at the various angles, and then rotated for times t min (as indicated) parallel to the horizontal axis of the klinostat. A: roots not inverted prior to stimulation. B: roots kept inverted for 16 min prior to stimulation. - To avoid interference with other symbols at 15° stim. angle, the following points are not marked: A : t = 30 : 5.0°; t = 20 : 4.6°; B : t = 30 : 9.8°. - The least significant differences between curvatures at 45° and 135° are entered in Fig. 40.

probably anomalous. It is very unlikely that curvatures should be lower at 135° than at 165°; and in a series of control experiments (Fig. 39), the values at 135° fall in line with results obtained at the other stimulation angles.

After 10, 20, and 30 min on the klinostat, it is evident that stimulation at 45° yielded considerably larger curvatures than at 135°. Similarly, stimulation at 15° yielded somewhat larger curvatures than at 165°, but only after 10 and 20 min of klinostat rotation. The optimum is near 45°, in complete agreement with the hypothesis of the superior effect of amyloplasts sliding along the cell walls.

Later, however, after 40 and 60 min on the klinostat, something happens which gives us back the type of curve we get with not previous inverted roots, having their optimum around 135° or higher. This phenomenon will be discussed later.

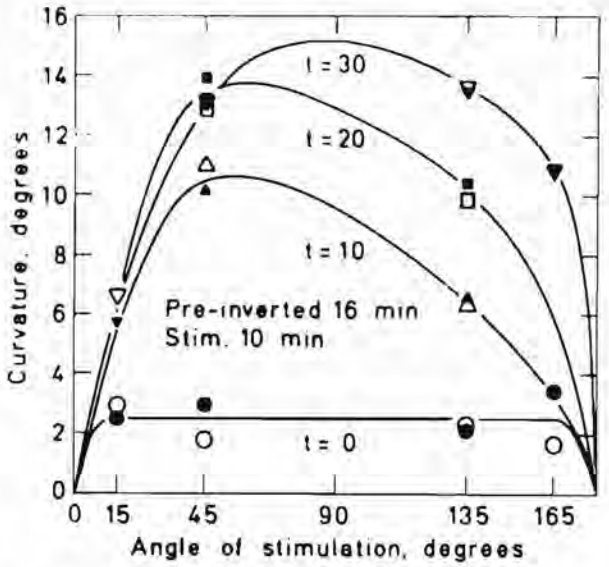


Fig. 39. *Control experiments.*

Cress seedlings inverted for 16 min and stimulated for 10 min at various angles as in Fig. 38 B. Then turned the shorter way (open symbols) or the longer way (filled-in symbols) to the horizontal position before starting the rotation of the klinostat axis. Readings after various times t (min) as indicated.

3. Control experiments

A series of control experiments were carried out.

(a) It was verified that the average curvature of roots which had been kept inverted for 16 min did not, at the end of the inversion period, differ significantly from the average curvature (zero degrees) of roots kept in the normal position.

(b) Curvatures might, however, develop in unstimulated roots rotating on the klinostat. This possibility was tested in a special series of experiments, using both inverted and not inverted plants. These were rotated parallel to the horizontal axis of the klinostat for 30 min and their curvatures recorded. This test was combined with an investigation of the possible influence of the process of turning the seedlings from the stimulation position to the horizontal position before starting the rotation of the klinostat. This can be done in different ways. When, for instance, a root has been stimulated at 45°, it may be moved up 45° to the horizontal position (shorter way), or it may be moved 315° in the opposite direction to the same position (longer way). In the experiments reported so far, the plants were always turned the shorter way. The effect of the method of turning the plants to the horizontal position

was studied both on unstimulated roots and on stimulated ones. Seedlings were turned from the starting position (normal or inverted) to one of the four angles 15°, 45°, 135°, or 165°; and from there they were either immediately or after 10 min turned to the horizontal position, either the shorter or the longer way. The curvatures were recorded after 30 min of klinostat rotation (for stimulated, pre-inverted plants also after 0, 10, and 20 min).

The results of these control experiments are shown in Table 6 and (for stimulated, pre-inverted plants) in Fig. 39. The method of turning the plants to the horizontal position before klinostat rotation appeared to have no significant influence on the size of the curvatures in any of the four cases (± inversion, ± stimulation). Likewise, there was no definite trend related to the magnitude of the stimulation angle. So it may be concluded that the turning itself has no significant effect on the results.

Table 6. *Curvatures in control experiments.*

Effect of two different methods of turning cress seedlings from stimulation position to horizontal position before klinostat rotation. Stimulation at various angles for 0 min and 10 min. Curvatures (Z_{30}) measured in degrees after 30 min of klinostat rotation

Treatment	Parameter	Method of turning plants from stimulation position to horizontal position before klinostat rotation							
		Shorter way				Longer way			
Not inverted Stim. 0 min	Angle of stimulation	15°	45°	135°	165°	15°	45°	135°	165°
	Number of plants	7	8	9	9	9	7	8	8
	Curvature, Z_{30}	0.6	1.0	-0.1	1.1	0.3	0.1	0.4	0.9
	Mean errors (d.f. = 57)	0.65	0.61	0.57	0.57	0.57	0.65	0.61	0.61
	Mean curvature, Z_{30}			0.6				0.4	
	Mean error (d.f. = 57)			0.30				0.30	
Inverted 16 min Stim. 0 min	Angle of stimulation	15°	45°	135°	165°	15°	45°	135°	165°
	Number of plants	28	19	17	24	15	20	16	19
	Curvature, Z_{30}	2.3	2.4	1.4	1.8	2.4	1.6	2.7	2.9
	Mean errors (d.f. = 149)	0.71	0.86	0.91	0.78	0.96	0.83	0.93	0.86
	Mean curvature, Z_{30}			2.0				2.4	
	Mean error (d.f. = 149)			0.40				0.45	
Not inverted Stim. 10 min	Angle of stimulation	15°	45°	135°	165°	15°	45°	135°	165°
	Number of plants	9	8	8	9	7	8	7	7
	Curvature, Z_{30}	8.3	14.9	18.1	13.9	8.4	14.3	20.0	13.1
	Mean errors (d.f. = 55)	1.01	1.07	1.07	1.01	1.14	1.07	1.14	1.14

The klinostat rotation as such may, however, have some effect. Small curvatures, which on an average were positive (0.6° and 0.4°) developed in unstimulated, not inverted plants during 30 min of klinostat rotation (upper part of Table 6). In no case did the curvatures at the individual stimulation angles differ significantly from zero, but the

mean of curvatures in the whole "shorter way" series (0.6°) did reach the 5% level of significance. Still, this curvature is small, and the curvatures must be assumed to have been smaller after 10 and 20 min of rotation than at 30 min.

A similar phenomenon was observed in unstimulated, pre-inverted seedlings, but in this case the curvatures were larger (means: 2.0 and 2.4°) and reached significance at several of the individual stimulation angles.

The very manipulation of the plants thus seems to give rise to small positive curvatures. Part of this effect may be due to the fact that the plants were indeed kept briefly at a geotropically stimulating angle. As shown by Larsen (1969; Fig. 3) a stimulation as short as 14 sec is enough to give measurable curvature in *Lepidium* roots. The possibility that some unnoticed mechanical property of the klinostat may contribute to a slight bias toward positive curvatures is not excluded. These curvatures were recorded after 30 min of klinostat rotation of "unstimulated" plants. In the case of stimulated plants, it is unknown if such "background" curvatures are involved, and if so, whether they extrapolate back only to the beginning of rotation or to the beginning of stimulation; but they must undoubtedly be smaller at 0, 10, and 20 min of rotation than at 30 min. No correction has been made for such curvatures.

The responses of not inverted roots which had been stimulated for 10 min at the various angles and then turned either the shorter or the longer way to the horizontal position and rotated for 30 min are listed in the lower part of Table 6. Although only one series of experiments was carried out, the results indicate clearly that the position of the optimum around 135° is uninfluenced by the manipulations described.

This problem was investigated more thoroughly in the case of pre-inverted plants. The results are shown in Fig. 39. For unknown reasons the absolute values of the curvatures differ somewhat from the ones shown in Fig. 38 B, but the presence of an optimum near 45° after 10 and 20 min of rotation was verified. The differences between mean curvatures at 45° and 135° were very highly significant (0.1% level or better) both at 10 min and at 20 min. The transition of the optimum toward angles larger than 90°, however, has started already at 30 min in Fig. 39, whereas this change in Fig. 38 B did not occur until some time between 30 and 40 min on the klinostat.

The main purpose of this experiment was to check the effect, if any, of the method of turning the plants, and the results indicate clearly that there is no significant difference in the geotropic responses between plants turned the shorter and the longer way to the horizontal position before being rotated on the klinostat.

D. Discussion

If the normal location of an optimum for geotropic stimulation at angles around 135° is to be explained by amyloplast sliding, then, as pointed out in the introduction, one would predict that roots which had been kept inverted long enough for their amyloplasts to accumulate in the opposite end of each functional statocyte would show an optimum around 45°. The demonstration of such an optimum in pre-inverted roots would thus serve as a support for the starch statolith hypothesis. Approaches towards the expected shift of the response curve by pre-inversion of the roots were reported by Larsen (1965, 1969), but the results reported in the present chapter demonstrate the shift much more clearly. The starch statolith hypothesis has thus received additional support. But as pointed out previously by Larsen, some factor other than statolith movements also has a strong influence on the development of the geotropic curvatures. Among other things, this is evidenced by the fact that in pre-inverted roots, the optimum does not remain at its initial position around 45°, but sooner or later is found around 135° as in not pre-inverted roots. In Fig. 38 B, this happens between 30 and 40 min after the beginning of klinostat rotation, in Fig. 39 somewhat sooner. The time of this transition seems to be influenced by small changes in the experimental conditions.

If we compare the time courses of the curvatures at 45° and 135° in normal and in pre-inverted plants (Figs. 40 A and 40 B), we notice that in both cases the curvatures at 45° come to a stop, or even decrease, within the chosen duration of the experiment. In Fig. 40 A this happens between 40 and 60 min, in Fig. 40 B already at about 30 min. At 135°, on the other hand, the curvatures continue to increase at least for a whole hour. A similar pattern emerges if the responses to stimulation at 15° and 165° are compared in the same way. These effects, except for the actual time of the stop at the lower angle of stimulation, are independent of the pretreatment of the roots and are

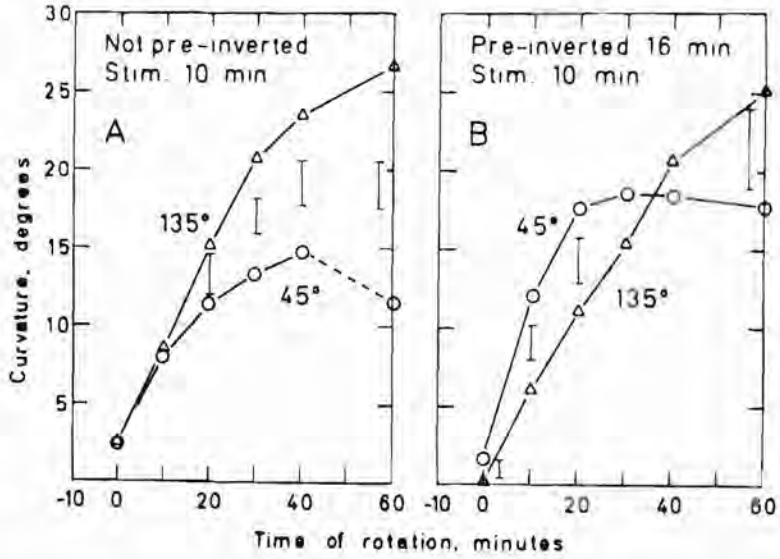


Fig. 40. *Geotropic curvatures of cress roots plotted against time of rotation on the klinostat after the end of 10 min stimulation at 45° and 135°, respectively, as indicated.*

Minus 10 on the abscissa marks the beginning of stimulation. Where the differences are significant, vertical bars indicate the least significant difference at the 5% level. A B as in Fig. 38. The curvature in B at zero time and stimulation at 135° is regarded as anomalously low.

in agreement with results obtained and discussed by Larsen (1965). In that paper it was shown that after-treatments consisting in placing the plants for 5 min in the normal position after stimulating them in the horizontal position, but before rotating them on the klinostat, had a strong, inhibitory effect on the development of geotropic curvatures in the roots. If, on the other hand, the plants were kept in the inverted position during the after-treatment, there was little inhibition or even some enhancement of the curvatures as compared with plants that were rotated directly without any after-treatment in a vertical position. The after-treatments had practically the same effects on normal roots and on roots that had been kept inverted for 15 min prior to stimulation. The inhibitory effect of the after-treatment in the normal position was interpreted as tonic (i.e. interfering with the reacitivity) rather than tropistic. [von Ubisch (1928) was evidently studying only the tonic effects, and her results have no bearing on the starch statolith hypothesis.]

In the experiments referred to in Figs. 38 A and B, no after-treatment in a vertical position were applied, but the tonic effects

have not been eliminated except at 90°. The inhibitory, tonic effects must be present during the stimulation, although decreasing when the angle of stimulation is increased.

So a reasonable interpretation of the shape of the curves in Figs. 38 A and B is that they are the results of at least these two effects: (1) a stimulation due to the movement of amyloplasts, which is enhanced if these are allowed to slide along the cell walls, and (2) a modification of the development of the resulting curvatures by tonic effects, which are inhibitory between the angles of stimulation 0° and 90°, and absent or enhancing between 90° and 180°.

It should be remembered that the curvatures, after the zero-minute reading, are developing on the rotating klinostat. The inhibitory, tonic effects are thus after-effects, induced by the position of the plants during the stimulation. The roots are not getting closer to their normal position during the experiment. In nature, on the other hand, under continuous geotropic stimulation, the roots will approach their normal position, the inhibitory, tonic effect getting stronger and stronger and serving to counteract or prevent "overshoot" of the curvature.

The nature of the tonic effects seems to remain unknown. At least, contrary to Zimmermann's suggestion (1927), they cannot be explained by statolith movements.

E. Summary

When roots of cress seedlings (*Lepidium sativum* L.) are stimulated for 10 min at an angle of 135° (i.e. the root tips are pointing obliquely upward), the resulting geotropic curvatures become larger than after 10 min stimulation at 45°. This well-known behaviour has been explained by the better conditions for statoliths, initially located in the floor end of the statocytes, to slide along the cell walls when root tips are pointing upward at 135° than when pointing downward at 45°. Accepting this explanation, one would predict the optimum angle of stimulation to be near 45° when roots had first been kept inverted long enough for their statoliths to accumulate in the opposite end of each functional statocyte. This prediciton has been verified in experiments with cress seedlings which were first kept inverted for 16 min, then stimulated for 10 min at given angles, and subsequently rotated parallel to the horizontal axis of the klinostat at 2 rpm. Under these conditions, roots stimulated at 45° curve faster during a 20 to 30 min period on the klinostat than roots stimulated

at 135°, but thereafter they stop curving. Roots stimulated at 135°, on the other hand, although initially curving slower than those at 45°, continue curving for at least a whole hour, and attain larger curvatures than the others after 40 min. The optimum shifts from near 45° to near 135° during the course of the klinostat rotation. The behaviour of normal and pre-inverted roots is interpreted as the result of at least two effects: (1) a stimulation due to the movement of amyloplasts, which is enhanced if these are allowed to slide along the cell walls, and (2) a modification of the development of the resulting curvatures by tonic effects, which are inhibitory between stimulation angles 0° and 90°, and absent or enhancing between 90° and 180°.

Chapter VI

EXPERIMENTAL REMOVAL OF STATOLITH STARCH

A. Introduction

Previous attempts to remove the statolith starch from various plant organs have been reviewed in Chapter I. More recently Pickard & Thimann (1966) have succeeded in making wheat coleoptiles apparently starch-free by incubation with gibberellic acid plus kinetin at 30°C for 34 hours. The starch-depleted coleoptiles curved and elongated more slowly than freshly excised, untreated coleoptiles. The ratio of curvature to growth, however, was about 50° per mm of elongation regardless of whether the pretreatment was done in water, in 0.03% sucrose, or in gibberellic acid plus kinetin. The wheat coleoptiles treated with gibberellic acid and kinetin were examined (1) freshly squashed, (2) sectioned semithin from material embedded in glycol methacrylate, and (3) as electron micrographs. In the starch-depleted tissue Pickard & Thimann observed shrunken starch-free amyloplasts, but these did not seem to move in response to gravity. The authors conclude that starch grains are not necessary for graviperception in wheat coleoptiles. They suggest that perhaps the pressure distribution of the entire cell contents may be essential. They do not, however, dismiss the general concept of the statolith theory, but they conclude that the classical statolith hypothesis is in need of revision.

Attempts have also been made recently to obtain starch-depleted roots. Pilet & Nougarède (1971), Nougarède & Pilet (1971), and Pilet (1972) working with roots of *Lens*, reported that the content of amyloplast starch was supressed when the roots were treated with gibberellic acid. It was concluded that gibberellic acid significantly reduced the georeaction and decreased the statolith density when used at concentrations which did not influence root elongation.

As mentioned in the preface the writer had previously (Iversen 1969) reported experiments in which it was attempted to duplicate Pickard & Thimann's experiments using roots of cress. In the present chapter these results are summarized and details of observations on similar work with other plant roots is presented. It was felt necessary for various reasons to repeat Pickard & Thimann's experiments with wheat coleoptiles and observations from these experiments are also discussed here.

B. Materials and Methods

Plant material and cultivation

For the root experiments seeds of cress (*Lepidium sativum* L.), red clover (*Trifolium pratense* L.), and white clover (*Trifolium repens* L.) were sterilized with at 3% calcium hypochlorite solution for 15 min, germinated on moist filter paper in petri dishes, and grown in the normal, vertical position in the dark at 21 ± 0.5°C. After approximately 48 and 54 h for cress and clover, respectively, seedlings having straight roots were selected for the GA_3 plus kinetin treatment.

To prepare plant material for the wheat coleoptiles experiments, seeds of bearded hard red spring wheat (*Triticum durum* Desf. var. Henry from the L.L. Olds Co., Madison, Wisconsin) were soaked for 2 h and germinated in the dark at 21°C for about 36 h. After that time coleoptiles which had attained a length of 2 to 3 mm were selected and excised for the hormonal treatment.

Hormonal treatments

The seedlings for the root experiments were transferred to petri dishes (diam. 17 cm) containing 2 disks of filter paper which for routine work were moistened with 7 ml of one of the following solutions in distilled water:

1. Gibberellic acid (abbreviated GA_3), 4.3×10^{-5} M; kinetin (abbreviated K) 4.3×10^{-5} M; Penicillin Novo, 1.4×10^{-5} M.
2. Sucrose, 7.7×10^{-4} M; Penicillin Novo, 1.4×10^{-5} M.
3. Penicillin Novo, 1.4×10^{-5} M (water control).

In the treatments of the clover seedlings the sucrose solution was generally omitted in the subsequent geotropical experiments. At intervals of 12 h the seedlings were rinsed and transferred to dishes with filter paper moistened with fresh solutions. The dishes were kept in the vertical position in the dark at varying temperatures and for different periods of time. In cress and clover seedlings the disappearance of starch was judged complete after 29 h at 35°C and after 30 h at 34°C, respectively.

The starch-depletion of the wheat coleoptiles was performed in cotton-plugged 50 ml flasks containing 2.5 ml of the incubation

solution. The composition of these solutions has been described by Pickard & Thimann (1966). Experiments were carried out using two control groups of plants, one group incubated in 0.03% sucrose, the other in plain water, in addition to the groups incubated in solutions of gibberellic acid (10^{-5} M) and kinetin (7×10^{-5} M). All solutions contained 10^{-4} M Penicillin Novo. The flasks were incubated in the dark at 30°C and 34°C but they were not shaken as this occasionally resulted in displacement of the coleoptiles away from the solution in the flasks. The coleoptiles were rinsed and transferred to fresh flasks of solution at intervals of approximately 10 h to minimize microbial contamination.

Microscopical examination

Starch depletion both in roots and coleoptiles was judged by light microscopical and electron microscopical examination. The roots were stained with I_2-KI and flattened with a cover slip. All the cells in the root tip could be inspected as the tissue is thin. The coleoptiles were cut longitudinally into two halves and flattened gently with a cover slip. Almost all cells could be inspected by observing from both inner and outer surfaces.

For confirmation, both roots and coleoptiles were fixed in Nawaschin II solution for 12 h and then carried through the embedding process to paraffin, sectioned to 9 μm thickness and stained with the PAS-reagent, before examination in the light microscope.

Electron micrographs were prepared to confirm the extent of the starch depletion. Segments, 10 and 2 mm long, respectively, were removed from selected roots and coleoptiles and fixed according to one of the procedures described in Chapter II. Dehydration was carried out in ethanol of increasing concentration at room temperature. The tissue was finally transferred in two baths of propylene oxide before embedding in Epon. Ultrathin sections were made from near the median plane of the root tips. The sections were stained for 15 min with 1% uranyl acetate in water and thereafter for 10 min with lead citrate. The sections were examined in different electron microscopes (Siemens Elmiskop I, Siemens Elmiskop 101, Philips EM 300, and JEOL 100 B), at 60 KV and 80 KV.

Geotropical experiments

a. Roots

The starch-depleted, straight GA_3 + K-treated intact seedlings and control seedlings pretreated in plain water at 21°C and at 35°C (34°C) were rinsed and placed, 8 to 10 parallel to one another, with their roots between two agar slices, 35 x 75 x 1 mm, consisting of 1.25% agar without nutrients. The agar slices rested on a glass plate and were protected by a plastic cover.

The cress seedlings were given a 3 h adjustment period at 21°C with their roots in the normal, vertical postion. After that the plants were stimulated for 30 min in the horizontal position and then rotated at 2 rpm parallel to the horizontal axis of the klinostat. The clover seedlings were given various adjustment periods at 21°C with their roots in the normal position. For routine work, however, the adjustment period was 2 h and the roots were continuously stimulated in the horizontal position for 6 h (red clover) or 14 h (white clover). The development of the geotropic curvatures was followed by photographing the seedlings at various times. Angles of curvature and the lengths of the roots were measured following the procedures described in Chapter III.

After the geotropical experiments all the GA_3 + K-treated roots were examined microscopically as a routine test, to check that the cells were still devoid of starch.

b. Coleoptiles

After the incubation period at 30°C or 34°C, coleoptiles were selected for the geotropical experiments; those with large curvatures were discarded. The coleoptiles were rinsed and placed in groups of 10 on a glass plate between agar sheets made up to a final concentration of agar containing 10^{-5} M GA_3, 7×10^{-5} M K, and 10^{-4} M penicillin. The agar in the two control groups contained 0.03% sucrose and plain water, respectively. The three groups (1) GA_3 + K, (2) sucrose control, and (3) water control, were placed on the same glass plate in each experiment. Coleoptiles which had small curvatures were oriented so that their tips bent downward. The coleoptiles were adapted to the agar medium for one hour in the normal position before they were stimulated

continuously in the horizontal position. The development of curvatures and elongation was followed photographically for 24 h. The experiments were performed in the dark at 21°C. At the end of the experiments all the coleoptiles were examined microscopically.

C. Results

The effectivity of the hormonal treatment in removing the starch from the root tip

In all types of roots examined the size and numbers of the starch grains in the root cap had decreased. Fig. 41 A illustrates the starch content in a root of cress which has been treated for 24 hours at 35°C. The Storeys I, VI, and VII are depleted but the rest of the root cap cells contain very small residual starch grains. Fig. 41 B shows a root treated for 36 hours at 35°C. No starch grains are visible either in the columella or in its surrounding cells. In Storeys II, III, and IV plastids can be seen without starch grains.

By keeping intact cress seedlings in the dark at 35°C on filter paper moistened with water (witout nutrients added) the roots could gradually be made starch-free. The root in Fig. 41 C was treated in this manner for 48 hours. Residual starch grains can be seen in the columella cells but as shown in Table 7, all the cells are depleted of starch by incubation for more than 60 hours.

A cress root incubated in 7.7×10^{-4} M sucrose for 48 hours at 35°C is shown in Fig. 41 D. Only the columella cells contain starch, but the size and number of starch grains have decreased, although the decrease is considerably smaller than in the starved roots (Fig. 41 C). Even after 72 hours, starch grains were found in the cells when sucrose was present in the incubation medium (Table 7). The absence of starch in properly treated roots was confirmed by other staining methods.

The micrographs of PAS-stained GA_3 + K-treated cress roots give an overwhelming impression that the treatment is extremely effective. Various reasons make desirable an independent check of the PAS-stained plants in the electron microscope. Fig. 42 A illustrates a part of a columella from an untreated root fixed in osmium tetroxide. The amyloplasts appear as somewhat irregular or ovoid sacs.

Table 7. *Influence of various treatments at 35°C on the starch content in roots from seedlings of cress.*
The results are based on series of micrographs.

Experimental treatment	24 hours	36 hours	48 hours	60 hours	72 hours
GA_3 + K	Residual starch grains in Storeys II, III and IV.	No starch visible	No starch visible	No starch visible	No starch visible
Water	Large amyloplasts with starch	Large amyloplasts with starch	Moderate starch content in Storeys II to VI. Storeys I and VII starch-free	No starch visible	No starch visible
Sucrose	Large amyloplasts with starch	Large amyloplasts with starch	Large amyloplasts with starch except in Storeys I and VII	Moderate starch content in Storeys II to VI. Storeys I and VII starch-free	Moderate starch content in Storeys V and VI

Each amyloplast contains two to several starch grains. Roots incubated in GA_3 + K at 35°C for 36 hours are free from starch in the plastids, but the plastids are normal in other respects (Fig. 42 B and D). When the hormonal pretreatment period is decreased, occasionally residual starch grains can be observed in the amyloplasts (Fig. 42 C). The membranes in the cells are unaffected by the GA_3 + K-treatment (Fig. 42 C and D) but when preserved by the double-fixation procedure a fixation artefact appears which results in apparently membrane-free organelles (Fig. 42 B). The cell organelles such as mitochondria, ER, and Golgi apparatus are otherwise normal in morphological detail and appear unchanged.

The starch-depletion has also been followed in detail in roots of clover. In these plants each amyloplast contains several starch grains (Fig. 42 E). The starch content decreases in the clover root cells on incubation with GA_3 + K but the extent of the starch breakdown is more dependent on the incubation temperature than was the case for cress roots. When incubated at 30°C it can be shown that the size and abundance of the starch grains decrease during the GA_3 + K-treatment but even after more than 40 hours a number of starch grains are still present. Incubation at 34°C increases the destarching process considerably; after 30 hours more than 95% of the roots are starch-depleted (Fig. 42 F). In a small number of these starch-depleted roots, amyloplasts with a few starch grains remaining can be observed.

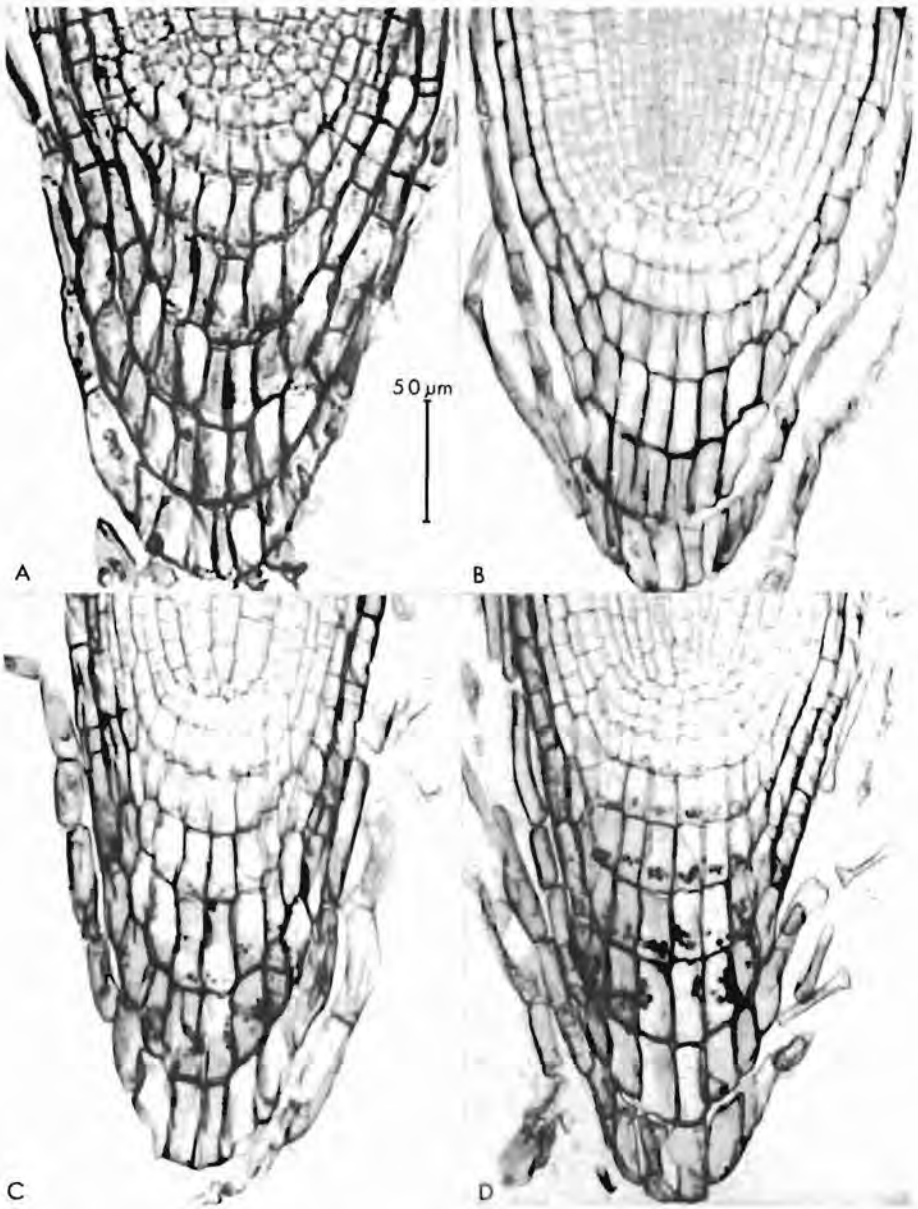


Fig. 41. *Light micrographs of root tips of cress after different pretreatments at 35°C as follows:*

A: 24 hours in GA_3 + K, B: 36 hours in GA_3 + K, C: 48 hours in water, and D: 48 hours in 7.7×10^{-4} M sucrose.

Whether these partly starch-depleted amyloplasts are movable or not has not yet been verified. In the geotropical experiments, the roots were subsequently examined under the microscope and presence or absence of residual starch in their amyloplasts taken into consideration.

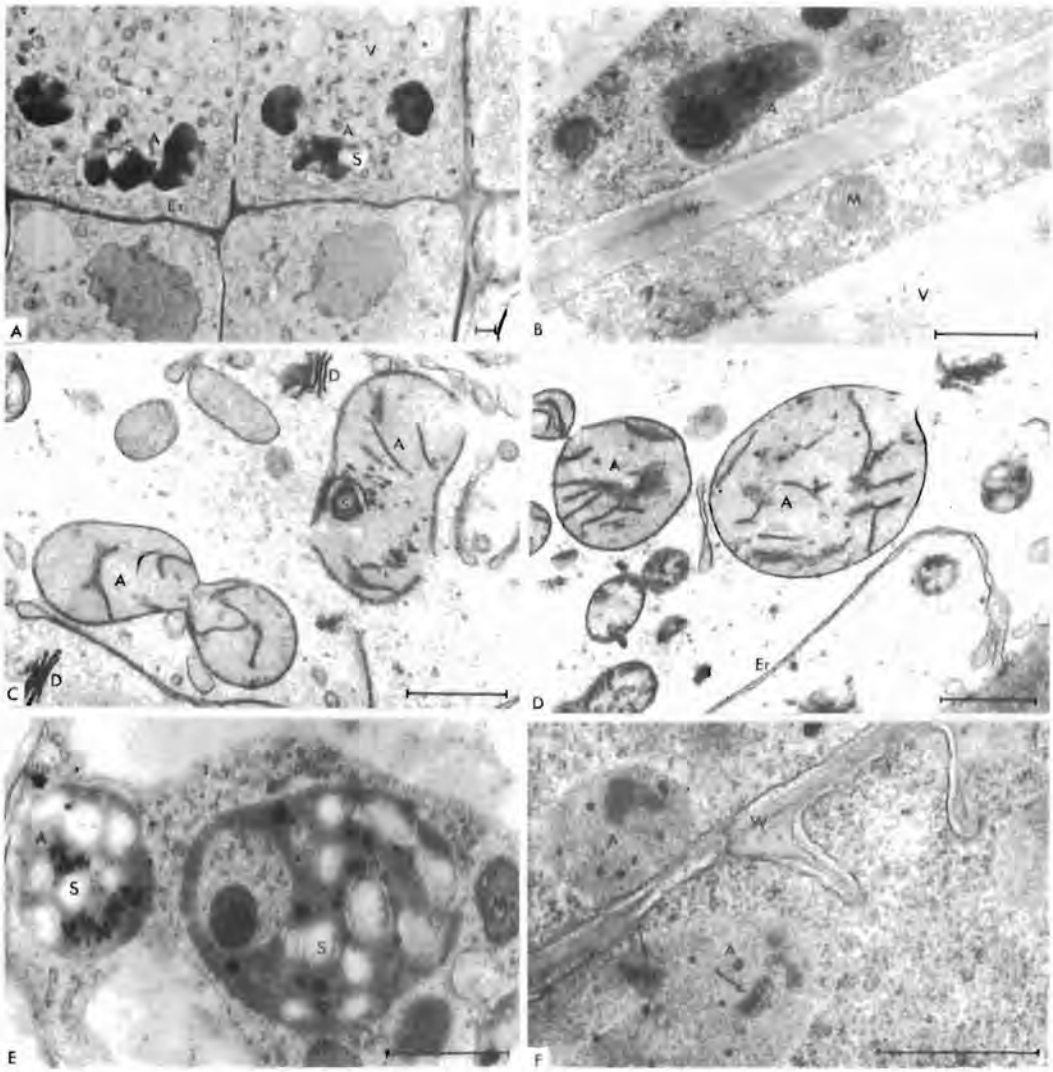


Fig. 42. *Electron micrographs of root tip cells of cress (A to D) and clover (E and F) seedlings.*

A: Untreated plant cell containing amyloplasts (A) with starch grains (S). OsO_4-fixation. B: Cell from the same region after GA_3 + K-treatment at 35°C for 36 hours. A starch-depleted amyloplast (A) which contains osmiophilic grains is indicated. Glutaraldehyde-OsO_4-fixation. C and D: Columella cells after GA_3 + K-treatment at 35°C for 28 and 36 hours, respectively. In one of the amyloplasts (A) in C a residual starch grain (S) can be observed. Permanganate fixation. E: Untreated root tip cell from white clover. Amyloplasts (A) with starch grains (S) are indicated. Glutaraldehyde-OsO_4-fixation. F: Root tip cells from white clover after GA_3 + K-treatment for 30 hours at 34°C. Note the crystalloid inclusions (arrows) in the starch-depleted amyloplasts (A).

On the micrographs are also indicated dictysomes (D), endoplasmic reticulum (ER), mitochondria (M), cell walls (W), and vacuoles (V).

Cell elongation and geotropic responses in starch-free roots

As indicated by cytological investigations the root tip cells are not visibly damaged by the hormone-treatment. To examine the elongation of the starch-depleted cress roots, groups of seedlings incubated at 35°C with GA_3 + K, water, and 7.7×10^{-4} M sucrose solution, respectively, were measured at 12-hour intervals. Fig. 43 shows the results of these experiments. At 48 hours from the start of the treatments, the growth of the GA_3 + K-treated roots has been reduced to a minimum, but the roots from the other groups are still elongating after 72 hours at 35°C. When illuminating the hormone-treated seedlings after 72 hours with a fluorescent tube (Philips 40W/55) for 24 hours starch was reformed.

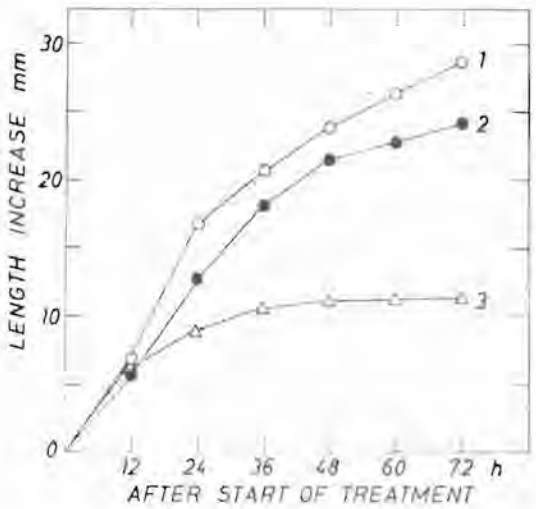


Fig. 43. *Length increase at 35°C of cress roots during incubation in one of the following media:*
(1): 7.7×10^{-4} M sucrose, (2): water, and (3): GA_3 + K. The lengths were measured at 12-hour intervals after the treatments at 35°C.

A comparison among the geotropic reactivities of the variously treated roots is meaningful only if the roots are still able to elongate. Such comparisons, therefore, were made with roots which had been treated for 29 hours at 35°C in which case there was still some elongation even in the GA_3 + K-treated cress roots at 35°C, indicating that they would elongate also at 21°C.

Table 8. *Growth and curvature of cress roots after three different pretreatments.*

The incubation temperature is indicated. After the pretreatments all the roots were stimulated and rotated at 21°C.

Pretreatment	No. of exp.	Curvature recorded after 60 min, degrees	Initial length, mm	Rate of elongation mm ± mean error *e* per hour during first 3 hours	Curvature-rate, degrees per mm elongation during first 30 min after end of stimulation
Plain water at 21°C	2	21.4	44.0	0.64 ± 0.068	34
GA_3 + K at 35°C ..	6	0	33.3	0.48 ± 0.054	0
Plain water at 35°C (control)	5	22.0	40.4	0.33 ± 0.050	65

After the pretreatments at 35°C, the GA_3 + K-treated and control cress seedlings were transferred to 21°C and kept in their normal position at that temperature for 3 hours before being stimulated in the horizontal position for 30 min. After stimulation, the seedlings were rotated parallel to the horizontal axis of the klinostat at 2 rpm and photographed every 30 min. Curvatures and total lengths of the roots were measured on the negatives. The results are given in Table 8 and Fig. 44. The starch-depleted roots (Curve 3 in Fig. 44) were unable to respond to gravity, but elongated 0.48 mm per hour during the first 3 hours after beginning of stimulation. Under the same conditions, the control roots pretreated in plain water at 21°C and 35°C elongated 0.64 mm and 0.33 mm, respectively, per hour. The curvatures measured 60 min after the beginning of stimulation (= 30 min after the beginning of klinostat rotation) were 22 and 21.4 degrees, respectively, in the 21°C and 35°C water controls. Assuming the growth rate to have been constant during the first 3 hours after the beginning of the stimulation, and using the increase in curvature during the first 30 min period after the end of the stimulation, one finds that these roots curved 34 degrees and 65 degrees, respectively, per mm of elongation.

The elongation and geotropic curvature of starch-depleted clover roots has also been studied. The results of these experiments are presented in Table 9 and Figs. 45 and 46. The table shows the increase in length and rate of curvature in degrees per millimeter extension both for red and white clover. The red clover roots which have benn pretreated in GA_3 + K at 34° for 30 hours were unable to respond to gravity when they after a 2-hour adaptation period, were stimulated

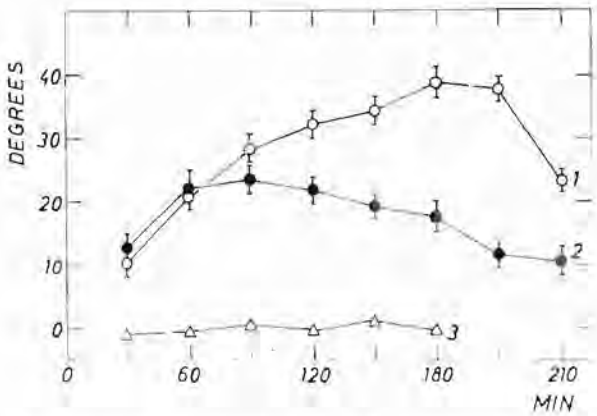


Fig. 44. *Geotropic curvatures in cress roots at 21°C as a function of time.*

Ordinate: Curvature in degrees.

Abscissa: Time from the start of 30 min stimulation. Standard deviation indicated.

Pretreatments: Curve (1): water at 35°C; Curve (2): water at 21°C; Curve (3): GA_3 + K at 35°C.

in the horizontal position at 21°C in the dark (Fig. 45). The growth of these roots was followed for 6 hours at 21°C and the results are based on measurements of 64 roots from 7 different experiments (Table 9 B). The elongation of roots in two control groups was also followed. The growth of roots which have been kept in water at 34°C for 30 hours before stimulation at 21°C, was higher than in the GA_3 + K-treated group; the increase in length during the 6-hour period was 0.73 mm and 0.50 mm, respectively. The elongation in these groups, however, was greatly reduced compared to the growth of the untreated roots (2.19 mm) which had been growing continuously in water at 21°C (Table 9 A).

The geotropic curvature followed the same pattern (Fig. 45). The GA_3 + K-treated red clover roots did not show any curvature upon geotropic stimulation. The extent of starch-depletion was examined after the geotropic experiments and occasionally when traces of starch could be observed in one or more cells, such seedlings were rejected.

Upon illumination the depleted roots regenerated the starch after 10 hours; at the same time the roots regained their geotropic responsiveness. The curvature of the control roots which had been incubated at 34°C (Fig. 45; curve 1) was significantly lower than for the roots at 21°C (Fig. 45; curve 2). Assuming the growth rates to have been constant during the 6-hour period, one finds that the red clover

water controls at 21°C and 35°C curved 35.7 degrees and 17.7 degrees, respectively, per mm of elongation (Table 9 A).

The white clover roots do not show the same high growth rate as the red clover. The water controls at 21°C elongated slower than the 35°C control roots; 0.87 mm and 1.07 mm during the first 6 hours, respectively (Table 9 B). The GA_3 + K-treatment significantly reduced the growth rate as the hormone pretreated roots elongated 0.54 mm in the same period. The geotropic curvature in the 34°C control group was also higher than in the other. The GA_3 + K-treated roots did not respond upon geotropic stimulation. The curvature per mm of elongation was slightly higher in the 34°C control group than in the group pretreated in water at 21°C.

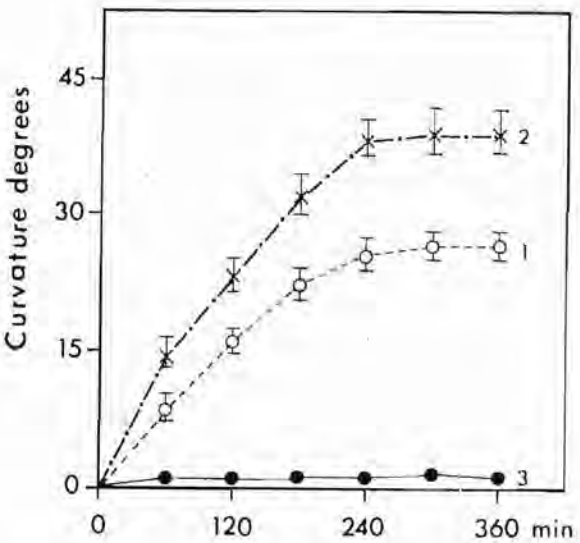


Fig. 45. *Geotropic curvatures in red clover roots at 21°C as a function of time.*

Ordinate: Curvatures in degrees.
Abscissa: Time from start of continous stimulation.
Mean errors indicated.
Pretreatments: Curve (1): water for 30 hours at 34°C; Curve (2): water at 21°C; Curve (3): GA_3 + K for 30 hours at 34°C.

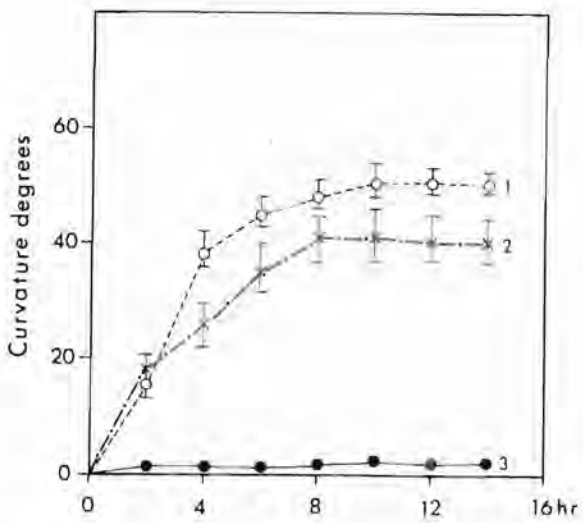


Fig. 46. *Geotropic curvatures in white clover roots at 21°C as a function of time.*

The same indications as for Fig. 45.

The extent of starch-depletion in coleoptiles

Pickard & Thimann (1966) have given a detailed description of the ultrastructure of GA_3 + K-treated and untreated wheat coleoptiles. Most of their results have been confirmed but the fact that a few discrepancies have been found made it seem necessary to study this in more detail.

In Fig. 47 electron micrographs of coleoptiles which have been pretreated in GA_3 + K are presented. When the coleoptiles were incubated in GA_3 + K for 36 hours at 30°C residual starch grains could be observed in the subepidermal parenchymal cells about one-half mm from the apex and close to a vascular bundle (Fig. 47 A and B).

Table 9. *Influence of various treatments on the growth of roots from seedlings of red (A) and white (B) clover.*

The results are based on measurements of the length of enlarged root images by means of a chartometer. The growth and stimulation period lasted 6 hours and the curvature per mm of elongation was determined at the end of this period. Mean errors (± e) are indicated.

Plant Material	Pretreatment	Increase in length (mm ± e)	Curvature per mm of elongation ($degrees \cdot mm^{-1}$)
A. Red clover	GA_3 + K	0.50 ± 0.07	-
	Water at 34°C	0.73 ± 0.15	35.7
	Water at 21°C	2.19 ± 0.19	17.7
B. White clover	GA_3 + K	0.54 ± 0.03	-
	Water at 34°C	1.07 ± 0.06	47.7
	Water at 21°C	0.87 ± 0.12	41.4

The neighbour cells, however, contained mainly starch-depleted amyloplasts of about the same shape and size as the starch-containing bodies. This observation is not in accordance with the observations reported by Pickard & Thimann (1966) who studies a closely related region after pretreatment of the coleoptiles under similar conditions. Studies in the light microscope with PAS-stained sections have confirmed the EM-observations. On 10 µm thick sections amyloplasts with starch grains can be seen after this GA_3 + K-treatment especially frequently in the

subapical portion of the coleoptile.

A more detailed study of the starch-depletion in coleoptiles has revealed that the GA_3 + K-treatment has to be done at 34°C for 36 hours to ensure that all the cells are completely starch-depleted. Even if this increase in temperature slightly influences the elongation rate of the coleoptiles (Table 10) it apparently does not change the ultrastructure of the cells. As indicated on Fig. 47 C and D, starch-depleted amyloplasts with an appearance as in untreated cells can be seen. The depleted plastids are normal in morphological detail; membranes, lamellae, osmophilic particles, and the groundplasm appears unchanged (cf. Pickard & Thimann 1966; Fig. 4).

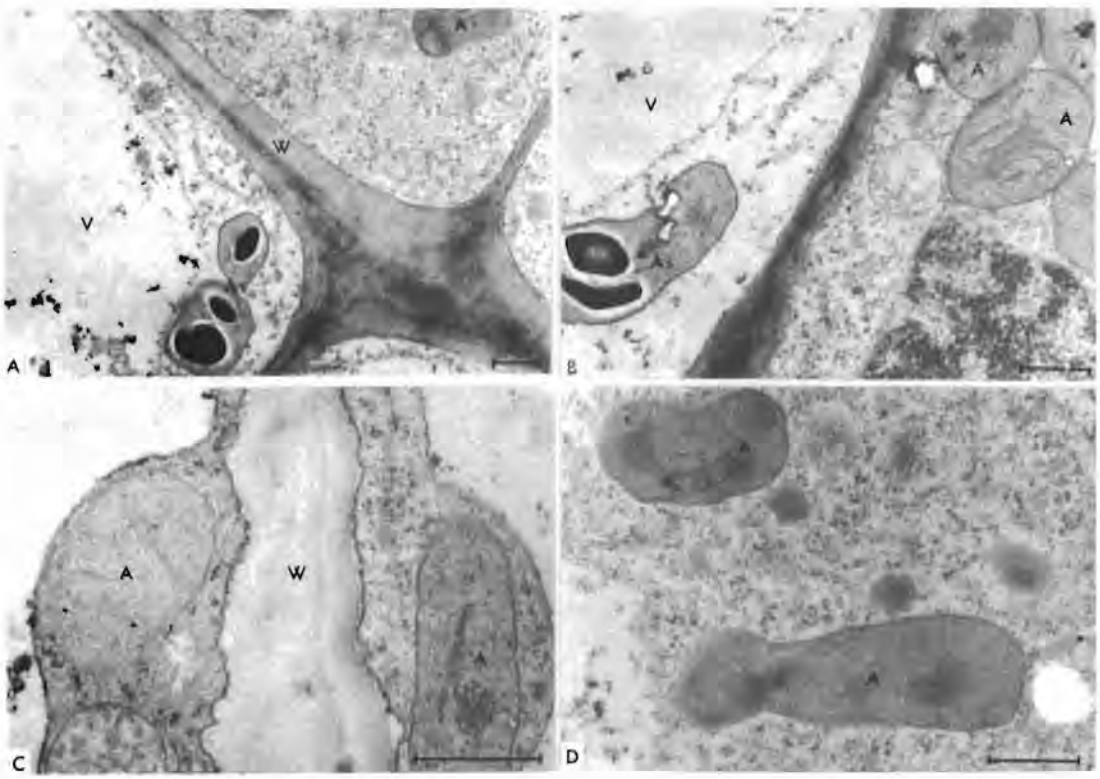


Fig. 47. *GA_3 + K-pretreated wheat coleoptiles.*
Subepidermal cells containing starch-depleted (A) and starch-containing (As) amyloplasts. A and B: Incubated in GA_3 + K for 36 hours at 30°C. C and D: Incubated in GA_3 + K for 36 hours at 34°C. No starch-containing amyloplasts are visible. Cell walls (W) and vacuoles (V) are also indicated.

Cell elongation and geotropic response of GA_3 + K-pretreated coleoptiles

In the present study the geotropic response of GA_3 + K-treated wheat coleoptiles incubated at 30°C and at 34°C for 36 hours has been compared. In the first case the experimental procedure was as close to the methods used by Pickard & Thimann (1966) as possible and therefore the results should be comparable. As demonstrated, however, in the microscopical studies, the coleoptiles still do contain residual starch in some amyloplasts after GA_3 + K-pretreatment at 30°C for 36 hours. The results from the geotropic experiments with this group of coleoptiles are presented in Fig. 48 A and Table 10 A. In the figure a comparison between the partly depleted coleoptiles and control groups has been made. Upon continuous horizontal stimulation of the GA_3 + K-treated coleoptiles a slight geotropic curvature starts after a few hours and continues until it reaches a maximum of 9.4 degrees after 24 hours. For the freshly excised coleoptiles (Fig. 48 A; curve 4) the development of the curvature follows the same pattern as for those incubated in sucrose (curve 2). The final curvature (approx. 36 degrees) is, however, significantly lower than for the control group incubated in water at 30°C (curve 1); these roots attain a final curvature of 51 degrees after 24 hours. The elongation rate is highest in the water control (0.8 mm in 24 hours) and lowest in the GA_3 + K-treated coleoptiles.

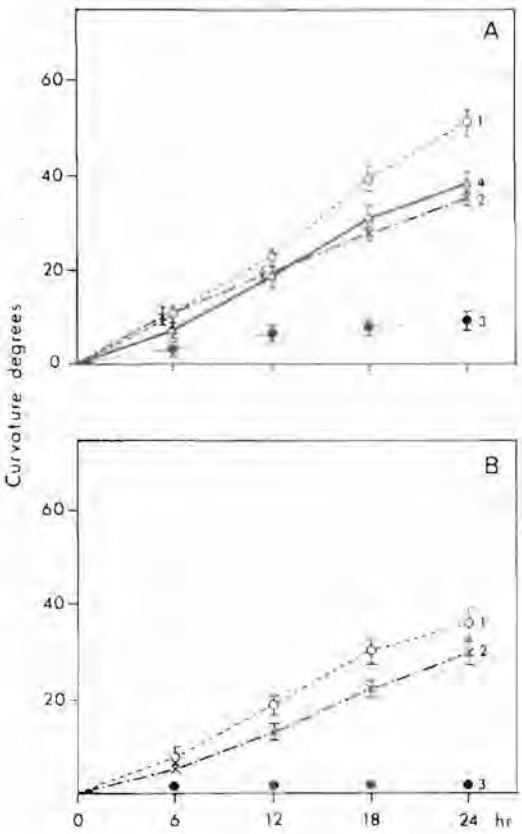


Fig. 48. *Geotropic curvature of wheat coleoptiles as a function of time.*

The numbers indicate: (1) water for 36 hours at 30°C and 34°C, (2) 0.03% sucrose for 36 hours at 30°C and 34°C, (3) GA_3 + K-treatment for 36 hours at 30°C and 34°C, and (4) freshly excised coleoptiles.

A: coleoptiles incubated for 36 hours at 30°C, B: coleoptiles incubated for 36 hours at 34°C. Each point on the curves represents at least 50 coleoptiles (except curve 4 where each point represents 30 coleoptiles) from more than 6 replicate experiments.

Mean errors are indicated.

Table 10. *Growth and curvature of wheat coleoptiles after three different pretreatments.*

The results are based on experiments performed for 36 hours at 30°C (A) and 34°C (B). The curvatures are measured after 24 hours (e = mean errors)

A.	Pretreatment		
	Sucrose	Water	GA_3 + K
Length (mm)			
Initial	3.58	3.53	4.87
Final	4.20	4.33	5.40
Increase	0.62	0.80	0.53
Visible starch grains	Plentiful	Moderate numbers	A small number
Increase in curvature (degrees ± e)	37.4 ± 3.5	51.1 ± 3.5	9.4 ± 1.2
Curvature (degrees) per mm of elongation	60.3	63.8	17.7
B.			
Length (mm)			
Initial	3.08	3.37	4.15
Final	3.53	4.01	4.64
Increase	0.45	0.64	0.49
Visible starch grains	Plentiful	Moderate numbers	None
Increase in curvature (degrees ± e)	29.1 ± 2.9	35.7 ± 3.8	1.6 ± 0.4
Curvature (degrees) per mm of elongation	64.7	55.8	3.2

The rate of curvature (in degrees per mm extension) is approximately the same in the sucrose and water control group (Table 10 A), while this rate is greatly reduced in the GA_3 + K-group of partly starch-depleted coleoptiles.

When the pretreatment temperature is increased the subsequent geotropic response of the coleoptiles in all three groups (water control, sucrose, and GA_3 + K) is decreased (Fig. 48 B and Table 10 B). After this pretreatment the GA_3 + K-group is completely starch-depleted and upon geotropic stimulation the coleoptiles only show a negligible

response; 1.6 degrees in 24 hours. The decrease in geotropic curvatures of the two control groups is likely to be due to a decrease in elongation rate as this has been reduced by 27% and 20%, respectively, in the sucrose and water control probably as a result of the increase in pretreatment temperature. The geotropic responsiveness, however, is not influenced by the temperature increase as the ratio of degrees of curvature to increment in length only shows small modifications in the two control groups (Table 10): The value for the sucrose control coleoptiles has increased from 60.3 degrees to 64.7 degrees while the corresponding values for the water control is 63.8 degrees and 55.8 degrees, respectively. The similarity between these values indicates that the change in incubation conditions does not change the pattern of response. The reduction in geotropic responsiveness in the GA_3 + K-group can therefore not be explained as a result of the increase in the pretreatment temperature but it is rather a consequence of the decrease in starch content of the coleoptiles.

D. Discussion

As emphasized again by Wilkins (1966) in studies where the experimental removal of statolith starch is related to the geotropic reactivity of plants, it is of great importance that the experiments are carried out without permanent damage to the plant material. The treated organs should be capable of cell elongation after the destarching and measurements of growth rates and tests for geotropic reactivity should preferably be carried out simultaneously on the same plant material. When working with graviperception in stems and coleoptiles it is in addition necessary to perform phototropic tests but the demand for such tests has hardly any relevance to experiments with roots.

The method employed for starch depletion in the present study is primarily based on the reports where gibberellins were found to promote amylase activity in barley endosperm (Paleg 1960, 1961; Varner 1964). On incubation with gibberellic acid and kinetin, Boothby & Wright (1962) reported that the starch content of young wheat coleoptiles was decreased. These results encouraged Pickard & Thimann (1966) to employ this hormonal treatment on coleoptiles in an attempt to study the relation between starch grains and geotropic curvature. In a similar study on cress roots, the same method was used by the

present writer (Iversen 1968, 1969) who found that apart from the fact that the amyloplast did loose the starch content the general ultrastructure of the starch-depleted cells was unchanged. Experiments on similar lines have been performed by Pilet et al. (1971) on the action of gibberellic acid on the density of amyloplasts in *Lens* roots. They found using the method of zonal centrifugation, that two groups of amyloplasts with different specific density did occur after treatment with gibberellic acid. The action of gibberellic acid on the ultrastructure of lentil roots was also reported by Nougarède & Pilet (1971) who found that the destarching effect of GA_3 was closely related to the incubation temperature. At 35°C, 95% of the lentil roots were starch-depleted after 24 hours but at 30°C the same result was first attained after 40 hours. They concluded that the suppression of starch by GA_3 was the result of an activation of synthesis of α-amylases.

The results reported by Nougarède & Pilet (1971) are in accordance with observations reported previously from studies on cress roots (Iversen 1968, 1969). In the present study these results have been summarized and a closely related pattern has also been found when depriving clover roots of amyloplast starch. In these plants the incubation temperature is more critical for the destarching process than was the case in cress roots. It has not been possible to deprive cress or clover roots of starch by treatment within a 40-hour period with GA_3 alone as reported for lentil roots (Nougarède & Pilet 1971). For cress and clover it seems as if the combination of gibberellic acid and kinetin accelerates the destarching. It should, however, be emphasized that starch-depleted cress roots can be observed after keeping the seedlings on filter paper moistened with water for more than 60 hours at 35°C.

One of the demands which makes studies on these lines relevant to the starch-statolith hypothesis, is that the starch-deprived roots and coleoptiles are still able to elongate after the hormonal treatment. This has been shown to be the case for lentil roots when GA_3 was employed at certain doses to supress the amyloplasts (Pilet 1972). It was then found that GA_3 significantly reduced the georeaction while the roots were still elongating. The same observations have been made in the present study on cress roots which have been starch-depleted after GA_3 + K-treatment at 35°C for 29 hours. When these roots were returned to 21°C and allowed 3 hours for temperature adaptation they elongated at a rate of 0.48 mm/hour. Under the same conditions control roots pretreated in plain water at 21°C and at 35°C elongated at 0.64 and

0.33 mm/hour, respectively (Table 8). No geotropic curvatures occurred in the starch-depleted cress roots whether the roots were stimulated for 30 min and then rotated on the klinostat or whether they were stimulated continuously for several hours in a horizontal position.

A similar observation was made on starch-depleted clover roots. The most obvious difference in the elongation pattern of cress and clover roots is that the growth of the GA_3 + K-treated clover roots is decreased compared to the group pretreated in plain water at the same incubation temperature (Table 9). Both the starch-depleted white and red clover roots did not respond to gravity.

The absence of geotropic responsiveness in the GA_3 + K-pretreated, but still elongating, cress and clover roots is the result to be expected if the statolith function is performed by starch grains. This conclusion is also based on the observation that the roots reformed starch when the seedlings were illuminated. With the re-formation of starch; after 20-24 hours and 10 hours for roots of cress and clover, respectively, geotropic responsiveness was restored.

Pickard & Thimann (1966) succeeded in finding conditions under which both geotropic curvature and cell elongation did occur. They observed that wheat coleoptiles made starch-free by treatment with GA_3 + K bent upward after horizontal stimulation. The geotropic response and growth of these starch-depleted coleoptiles was much slower than that of freshly excised coleoptiles, but they did respond and curved about 45 degrees over the span of 10 hours. The geotropic responsiveness (curvature per mm of elongation), was the same both in the GA_3 + K-treated and control groups.

In the present study similar experiments have been carried out with the same cultivar of *Triticum durum* Desf. as used by Pickard & Thimann. When the procedure and conditions, including the incubation temperature ($30^{\circ}C$), were as close as possible to those employed by Pickard & Thimann, a similar reaction pattern was observed. Coleoptiles pretreated in GA_3 + K, plain water and sucrose at $30^{\circ}C$ for 36 hours elongated and curved more slowly than the corresponding groups presented by Pickard & Thimann. The ratio of curvature to growth was, however, higher in the sucrose and water control in the present study, while the corresponding value was lower for the GA_3 + K-pretreated coleoptiles. These differences might be explained by the small modifications in the experimental procedure *e.g.* the coleoptiles in the present study were kept in an agar medium when geotropically stimulated.

It has been demonstrated in the present study that conditions can be found under which completely destarched coleoptiles do not respond upon geotropic stimulation. It seems certain that there still exists amyloplast starch in GA_3 + K-treated coleoptiles which have been incubated for 36 hours at 30°C (cf. Fig. 47 A and B). In Pickard & Thimann's study these cells with residual starch seems to have escaped detection. Under more favourable destarching conditions (e.g. by increasing the incubation temperature from 30°C to 35°C) these cells can be deprived of their starch. This increase in temperature has a decreasing effect on the elongation rate of the control groups but their geotropic responsiveness is on the average unimpeded. In the starch-depleted coleoptiles the temperature increase has only a slight decreasing effect on the growth. The geotropic responsiveness, however, is almost completely eliminated.

The interpretation of these results in connection with the starch statolith hypothesis will be further discussed in "General Discussion" (Chapter IX).

E. Summary

Roots of garden cress (*Lepidium sativum* L.), red clover (*Trifolium pratense* L.), and white clover (*Trifolium repens* L.) seedlings were made starch-free by treatment in the dark with gibberellic acid and kinetin for 29 hours at 35°C (cress roots) and 30 hours at 34°C (clover roots). After 3 hours of temperature adaptation at 21°C the starch-depleted cress roots were unable to respond to gravity, but elongated 0.48 mm per hour. Under the same conditions control roots pretreated in plain water at 21°C and at 35°C elongated 0.64 and 0.33 mm per hour, respectively (at 21°C). After 2 hours of temperature adaption at 21°C the starch-depleted red and white clover roots were also unable to respond to gravity; they elongated 0.50 and 0.54 mm during 6 hours, respectively. The water control roots which had been incubated at the same pretreatment temperature, had during the same period elongated 0.73 and 1.07 mm, respectively. When the hormone-treated seedlings were illuminated, their roots reformed starch after 20 to 24 hours (cress) and 10 hours (clover); simultaneously the geotropic responsiveness was restored.

The extent of starch-depletion in wheat coleoptiles (*Triticum durum* Desf. var. Henry) has also been examined. In excised coleoptiles

which have been pretreated in gibberellic acid and kinetin for 36 hours at 30°C residual amyloplast starch can occasionally be observed in the apical part of the tissue. These coleoptiles upon geotropic stimulation curved 9.4 degrees and elongated 0.53 mm during a 24-hour stimulation period; this final curvature is only 18.4% of the value in the corresponding water control group. If, however, the coleoptiles were incubated with gibberellic acid and kinetin at 34°C for 36 hours the tissue was completely deprived of amyloplast starch. These coleoptiles only showed an insignificant curvature (1.6 degrees) during 24 hours horizontal stimulation period; they were, however, still able to elongate 0.49 mm during the same period. These results are discussed in relation to other recent reports concerning the starch statolith hypothesis.

Chapter VII

AUXIN TRANSPORT IN RELATION TO GEOTROPISM IN ROOTS

A. Introduction

It has been the aim of the work presented in this chapter, to demonstrate the orientation of auxin movements in roots, to follow the enzymatic and non-biological conversions of auxins, and to a certain extent study the role of auxin in geotropism in roots.

The transport of auxin in root and shoot segments of plants has been the subject of a large number of investigations. Much is known about the movement of auxins in shoots, but considerably less is known about the movement in the root (Kirk & Jacobs 1968, Wilkins & Scott 1968, Scott & Wilkins 1968, Morris et al. 1969) and the available information, even that concerning the most fundamental questions, is contradictory. For example, the values reported for the velocity of auxin transport in root segments vary from zero to about 20% of the reported shoot values. Also in question is whether there exists an active polar transport, and if so, whether the net flux is toward the apex of the root (acropetal transport) or toward its base (basipetal transport). For reviews see Kirk & Jacobs (1968), Scott & Wilkins (1968), and Scott (1972).

The classical type of polar transport experiment, using segments of coleoptiles, stems, petioles, and roots is artificial in several respects. In such experiments the auxin is generally supplied to the segments from donor agar blocks, and the transported auxin is trapped in receiver blocks. In most cases, when studying the movement of auxin in root sections using this method, ^{14}C-carboxyl-labelled indole-3-acetic acid (IAA) has been used. The transport is followed by measurements of radioactivity in the segments and/or the receiver blocks. There is, however, very little known about what happens to a labile auxin like IAA in the agar blocks when these are in contact with possible auxin-destroying agents at the cut surfaces of the root. Other events on the cut surface may also distort the transport. Auxin applied across the cut surface may be absorbed into tissues which, in the intact plant, would be out of the main paths of transport (McCready 1966). Also nothing is known about the form in which auxin is transported in the segments. Pilet (1964) working with lentil roots, has taken the problem with the possible sources of errors on the cut

surface into account in his experiments. More recently Raa (1971) has demonstrated considerable enzymatic decarboxylation of IAA on the cut surfaces of cabbage.

The technique with segments and agar blocks has also been used for demonstration of polar movement of auxin in shoot segments. The problems concerning the fate of auxin are the same in shoot as in root segments. Working with *Avena* coleoptiles, Goldsmith & Thimann (1962) stressed destruction and immobilization of IAA as possible sources of error in experiments with excised segments.

Despite the problem with segment transport experiments, relatively little attention has been directed towards the auxin transport mechanism in intact plants. It is not known to what extent results from the translocation experiments with segments are applicable to the movement and metabolism of auxin in whole plants. However, one would expect experiments with intact plants to provide data more directly related to natural conditions.

Only a few studies of the transport and metabolism have been conducted with intact plants. Eschrich (1968) detected labelled material in various parts of autoradiographs of *Vicia faba* L. plants when IAA-2-^{14}C had been fed to fully expanded leaves, indicating both upward and downward movement. He concluded that the applied IAA moved in the sieve tubes but two other water soluble, labelled derivates of IAA were formed in parenchyma cells and were phloem-immobile. After injection into cotyledons of *Phaseolus coccineus* L., Whitehouse & Zalik (1967) observed acropetal translocation of IAA-1-^{14}C in shoots and coleoptiles.

Morris et al. (1969) working with light-grown dwarf pea seedlings, found that only part of the IAA-1-^{14}C and IAA-2-^{14}C applied to the apices of the seedlings was transported unchanged to the root system. Several minor derivates of IAA and large quantities of a compound tentatively identified as indole-3-aldehyde were readily transported to the roots and could be extracted from these. In the roots, they also detected indole-3-acetylaspartic acid as an immobile conversion product of the applied IAA.

The effect of auxin on the geotropical curvature in the roots has been examined in quite a few studies in the past decades. Geiger-Huber & Huber (1945) observed that if decapitated roots of *Zea mays* L., which normally did not show geotropical reactivity, were uniformly supplied with auxin they would perform positive geotropic reactions. This and similar results (e.g. Pilet 1953) have been interpreted as indicating

that auxin is necessary for the geotropic response. In roots where the endogenous auxin concentration is thought to be supra-optimal one would expect externally supplied auxin to reduce the rate of curvature if supplied in concentrations which retard the elongation of roots. In accordance with this, Audus & Brownbridge (1957), working with roots of *Pisum sativum* L. seedlings found suppression of both growth and curvature. When the roots were uniformly supplied with high concentrations of indoleacetic acid (10^{-8} M) the reaction time was lengthened and the rate of curvature reduced by 50%.

In the present chapter results are presented from a systematic investigation of the role the biological and non-biological factors play in auxin movement in excised root segments. The destruction effects on IAA in transport experiments have been examined by chromatography and by the use of auxin labelled in different positions i.e. IAA-1-^{14}C and IAA-5-^{3}H. This combination of two labelled auxins (instead of one) was chosen in the hope that clearer information on the destruction effect could be obtained. When the IAA-1-^{14}C molecule is decarboxylated, radioactivity will not be found in the spots on the chromatogram which show colour reactions with p-dimethyl-aminocinnamaldehyde (DMCA). However, with IAA-5-^{3}H some of the spots showing colours due to the indole ring will also be radioactive. In this chapter results are given from a study where the influence of externally supplied, labelled IAA and its metabolic derivatives on geotropical response has been examined.

B. Materials and Methods

Plant material and cultivation

Seeds of sunflower (*Helianthus annuus* L. var *californicus*), cabbage (*Brassica oleracea* L. cv. Ditmarsker), and dwarf wax bean (*Phaseolus vulgaris* L. cv. "Gullhorn") were sterilized in a 3% calcium hypochlorite solution for 30, 15, and 15 min, respectively. The sunflower and cabbage seeds were then germinated on moist filter paper in petri dishes (diam. 9 cm), and grown in the normal, vertical position in the dark at 25°C for 48 h. Seedlings were then selected for uniformity and placed between two slices, 50 x 75 x 1 mm, of 1.4% agar without nutrients. The slices were placed on a glass plate 78 x 115 x 1 mm, and protected by a plastic cover kept in place by two rubber bands.

After another 24 h the roots were harvested and selected for experimental treatment. The sterilized seeds of dwarf wax bean were rinsed with sterile water and imbibed for 24 h in the water. Then the seeds were placed on germinating blocks (Boysen Jensen 1939, p. 313) in boxes with high humidity in the air for another 24 h. Seedlings having straight roots about 10 to 20 mm long were selected and placed, 5 parallel to one another, with their roots between two agar slices, 80 x 65 x 1 mm, made up of 2% agar without nutrients. The slices were placed on a glass plate protected by a plastic cover. The plants grew in the normal, vertical position for 16 h before injection of labelled auxin.

Indole-3-acetic acid

The auxins were obtained as the 3-indolyl-acetic acid-1-^{14}C ammonium salt from the Radiochemical Centre, Amersham, Bucks., England and 3-indolyl-acetic acid-5-^{3}H from Schwarz Bioresearch, Inc., Orangeburg, N.Y. The specific activites were 57 mCi $mmol^{-1}$ and 16 or 17 Ci $mmol^{-1}$, respectively. Stock solutions were kept at -20°C, and fresh samples were prepared by thawing and dilution from the stock solutions. After a few days chormatographic analysis of the ^{3}H- fresh solution revealed one minor spot with R_f value approximately 0.90, besides the major spot of IAA. Because of this destruction the IAA-^{3}H-stock solution was chromatographed on paper several times during the experiments and the purified IAA redissolved in water. The IAA-^{14}C samples were counted before and after the experiments.

Measurement of radioactivity

Radioactivity was determined in a Packard Liquid Scintillation Counter (Model 3320) using a scintillation fluid containing 4 g PPO in 1 l toluene. The ^{3}H- counts recorded were corrected both for background and for quenching using the method of automatic external standardization in association with an AES ratio/efficiency curve.

Decarboxylation of IAA

In order to determine the rate of decarboxylation of IAA due to biological activity or by non-biological means, the following technique was employed (Raa 1971). The test material was placed in a scintillation vial containing carboxyl-labelled IAA. The vial was stoppered with a cork stopper. Fastened to the stopper and protruding into the vessel was a pin with a circular piece of glass fibre paper (diam. 8 mm) at the end (Fig. 49 A). The paper was moistened with 5% sodium hydroxide to trap gaseous $^{14}CO_2$. At the end of the incubation period, usually 3 or 4 h, the paper was dried in air and transferred to a vial containing 5 ml of the scintillation fluid.

Transport experiments with root segments

The movement of radioactive auxin was followed in 5 mm segments cut 5 mm from the root apex (Fig. 49 A, D), unless otherwise stated. Cutting and handling of the segments was done under weak green "safelight" as previously described (Chapter III, p. 51). All experiments were run at 25°C in the dark with approximately 95% humidity in the darkroom. To avoid contamination of heavy metals from razor blades cover glasses were used for cutting agar blocks, and the segments were cut with glass knives (5 mm between the edges; Fig. 49 B) made on a LKB Knife Maker. Four strips of agar (Agar No 3 from Oxoid Limited, London S.E.1, England; 1.4% in glass-distilled water) 35 x 3 x 1 mm were placed on a glass plate and protected by a plastic cover. Five root segments were then transferred to each agar strip and covered with another strip of agar. The glass plate containing the 20 segments with their proximal ends up was kept in the vertical position. The labelled auxin was supplied to the segments in individual donor blocks of 1.4% agar (2 x 2 x 2 mm) and collected in receiving blocks of plain agar (same size; Fig. 49 C, D). The (labelled) auxin was added to the agar in aqueous solution just prior to gelation, with a final range of concentrations from 0.01 to 10 µM.

At the end of the transport period the receiver blocks were removed from the segments and both segments and blocks either placed on a glass-fibre filter and dried in air, or collected in one of the following solutions: 5% potassium hydroxide, 5% sodium hydroxide, 2% barium hydroxide, or 5% barium hydroxide. After 6 h the solutions

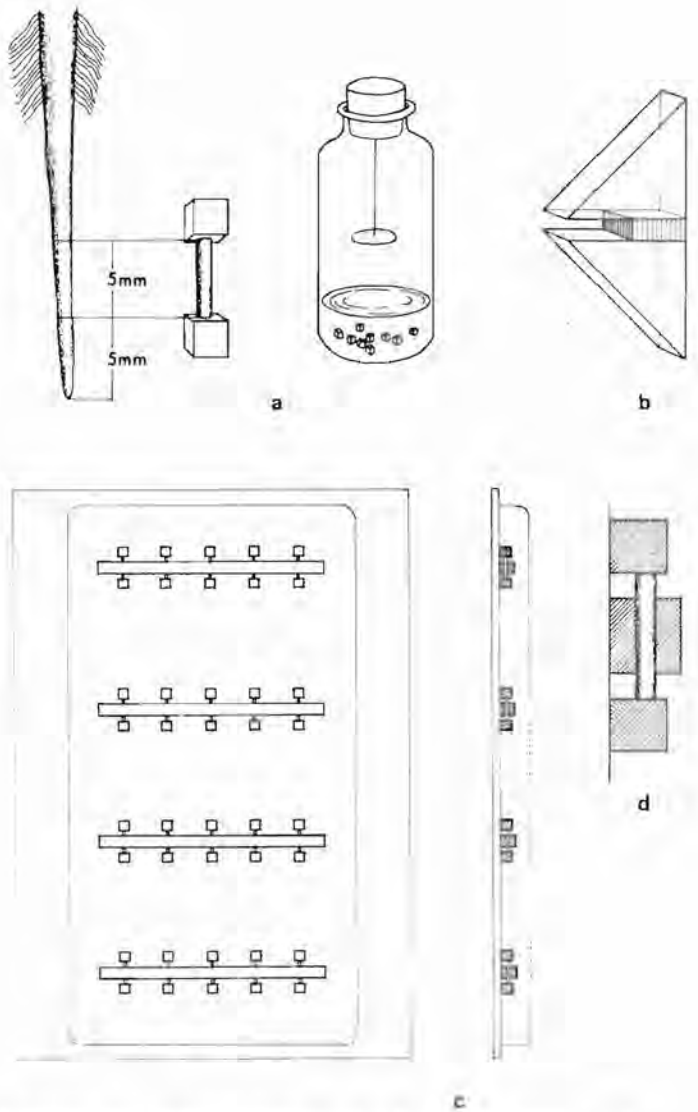


Fig. 49. *Transport experiments with root segments.*

A. The segments used were cut 5 mm from the root apex. The CO_2-collecting technique within a glass vial is also demonstrated. The small cubes at the bottom of the vial are either agar blocks or other test material.

B. The glass knives for cutting root segments.

C. The segments were placed in rows on a glass plate and protected by a plastic cover (seen in front view and from the side).

D. Close up view of the root segment between to agar plates (hatched) and in contact with agar receiver and donor blocks at the ends.

were evaporated to dryness and PPO was added before counting. In the case of $Ba(OH)_2$, which was found to be the most suitable trapping agent, the solution was filtered through a glass-fibre filter, the filtrate was

dried and the radioactivity of both filtrate and residue on the filter was measured. Any radioactive CO_2 produced by decarboxylation of IAA would be trapped as $BaCO_3$. In experiments with IAA-^{3}H the segments were cut transversely to yield two halves; these were counted separately after their fresh weight had been determined.

The contents of radioactive materials and of auxins in the receiver blocks and the segments were also analysed by thin layer chromatography. The blocks and the segments (20) were placed in methanol in a refrigerator, at +2 to 4°C. The extract was then reduced to a volume of approximately 0.1 ml by evaporation under low pressure. This volume was applied to the starting line of the chromatogram.

Transport experiment with intact plants

The movement of radioactive auxin was followed in the roots of intact bean seedlings. By means of a calibrated microsyringe the auxin was injected into the region root/hypocotyl in 10 µl aliquots from a 10^{-6} M IAA-5-^{3}H solution. Distilled water was injected into the control plants. After the injection the plants were returned to the vertical position for the required translocation period, after which the plants were either stimulated geotropically or had their roots cut into segments, which were placed in methanol and extracted at +2 to 4°C for 18 h. The extracts were either dried in warm air before scintillation counting or reduced to a volume of approximately 0.1 ml by evaporation under low pressure. This volume was applied to the starting line of the thin-layer chromatography plate.

Chromatographic analysis

The labelled IAA-stock solutions and the unlabelled IAA were analysed by paper chromatography (Whatman No 1). The solvent used was isopropanol : ammonia : water (8 : 1 : 1). In the chromatography of plant tissue and agar extracts thin-layer chromatography was used. The plate was coated with cellulose MN-Polygram Cel 300 (Macherey - Nagel & Co., Düren, Germany). Several solvent systems were used including isopropanol : ammonia : water (8 : 1 : 1 v/v), n-hexane, chloroform : acetic acid (95 : 1), n-butanol : glacial acetic acid : water

(5 : 1 : 2.2 v/v, a single-phase system), 70% ethanol, and quartz-distilled water. The developed thin-layer chromatograms were cut into 6, 10, or 20 sections, and the different zones were tested in the liquid scintillation counter. Comparisons were made with several authentic, unlabelled indole compounds which were added to the methanol extracts of unlabelled plant roots and chromatographed in parallel with the samples under investigation. Where possible, the auxin spots were made visible by spraying with a 1% solution of p-dimethyl-amino-cinnamaldehyde (DMCA) in HCl-ethanol. Fluorescence under UV was also used to determine the position of the compounds.

Geotropical experiments with plants treated with isotope-labelled auxins

After the translocation period (5 or 20 h) the dwarf wax bean plants were stimulated for 5 min in the horizontal position and then rotated parallel to the horizontal axis of the klinostat previously described (see Chapter III). Before start of stimulation a certain number of roots exhibited small curvatures. If these initial curvatures exceeded $\pm 5^{o}$ the respective roots were disregarded. Angles of curvatures were measured in the normal manner (see Chapter III) on images of the roots projected from the negatives onto a scale of degrees by means of a specially constructed Leitz Model SM microscope.

Root elongation was determined by projecting the negatives onto a wall and measuring the length of the enlarged root images by means of a chartometer.

Handling of the seedlings was done under weak, green "safe-light."

C. Results

Non-biological decarboxylation of IAA-1-^{14}C

Preliminary experiments had shown that non-biological destructions of auxin influenced the results of auxin transport studies. When IAA-1-^{14}C was dissolved in distilled water and a glass-fibre disc with a trapping agent for CO_2 was supplied, the amount of $^{14}CO_2$ recovered from the gas phase was much higher than the average counts determined when

IAA-1-^{14}C was dissolved in glass-distilled water. In phosphate-buffered IAA-solutions (pH 5.8) of different concentrations the relative rate of IAA decarboxylation was low when the concentration was 0.1 M phosphate (100 dpm/hour), but increased to 410 dpm/hour with 0.5 M phosphate. The decarboxylation effect is undoubtedly caused by impurities, in all probability mainly heavy metals, in the solutions. On the basis of these results glass-distilled water was preferred for routine experiments.

Enzymatic decarboxylation of IAA-1-^{14}C

Segments cut from the same zone of roots of sunflower and cabbage exhibited a striking difference. The tissue of both cut surfaces of sunflower roots turned brownish immediately upon cutting; in the cabbage segments no visible colour change was apparent. The colour change in sunflower segments may be explained by activation of polyphenol oxidases in the damaged cells. The activation effect on other oxidases (possibly IAA oxidases) is shown in Fig. 50 A. When segments from sunflower roots were placed in IAA-1-^{14}C solutions either undivided or cut into four equal parts, the biological destruction of auxin increased significantly. The same increase in enzymatic destruction of IAA after subdivision of root segments was observed in cabbage segments. The destruction capacity in this material was 3-4 times higher than in sunflower root segments.

In some experiments plain agar blocks were placed at the basal and apical ends of the root segments, and after various periods of time assayed for biological activity (Fig. 50 B). In the first 6 hours the destruction capacity was low; afterwards, it increased considerably. The enzymes have obviously diffused from the cut surfaces into the agar blocks. The highest enzymatic decarboxylation activity in sunflower roots was near the apical end, 5-10 mm behind the tip. Segments approximately 20 mm from the root tip exhibited lower decarboxylation activity, and segments 30 mm from the tip the lowest ones. In cabbage roots destruction of IAA was largest in the basal root segments 25 mm from the tip; it was less in the segments used for the transport experiments.

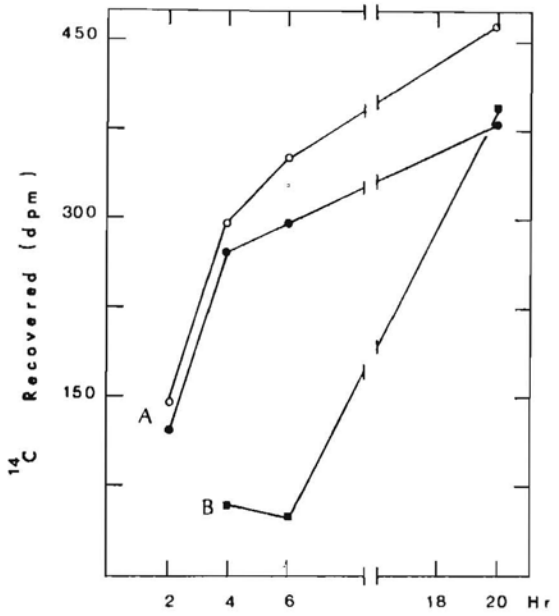


Fig. 50. *Enzymatic decarboxylation of IAA-1-^{14}C.*

A. Enzymatic destruction of IAA at the cut surfaces of sunflower as a function of time. The segments were either kept intact (●) or divided into four equal parts (o) before being placed in the IAA-^{14}C solution. Abscissa: Destruction period in hours.

B. Enzymatic decarboxylation of IAA-^{14}C after the diffusion of enzymes from the cut surface (■) into agar blocks. The blocks were placed in IAA-^{14}C solution for 3 hours and $^{14}CO_2$ recovered on filter paper with NaOH. Abscissa: Diffusion period in hours. All values corrected for non-biological activity.

Transport of auxin through root segments of *Helianthus* and *Brassica*

Donor blocks containing 10^{-6} M IAA-1-^{14}C were applied to the lower surface of root segments of sunflower and cabbage cut 5 mm behind the root tip. After transport periods of 6 to 20 hours in the vertical position the radioactivity in the receiver blocks was determined after drying the latter in air on glass-fibre filters. The results from sunflower are shown in Fig. 51 (columns A and B). During the first 6 hours 19 and 9 dpm of labelled auxin moved into the apical and basal receiver blocks. When the transport period was extended to 20 hours the recovered values were 25 and 19 dpm, respectively. A marked increase in the collected ^{14}C in the receiver blocks is evident when the receiver agar blocks are placed in 2 ml barium hydroxide and the released $^{14}CO_2$ trapped as barium carbonate (Fig. 51, columns C and D). The ratios of