Elongation and Contraction of the plant axis and Development of spongy tissues in the Radish Tuber (*Raphanus sativus* L. cv. Saxa Nova)

J. F. C. Magendans
*Department of Plant Cytology and Morphology,*
*Agricultural University,
Arboretumlaan 4, 6703 BD Wageningen, The Netherlands*
Magendans, J. F. C.

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Abstract

The development of the tuber of *Raphanus sativus* L. cv. Saxa Nova is rather indefinite; morphologically this tuber is interpreted as a mass of tissue that is continuously remodelled. Strong elongation and contraction of upper half, lower half of the hypocotyl and the upper part of the taproot occur independently of each other and at different times of development. Elongation and contraction of these parts also vary enormously among individual plants. This behaviour is evident in clay soil and in nutrient solution. When growing in clay soil, the lower half of the hypocotyl shows very strong elongation, while in nutrient solution, the upper half elongates the most. The radish tuber originates due to the activity of the vascular cambium in radial, tangential and in longitudinal direction (cambial secondary growth), but also due to the expanding and dividing of the xylem parenchyma cells (diffuse secondary growth). In healthy tubers all these meristematic activities occur completely in harmony with each other. As a result of elongation of part of the tuber axis the living elements in the secondary xylem expand and divide in longitudinal direction, including the phloem groups. However, the walls of xylem vessels are torn in different ways, at first in the central, older part of the tuber. Spongy tissue originates in the largest apotracheal parenchyma cells beyond the range of influence of the nearby strands of vascular tissue with vasicentric parenchyma cells around them and in a circular zone around the centre of the tuber. Sponginess is characterized by dead cells in which gas emboli are formed. This cellular phase of sponginess is followed by the lacunar phase because the dying spongy tissue will be torn by further elongation of the tuber. Sponginess occurs only after 7-8 days after the beginning of the process of elongation of the tuber. Measurements of transpiration and absorption rates of tubers with spongy tissues show that absorption takes place in proportion to the number of groups of vessels that is still functional, i.e. is situated outside of the area of spongy tissues. Consequently sponginess is caused by breaking of the vessels in the tuber by elongation. Tubers cultivated in nutrient solution show spongy tissues about 10 days later than after cultivation in clay soil, probably because of differentiation of new cambia and initiation of new tertiary vessels.
1. Introduction

Golinska (1928) found much diversity between radish cultivars in the relative contributions of hypocotyl and root to thickening which leads to tuber formation. Ting (1978) and Ting and Wren (1980) also found the same type of diversity when comparing the radish cultivars Cherry Belle and Long White Icicle, and have presented a model of the tuber development for each. The models incorporate not only differences in contribution of hypocotyl and taproot in the tuber development, but also differences in the extension of the plant axis during thickening of hypocotyl and taproot. Fujimura (1957) and Takano (1966a) studied the anatomical development of spongy tissues and observed the development of schizogenous intercellular spaces with emboli. These intercellular spaces become larger and this process is accompanied by the dissolution of the pectic substances in the middle lamella. The first intercellular spaces originate between the largest parenchyma cells between the strands of vascular elements.

The cause of the development of spongy tissues in radish tubers is mostly attributed to environmental conditions. Most attention is given to fertilizers. Hagiya (1957a, b) concluded that the growth of radish plants cultivars Rapid Red and Osakashijunichi was more vigorous after fertilization and that the produced abrupt growth of the tubers goes together with the occurrence of spongy tissues in the tubers. The vigorous growth leads also to low concentration of soluble matter in the tuber and largeness of xylem parenchyma cells. Park and Fritz (1983) found that when the nutrient supply is kept in the optimum zone, an extra application of N, P or K does not statistically increase the sponginess in radish tubers cv. Rex. Hey and Kobryń (1988) studied the development of sponginess in radish tubers var. radicula Pers., cv. Novired at different salt concentrations in the nutrient solution. It appeared that increased electrical conductivities of the nutrient solution and a low day-time temperature reduced the incidence of sponginess, but no data are given of the growth rate of the tubers, except that the tubers were 6% smaller in diameter at a day temperature of 7°C as opposed to those in the 12°C treatment. Studies of Takano (1966c) and Kano (1987) about the effects of the soil conditioner, polyvinyl alcohol, and of a cooling treatment of the soil respectively, also indicated that better circumstances for tuber growth stimulated the occurrence of sponginess. Takano (1966c) concluded that the occurrence of sponginess might be ascribed to the fact that the accumulation of assimilates or nutritives into the fresh tuber lags behind the abrupt thickening growth.

An accurate study of the structural relation between a vigorous growth of the radish tuber and the developmental process of sponginess is necessary.
that purpose a detailed analysis is given of the elongation and contraction by
the hypocotyl and upper part of the taproot during tuber development in the
radish cultivar Saxa Nova. Special attention is given to the diversity of these
phenomena in individual plants. This analysis is made in close connection with
the study of the anatomical development of spongy tissue and with some cytolo-
gical and physiological data of tubers developing sponginess. The investigations
have been made in tubers grown in clay soil mixture and in nutrient solution.
2. Materials and methods

2.1. Plant material and culture conditions

Seeds of the radish cultivar Saxa Nova, with a diameter of 2.75-3.00 mm, were obtained from Pannevis, Enkhuizen, The Netherlands.

One group of observations was made on 20 seedlings sown in a stainless steel box (Fig. 1). This box was placed in a greenhouse from 19th May till 23rd June, 1987 under natural lighting with a daily range of temperature and humidity of respectively 22 ± 4°C and 49 ± 26% r.h. The soil consisted of a mixture of clay and sand (indicated as clay soil for short) covered with a 4 cm layer of river sand. The hinged, long sides of the box could be folded down (Fig. 1A), the soil being kept in place by nylon gauze supported by stainless steel bars, 5.5 cm apart. The gauze could then be temporarily pulled away, allowing the tubers situated between the supporting bars to be brushed clean of sand with a paint brush and the seedling axis be marked by applying Edding 3000 waterproof drawing ink. Marks were placed in the middle of the hypocotyl, at the root/hypocotyl junction (collum radicis, cp. Fig. 10) and 5 mm below this position. Measurements of the developing seedling were taken with a ruler and sliding calipers according to Fig. 2. Every three days the measurements were taken and the marks renewed.

Figure 1. Culture of radish plants in a stainless steel box (A, transverse section). After one of the sideplates is turned forward (B) the sand and a mixture of clay and sand is kept in position by the nylon gauze that in its turn is fixed by stainless steel bars.

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Another group of observations was made on 50 seedlings cultivated on river sand fertilized with a Hoagland nutrient solution. After 8 days, these seedlings were transplanted to hydroculture in a stainless steel reservoir containing 140 l aerated Hoagland nutrient solution. This reservoir was placed in a greenhouse at the Centre for Agrobiological Research from 27th April to 9th June 1987 under natural light conditions and with a daily range of temperature and humidity of respectively 15 to 28°C and 70 ± 30% r.h. Determinations of transpiration and absorption were made with other ‘Saxa Nova’ plants cultivated in the greenhouse.

2.2. Physiological methods

In plasmolysis studies, sections of fresh radish tubers with spongy tissues were allowed to come to osmotic equilibrium in 8 bar and 12 bar mannitol solutions. Observations were made with a Nikon Nomarski microscope. Transpiration and absorption were measured after placing ‘Saxa Nova’ plants of about the same age and thickness of tuber in a growth cabinet (Weiss, W. Germany). The roots were rinsed with water and the thick taproot of the plants was cut under water, the tuber quickly dried again and put into a small beaker with a 4% eosin solution (Gaff, Chambers and Markus, 1964). Measurements were carried out with the potometer method (Kramer, 1959), weighing the plant and the absorbed solution of eosin before and after the test period. After different periods of time the tubers were sectioned transversely and the functioning strands of vascular...
tissue in the tubers determined microscopically by the red colour of the vessels. The light intensity in the culture chamber was about 35,000 lux at plant level, the temperature was controlled at 24°C and the air velocity was 0.4-0.5 m/sec. Determination of the osmotic value of the cell sap obtained by crushing of different parts of the tuber tissue in a garlic press, was done with a Knauer semi-micro osmometer type M.

2.3. Histological techniques and microscopy

Young tubers and parts of older tubers were fixed in a Nawaschin fixative (Craff I and II, Berlyn and Miksche, 1976) after all air in the tissue was removed in an exsiccator. The choice of the fixative was based on its high content of water and the results of preliminary experiments which showed no shrinkage of the treated tissues. After fixation, the material was passed through an alcohol series, stopping at 80% (v/v) for storage. Part of this material was dehydrated with the TBA method and embedded in paraplast (Lancer, Sherwood) paraffin wax. Sections of 7 µm were made with a Leitz rotary microtome and stained with safranin and fast green. Pectic substances were localized with the highly specific hydroxylamine-ferric chloride reaction (Jensen, 1962; Takano, 1966a) carried out on hand-prepared sections of the fixed material. Viability tests on cells in hand-made sections of fresh tubers with spongy tissue were performed with a combination of fluorescein diacetate and lissamine green (Van Lammeren, 1988) and sustained by observing cytoplasmic streaming, plasmolysis and the difference between smooth cell walls of dead cells and cell walls with a fine granular cytoplasm against them, of living cells. The hand-made sections were studied in a solution of 6 g sorbitol in 100 ml distilled water. Observation was done with a Nikon Labophot fluorescence microscope with mercury lamp, 365 nm excitation filter and 420 nm barrier filter, and with Nikon Nomarski optics. Other fixed and fresh material was sectioned by hand and observed by a Wild zoom stereomicroscope M7S with incident illumination generated by a Volpi Intralux 150H fibre ring illumination, creating a black background by means of water between the glass slide and a solid black plate as support for the slide. 'Camera lucida' equipment was used in determining cell sizes. Clearing of tissue was done with NaOH and chloral hydrate (Berlyn and Miksche, 1976).

2.4. Statistical methods

Statistical analysis and many tests of significance were by Wilcoxon's test. The rank correlation coefficient test (Spearman's test) was also applied.
3. List of abbreviations

<table>
<thead>
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<th>Symbol</th>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>ap</td>
<td>apotracheal xylem parenchyma</td>
</tr>
<tr>
<td>c</td>
<td>cotyledon(s)</td>
</tr>
<tr>
<td>ca</td>
<td>cambium</td>
</tr>
<tr>
<td>co</td>
<td>flap-like segment of (partially) split off cortical tissue</td>
</tr>
<tr>
<td>cp</td>
<td>cellular phase of sponginess</td>
</tr>
<tr>
<td>cr</td>
<td>collum radicis</td>
</tr>
<tr>
<td>d</td>
<td>day(s)</td>
</tr>
<tr>
<td>DAS</td>
<td>day(s) after sowing</td>
</tr>
<tr>
<td>f</td>
<td>fissure in stele of hypocotyl and/or root</td>
</tr>
<tr>
<td>h</td>
<td>halo of living, often radially extended parenchyma cells around sve</td>
</tr>
<tr>
<td>H</td>
<td>length of hypocotyl</td>
</tr>
<tr>
<td>H₁, H₂</td>
<td>resp. upper and lower half of H at 8 d</td>
</tr>
<tr>
<td>ip</td>
<td>interxylary phloem</td>
</tr>
<tr>
<td>L</td>
<td>H + R</td>
</tr>
<tr>
<td>l</td>
<td>libriform fibres</td>
</tr>
<tr>
<td>LM</td>
<td>light micrograph(s)</td>
</tr>
<tr>
<td>LMNo</td>
<td>light micrograph(s) with Nomarski optics</td>
</tr>
<tr>
<td>lp</td>
<td>lacunar phase of sponginess</td>
</tr>
<tr>
<td>mx</td>
<td>metaxylem</td>
</tr>
<tr>
<td>pe</td>
<td>periderm</td>
</tr>
<tr>
<td>ph</td>
<td>phloem</td>
</tr>
<tr>
<td>px</td>
<td>protoxylem</td>
</tr>
<tr>
<td>r</td>
<td>mark of 5 mm below cr at 8 d</td>
</tr>
<tr>
<td>R</td>
<td>upper 5 mm of root at 8 d</td>
</tr>
<tr>
<td>s</td>
<td>sieve plate</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopic view(s)</td>
</tr>
<tr>
<td>se</td>
<td>sieve element(s)</td>
</tr>
<tr>
<td>sp</td>
<td>secondary phloem</td>
</tr>
<tr>
<td>StM</td>
<td>stereomicroscopic view(s)</td>
</tr>
<tr>
<td>sve</td>
<td>strand of vascular elements</td>
</tr>
<tr>
<td>sve'</td>
<td>idem lg sectioned</td>
</tr>
<tr>
<td>sx</td>
<td>secondary xylem</td>
</tr>
<tr>
<td>T</td>
<td>thickness of H at cotyledonary node</td>
</tr>
<tr>
<td>T_c</td>
<td>thickness of tuber at cr, measured in plane of cotyledons</td>
</tr>
<tr>
<td>T_l</td>
<td>maximum thickness of tuber, measured in plane of cotyledons</td>
</tr>
<tr>
<td>tr</td>
<td>tracheary element(s)</td>
</tr>
<tr>
<td>v</td>
<td>group of vessels</td>
</tr>
<tr>
<td>vp</td>
<td>vasicentric xylem parenchyma</td>
</tr>
<tr>
<td>vpr</td>
<td>vessel primordium</td>
</tr>
<tr>
<td>vt</td>
<td>new tertiary vessel</td>
</tr>
<tr>
<td>Z</td>
<td>distance from position of maximum thickness of tuber to cr</td>
</tr>
</tbody>
</table>

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4. Results and conclusion

4.1. Examples of variance in growth of the tuber

In Table 1 data are shown of two radish tubers after 8 and 27 d in clay soil (plants 1 and 2). Both plants represent the extremes in relative rate of elongation of upper and lower half of the hypocotyl and the upper part of the root in 20 plants; this is illustrated in Fig. 3. The total length of the hypocotyl after 27 d was almost the same in both plants, but the upper part of the hypocotyl became more than twice as long as in plant 1 and remained unchanged in length in plant 2 over 19 d. The relative contribution of the lower parts of the hypocotyl was the reverse of this. In plant 1 it increased by only 14.3% while in plant 2, the lower hypocotyl extended almost 3-fold in 19 d. Also the upper part of the root showed different percentages of increase 27 d after sowing; in plant 1 a shortening of 45% and in plant 2 this part did not show any change in length over 19 d. But the contraction of this root part is a process with a maximum, after which this part shows elongation again (Figs 4F, 5F). In plant 1 this root part was shortest probably at 27 d after sowing and in plant 2 this part was shortest at 20 d (−30%). The total length of the tubers did not differ much in both plants (25 and 28 mm) and the relative increase of the maximum width

![Diagram](image-url)
was not much different either (1311.1 and 1457.9%). The position of the maximum width of the tuber in relation to the collum radicis was in plant 2 a bit lower in the hypocotyl than in plant 1.

In Table 1 the same data are shown of two radish tubers after 8 and 22 d of development in nutrient solution (plant nos. 3, 4). These examples also represent the extremes in relative rates of elongation of upper and lower half of the hypocotyl and upper part of the root. The $H_2$ part of the hypocotyl in plant 3 elongated by 110%, more than twice as much than the $H_1$ part (only 30%). In plant 4, the opposite was found; the $H_1$ part elongated 75% while in the $H_2$ part elongation was negative (−22.2%). In plant 3, the upper part of the root (R) had elongated by 50% over 22 d while in plant 4 this part had contracted by 14.0%. When comparing these data with those of plants 1 and 2 one can conclude that the increase in length of the hypocotyl is greater in the plants cultivated in clay soil. However, the variation in change in length of the hypocotyl of the radish seedling is striking under both sets of conditions.

4.2. Graphic representations of the growth of the tuber

Because of large variations in elongation, both positive and negative, of the hypocotyl and upper 5 mm of the root, the growth of the $H_1$, $H_2$ and R parts of the seedling has been represented as graphs in two groups. In one group the development is shown of the different parts of hypocotyl and root in which no shortening below that measured on day 8 occurred, and in the other group the development of these parts is shown in which a period of contraction below the original length took place. In Fig. 4A, B, the change in length of the upper half of the hypocotyl of the seedling ($AH_1$) of 17 plants after 8−29 d is shown. Results for the 10 plants in which no shortening of the $H_1$ part occurred within the period 8-29 d after sowing, are given in Fig. 4A. In one plant, however, some contraction did occur after elongation of about one week. The growth pattern of the remaining 7 plants in which contraction of $H_1$ was seen, is given in Fig. 4B. The maximum contraction from day 8 values −30%, measured in one plant at 17 d. In a second plant the contraction occurred only after 26 d. At 29 d, only 2 plants were available yet due to chemical fixations for histology, versus 7 plants for the previous dates. In Fig. 4C, D, the change in length of the lower half of the hypocotyl ($AH_2$) is shown. In 6 plants, no contraction of $H_2$ took place (Fig. 4C). However, the remaining 11 plants did show contraction (Fig. 4D, 65%), that was more severe than in $H_1$ (41%). The maximum contraction was −29% in a single plant at 17 d and at 29 d only 5 plants were available yet versus 11 plants at the previous dates. Again it is shown, as in Fig. 4B, that contraction of parts of the hypocotyl is a temporary phenomenon as it is in the taproot.

In Fig. 4E, F, the change in length of the upper 5 mm of the taproot ($AR$) after 8 d is shown. The growth of 17 plants in clay soil is analysed. Only in one plant was no contraction seen in this section between 8-26 d, while 16 plants
Table 1. Data of tubers of two plants in clay soil (nos. 1, 2 at 8 and 27 days) and of two plants in nutrient solution (nos. 3, 4 at 8 and 30 days). Plant nos. 1, 2 show opposite extremes in longitudinal increase of H1 and H2 (see Fig. 4) as do plant nos. 3, 4. Explanation of symbols see Fig. 2; L1, estimated length of tuber.

<table>
<thead>
<tr>
<th></th>
<th>Plant 1 clay soil</th>
<th>Plant 2 clay soil</th>
<th>Plant 3 nutrient solution</th>
<th>Plant 4 nutrient solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>seedling 8 d old</td>
<td>measurements of</td>
<td>seedling 8 d old</td>
<td>measurements of</td>
</tr>
<tr>
<td></td>
<td>length (mm)</td>
<td>tuber after 27 d</td>
<td>length (mm)</td>
<td>tuber after 27 d</td>
</tr>
<tr>
<td></td>
<td>length (mm)</td>
<td>increase (%)</td>
<td>length (mm)</td>
<td>increase (%)</td>
</tr>
<tr>
<td>H1</td>
<td>15.0</td>
<td>25.5</td>
<td>11.5</td>
<td>24.5</td>
</tr>
<tr>
<td>H2</td>
<td>8.0</td>
<td>17.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>H3</td>
<td>7.0</td>
<td>14.3</td>
<td>6.5</td>
<td>19.0</td>
</tr>
<tr>
<td>R</td>
<td>5.0</td>
<td>-45.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>L1</td>
<td>25</td>
<td>28</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>width (mm)</td>
<td>width (mm)</td>
<td>increase (%)</td>
<td>width (mm)</td>
<td>width (mm)</td>
</tr>
<tr>
<td>T</td>
<td>1.4</td>
<td>4.5</td>
<td>1.4</td>
<td>5.5</td>
</tr>
<tr>
<td>T1</td>
<td>25.4</td>
<td>1311.1*</td>
<td>29.6</td>
<td>1457.9*</td>
</tr>
<tr>
<td>Tc</td>
<td>1.8</td>
<td>111.1</td>
<td>1.9</td>
<td>23.8</td>
</tr>
<tr>
<td>Z</td>
<td>0.47xH</td>
<td>0.36xH</td>
<td>0.09xH</td>
<td>0.65xH</td>
</tr>
</tbody>
</table>

* with Tc of seedling as reference value.
showed contraction, i.e. 94%, a much larger proportion than that observed in H₂ and H₁. The capacity of these organs to contract is therefore greater at lower positions; the maximum shortening was -45% in two plants at 21 and 26 d. Contraction proved to be temporary since by 33 d strong elongation (up to 140% at 33 d) was recorded.
The change in length of hypocotyl parts and the upper part of the root of plants cultivated in nutrient solution has also been analysed. In Fig. 5A, B, the change in length of the upper half of the hypocotyl of the seedling (ΔH₁) of 12 plants after 8-35 d is shown. Results for the 7 plants in which no shortening of the H₁ part occurred within the period 8-35 d after sowing, are given in Fig. 5A. In one plant, however, some shortening did occur after elongation of about 17 d. The growth pattern of the remaining 5 plants in which contraction of H₁ was seen is given in Fig. 5B. The maximum contraction from day 8 values -9%, measured in one plant at 11, 14 and 29 d. In a second plant the contraction occurred only after 23 d (Fig. 5C); it is shown that after a period of elongation a period of contraction occurs to a minimum length of -6% at 26 d. After 29 d very rapid elongation takes place again. The growth in length of the upper part of the hypocotyl is very irregular in this group, and this causes a flat curve in

Figure 5. Nutrient solution. Average increase of H₁, H₂ (as % of the length at 8 DAS and R (as % of the upper 5 mm (= 100%) of the root at 8 DAS); bars represent S.D.
A, ΔH₁ in 7 plants without contraction of H₁ below the length at 8 d; B, ΔH₁ in 5 plants with contraction of H₁ below the length at 8 d; C, ΔH₁ in one of the 5 plants in B in which contraction of H₁ occurred only after 23 d; D, ΔH₂ in 2 plants without contraction of H₂; E, ΔH₂ in 10 plants with contraction of H₂ below the length at 8 d; the ultimate contraction was -28% at 32 d; F, ΔR in 11 plants with contraction of this root part below the length at 8 d; the ultimate contraction was -35% at 23 d.

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Fig. 5B. Elongation may be very rapid after contraction; another plant of this group elongated already to 109% at 32 d after contraction till the 17th day.

In Fig. 5D, E, the change in length of the lower half of the hypocotyl (AH₂) of 12 seedlings when cultivated on nutrient solution after 8-32 d is shown. In two plants no contraction of the H₂ part within the period 8-32 d after sowing took place (Fig. 5D) and no significant shortening occurred in H₂ within this period. The remaining 10 plants did show contraction in H₂ (Fig. 5E), a greater percentage of plants (83%) than in the H₁ part (42%). The maximum shortening was −28% in one plant at 32 d, and 9 plants were available yet.

Again it is shown that contraction of parts of the hypocotyl is a temporary phenomenon as it is in the taproot. In Fig. 5F the change in length of the original upper 5 mm of the taproot (AR) of 11 seedlings after 8-32 d is shown. All plants showed contraction below the original length of 5 mm at 8 d, a greater percentage of plants (100%) than in the H₂ and H₁ parts. Also in this group of plants, cultivated on nutrient solution, the capacity to contract is greater at lower positions. The maximum shortening was −35% at 23 d. The contraction of this part of the root proves to be temporary again in this group of plants; it is followed by strong elongation (up to 190% at 42 d, i.e. almost 3 times as long as at 8 d). At 32 d 8 plants were still available for analyses.

It may be concluded that the capacity of temporary contraction of hypocotyl and upper part of the taproot generally increases from the upper part of the hypocotyl to below the collum radicis in plants cultivated in clay soil as well as in nutrient solution. Not only the percentage of contraction varies strongly, but also the timing of contraction in the plants. After the process of shortening the following elongation can be rapid enough for the hypocotyl or root parts to become equal to the length of the same parts in plants without contraction (Fig. 4C, D).

In Fig. 6A, B, the increase of the diameter of the tuber of the radish plants is shown in clay soil and on nutrient solution. The difference of about 6 d in appearance of the tuber after 20 d can be ascribed to the fact that the plants on nutrient solution have been transplanted from sand to the solution after 8 d, while the other plants remained in clay soil. The increase in thickness of the tubers in both groups is not really different after 26 and 32 d when leaving the delay in growth aside (resp. clay soil and nutrient solution, Wilcoxon's test, P = 0.1, two-tailed probability).

Considering the position of the maximum diameter of the tuber along the plant axis, Fig. 7A, B shows that the tuber slowly comes down in clay soil but that this change is much more striking in the plants growing on nutrient solution. In this latter group the maximum diameter comes down under the collum radicis but later, after 40 d of culture, the tuber rises markedly again.

4.3. Increase in length of hypocotyl and diameter of tuber

From Fig. 6A, B, it can be derived that the difference of increase of the maximum
Figure 6. Average diameter of tuber ($T_t$) in mm; bars represent S.D. A, $T_t$ of 17-19 plants at the age of 14-26 d, clay soil; B, $T_t$ of 12 plants at each date at the age of 20-32 d, nutrient solution.

Figure 7. Position of maximum diameter of tuber ($Z$, in % of $H$, see Fig. 2) calculated at every indicated DAS as averages of the indicated number of plants. A, clay soil; B, nutrient solution; bars represent S.D.

diameter of the tubers amounts to 7.5 d (the average diameter of 20 mm is reached at 23 d in clay soil and at 30.5 d on nutrient solution). When comparing the maximum diameters of the tubers ($T_t$) developed in clay soil and on nutrient solution at 9 and 6 d difference in time of growth (resp. Tables 2, 3 and 4) no statistically significant difference between the maximum diameters can be found (Wilcoxon's test, see average values in these tables).

In Tables 2 and 3 the lengths and increases of length in % of hypocotyl and upper and lower part of each hypocotyl of 20 plants at 8 d and 21 d (clay soil) and of 12 plants at 8 d and 30 d (nutrient solution) are shown. These data are arranged in the sequence of longer hypocotyls of the seedlings at 8 d. In the tenth column the differences between the increases of length of upper and lower part of the hypocotyls have been recorded, and in the last column the maximum diameter of the tuber. Applying Spearman's rank correlation test the length of the hypocotyls at 8 d ($H$) has been compared with the increase of the length of the hypocotyls in % ($\Delta H$) in the two groups of plants. In the group of plants on nutrient solution the null hypothesis 'no rank correlation' can be rejected with a probability < 0.01 critical level (two-sided probability). One may conclude that the negative rank correlation indicate's that the shorter the hypocotyl of the seedling on nutrient solution the more the increase of this hypocotyl during the next 22 d will be. In the plants on clay soil no such a rank correlation could be demonstrated, however. Also applying Spearman's rank correlation test, the maximum diameter of the tubers of the clay soil plants has been compared with the length $H$ at 8 d of the seedlings. It appeared that no relation will exist between
Table 2. Comparison of hypocotyl length (H) and upper and lower part of the hypocotyl (H_1, H_2) of 20 plants at 8 d and at 21 DAS in clay soil. Also the maximum diameter of the tuber (T_1) at 21 d is indicated.

<table>
<thead>
<tr>
<th>H (mm)</th>
<th>H_1 (mm)</th>
<th>H_2 (mm)</th>
<th>H (mm)</th>
<th>H_1 (mm)</th>
<th>H_2 (mm)</th>
<th>ΔH</th>
<th>ΔH_1</th>
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<th>T_1 (mm)</th>
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Average ± S.D.
Table 3. Comparison of hypocotyl length (H) and upper and lower part of the hypocotyl \((H_1, H_2)\) of 12 plants at 8 d and at 30 d. From 8 to 30 d cultivation on nutrient solution. Also the maximum diameter of the tuber \((T_1)\) at 30 d is indicated.

<table>
<thead>
<tr>
<th>At 8 d (mm)</th>
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<th>At 30 d, increase (%)</th>
<th>At 30 d (%)</th>
<th>At 30 d (mm)</th>
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<td>9.5</td>
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13.4 ± 3.7 

17.8 ± 3.9 

37.0 ± 32.2 

39.2 ± 28.7 

31.4 ± 53.3 

2.1 ± 47.3 

15.7 ± 6.1 

average ± S.D.
the values of $H$ and the increase of $T$. The growth of the tubers on nutrient solution was more irregular and less plants were available; no test has been carried out.

![Graph](image)

Figure 8. Averages of the values $(\Delta H_1, \Delta H_2)$ in %, ordinate) of all tubers are shown every three d for the clay soil culture (○ ○ ○) and on nutrient solution (● ● ●). The values for nutrient solution have been corrected with 7.5 d (+ + +) because of delay of growth of tuber (cp. Fig. 6A, B) after transplanting.

Insert: schematic drawings of the average difference of $\Delta H_1$ and $\Delta H_2$ after development of the tubers in clay soil (at 29 d) and on nutrient solution (at 36.5 d). The calculation is done with the formula: $[(H_1 \times \Delta H_1) + (H_2 \times \Delta H_2) + (R \times \Delta R)] / L = \Delta L$, with average rounded up and down values of $H_1$, $H_2$, $L$ and $R$ at 8 d and of $\Delta H_1$, $\Delta H_2$, $\Delta L$ and $\Delta R$ in % at 29 d (clay soil) and at 36.5 d (nutrient solution).
The value \((\Delta H_1 - \Delta H_2)\) indicates the difference of increase of \(H_1\) and \(H_2\) in a growing tuber (Fig. 8, insert). By way of Wilcoxon's test these values of plants in clay soil are compared with those on nutrient solution. Between tubers at 21 d in clay soil and at 30 d on nutrient solution no significant difference could be found. For an explanation of this result see Fig. 8. In the graph the average values of \((\Delta H_1 - \Delta H_2)\) of the tubers in clay soil and on nutrient solution are shown in relation to time (DAS). As the delay of growth of the tubers on nutrient solution amounts to 7.5 d, the curve of \((\Delta H_1 - \Delta H_2)\) of plants on nutrient solution has been shifted to a time scale 7.5 d earlier in order to get comparable development of tubers in both groups of plants. When comparing \((\Delta H_1 - \Delta H_2)\) (clay soil) at 21 d \((-20.4 \pm 53.3\%)\) with \((\Delta H_1 - \Delta H_2)\) (nutrient solution) at 30 d \((2.1 \pm 47.3\%)\) (see Tables 2, 3) no significant difference will be found, because the curves do not diverge sufficiently enough at these points on the time scale. However, when the Wilcoxon's test is applied to these values at 29 d (clay soil) and 35 d (nutrient solution) (see Table 4), the difference is significant with a probability < 0.001 critical level (one-sided probability). This situation is illustrated in the insert of Fig. 8. The development of the tubers on clay soil is different from that on nutrient solution; in clay soil the lower half of the hypocotyl does mainly constitute the tuber while on nutrient solution the upper half of the hypocotyl has become longer after 29 d of growth. In Table 4 it is shown that the increase in thickness of the tuber in both groups of plants is comparable with a delay of 6 d in growth on nutrient solution.

The schematic drawings as insert of Fig. 8 are thus based on about equal aver-

Table 4. The average values of \((\Delta H_1 - \Delta H_2)\) in % and \(T_i\) in mm of every tuber in the period 14-29 DAS in clay soil and at 20-35 d in nutrient solution. These different periods are chosen because of comparable thickness of the tuber in these periods due to delay of growth after transplanting to nutrient solution (cp. Fig. 6A, B).

<table>
<thead>
<tr>
<th>No. of d</th>
<th>((\Delta H_1 - \Delta H_2)) clay soil (%)</th>
<th>No. of plants</th>
<th>(T_i) (mm)</th>
<th>No. of plants</th>
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<tbody>
<tr>
<td>14</td>
<td>(16.2 \pm 16.9)</td>
<td>17</td>
<td>(4.3 \pm 1.8)</td>
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</tr>
<tr>
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<td>(5.9 \pm 26.5)</td>
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<tr>
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<td>17</td>
<td>(14.7 \pm 4.3)</td>
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<tr>
<td>23</td>
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<tr>
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<tr>
<td>29</td>
<td>(-101.7 \pm 85.6)</td>
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<table>
<thead>
<tr>
<th>No. of d</th>
<th>((\Delta H_1 - \Delta H_2)) nutrient solution (%)</th>
<th>No. of plants</th>
<th>(T_i) (mm)</th>
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*Wageningen Agric. Univ. Papers 91-1 (1991)*
Table 5. Comparison of ΔH in plants on clay soil and on nutrient solution after 27.5 and 35 DAS resp.

<table>
<thead>
<tr>
<th></th>
<th>ΔH (%)</th>
<th>T (mm)</th>
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<td>nutrient solution</td>
<td>75.4 ± 37.0</td>
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</table>

average values of T, on clay soil and on nutrient solution after resp. 29 and 35 d (Table 4). When comparing exactly the increase of the length of the hypocotyl (ΔH) in plants on clay soil and nutrient solution, the average value of T, in both groups should also be exactly the same (Table 5). Besides the delay of growth of the tubers on nutrient solution of 7.5 d has been taken into account again (see Fig. 8). The value ΔH appears to be somewhat smaller in plants on nutrient solution, but this difference is not statistically significant. This difference does show correspondence however with the different values of ΔR in Figs 4F and 5F, resp. ± 20% and ± 55% in clay soil and nutrient solution at 29 and 36.5 d. Thus the contribution of the root to the total elongation of the tuber axis is greater in plants on nutrient solution at that moment. The within-group variance of measured values of ΔH is greatest in the plants on nutrient solution (Table 5), as with many other values; the plants on nutrient solution grow more irregular.

As the maximum root contraction in clay soil and in nutrient solution occurs at 20 d (ΔR, Figs 4F, 5F), and the percentages of contraction are not much different, this phenomenon may be less dependent on growth circumstances than the growth of the tuber in its entirety.

In conclusion it may be stated that great differences in growth take place between tubers developed in clay soil and grown on nutrient solution. The occurrence of differences is strongly dependent, however, of moment and place of measurement.

4.4. Anatomical aspects of tuber development

In the seedling (Fig. 9) the collum radicis (cr) is a morphologically sharply limited zone of the shoot-root axis, visible as an edge under which the young taproot with split cortex shows its red anthocyanin containing cells of pericycle and its primary phloem. This fissure is first visible in a plane perpendicular to that of the cotyledons. In Fig. 10 a transection of the middle of the hypocotyl of a seedling 9 d after sowing is shown. On both sides of the primary xylem with diarch structure, secondary xylem is formed. It is composed of vessels and libriform fibres. The metaxylem consists of vessels with spiral thickenings and parenchyma cells. In Fig. 11 a detail of Fig. 10 near the protoxylem is shown with one of the four first regular groups of interxylary phloem (ip) differentiating
Figure 9. Below c of the 15 d old seedling the 13 mm long hypocotyl is visible. cr is observed as an edge below which the young taproot with split cortex shows its pericycle. Scale bar 10 mm.

Figure 10. Transection of the middle of hypocotyl of a 9 d old seedling. It shows a diarch structure and the small quantity of l as the main component of the first sx. Scale bar 0.1 mm.

Figure 11. Detail of Fig. 10. Adjacent to the strand of px one of the four first strands of ip with se is visible. Scale bar 0.01 mm.

Figure 12. Transection of hypocotyl close to cr of 11 d old seedling. mx mainly consists of vessels, and sx of some vessels and much xylem parenchyma. Scale bar 0.2 mm.
on either side of each protoxylem strand (px). Fig. 12 shows a transection of
the hypocotyl close to the collum radicis with diarch structure 11 DAS. The
production of libriform fibres as the main component of the first secondary
xylem has stopped. After that only apotracheal parenchyma cells were formed
with scattered vessels. Periderm (pe) is formed in the pericycle isolating the cor-
tex, at first between the cotyledons; i.e. perpendicular to the plane of the primary
xylem. In Fig. 13 the flap-like segments of partially split off cortical tissue exactly
under each of the two cotyledons remain visible. Perpendicular to this plane
of the cotyledons the first deep fissure of the tuber may develop right under
the fissure of the cortex. In tubers grown in nutrient solution these fissures devel-
op very often and in this tuber younger, smaller fissures are also visible on the
right sight of the large fissure. All fissures are frequently filled with liquid (juice)
leading to entirely wet tubers; this liquid dries up after some days, but it may
occur once more when the fissure widens. Later most fissures originate on the
sunlit side. The upper mark on this tuber is the original collum radicis which
is morphologically not visible any more.

In Fig. 14 a radish tuber is shown after 24 d on nutrient solution. After a
first deep fissure, developed in the tuber perpendicular to the plane of the cotyle-
dons the tuber was split along this fissure due to further expanding and dividing
of the xylem parenchyma. Younger, smaller fissures are visible at the left side.
This left side of the tuber, connected to the largest part of the split taproot elong-
gated more than the right side; the two parts of the taproot and the lateral roots
are vigorously pulled apart. In Fig. 15 a radish tuber is shown after 21 d in
a clay soil mixture. The upper layer of river sand is brushed away for showing
the marks of the middle of hypocotyl at 8 d (upper mark), collum radicis (middle)
and the position of a level 5 mm below collum radicis at 8 d (lowest sign). The
increase in length of the upper and lower half of the hypocotyl amounts to resp.
0% and 125% between 8 and 20 DAS. In this normally developed tuber no fis-
sures occur; the expanding and dividing of the xylem parenchyma occurs in
total conformity with the dilating activity of the cambium.

The radish tuber originates due to the activity of the vascular cambium in
three dimensions, in radial, tangential and in longitudinal direction, but also
due to the expanding of the xylem parenchyma cells and mostly also a strong
dividing activity of the xylem parenchyma cells in all directions. In healthy tubers
all these meristematic activities occur completely in harmony with each other.
As long as the tuber grows rapidly this situation continues and no closed xylem
cylinder comes into being. Such an open xylem cylinder does also show dilatation
itself, and no rays will develop in this xylem (but cp. Hayward, 1938, and Joyce,
Aspinall and Edwards, 1983, who mention the existence of rays in radish tubers).
In Fig. 16 the expanding xylem parenchyma cells are clearly shown next to and
at the inside of the differentiating strands of vascular elements.

In Fig. 17 the central part of a transverse section of a young tuber, 11 mm
in diameter, is shown. The metaxylem has been scattered by the expanding and
dividing primary parenchyma cells. In Fig. 18 a detail is shown of a transverse
section of a young tuber, 12 mm in diameter, near the middle of the radius of
Figure 13. Radish plant, 30 d old, grown in nutrient solution. The upper mark on the tuber indicates cr, the lower mark the distance of 5 mm below cr at 8 d. Note first deep fi in the stele, perpendicular to the plane of c.

Figure 14. Drawing of 32 d old radish plant, grown in nutrient solution. Tuber deeply split by strong diffuse secondary growth. See text.

Figure 15. Radish plant, 21 d old, grown in clay soil. The upper layer of river sand is brushed away, showing the marks between H₁ and H₂ (arrow), of cr and of 5 mm below cr at 8 d. ΔH₁ = 0%, ΔH₂ = 125%. Figs 13, 14, 15 scale bar 10 mm.

Figure 16. SEM picture showing transection of tuber near ca. Expanding xylem parenchyma cells at the inside of ca and differentiating sxs: diffuse secondary growth. There is no closed xylem cylinder. Scale bar 100 μm.

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Figure 17. LM showing transection of young tuber, 11 mm in diameter. Expanding and dividing xylem parenchyma cells scatter metaxylem vessels. Parenchyma cells with amyloplasts, mx, helically thickened elements of mx.

Figure 18. LM of transection of young tuber, 12 mm in diameter. Expanding and dividing xylem parenchyma in radial direction near the middle of the radius, dispersing the tr. Arrow, direction of ca.

Figure 19. LM of transection of left part of tuber in Fig. 14 near centre on the level of cr. Cell division of xylem parenchyma occurs in all directions.

Figure 20. LM of transection of young tuber, 13 mm in diameter, near the middle of radius. The radiating cells (arrows) around the right centre with a small sve fit very well with the radiating cells (arrows) from the left centre (not visible), without showing a clear boundary line. Direction of ca is indicated with arrow. Figs 17-20 scale bar is 100 μm.

Figure 21. LM of transection of young tuber, 12 mm in diameter. A sve in the ground mass of xylem parenchyma consisting of a few vessels (tr) and nearly always some groups of ip. This strand is situated about 600 μm from ca. Direction of ca is indicated with arrow. Scale bar 20 μm.

the tuber. This healthy tissue shows expanding and dividing xylem parenchyma cells chiefly in radial direction, dispersing the tracheary elements (tr). In Fig. 19 a detail is shown of a transverse section of the left part of the tuber in Fig. 14 on the same level as cr. Cell division of xylem parenchyma occurs in all directions. In Fig. 20 a transverse section of a young radish tuber, 13 mm in diameter, is shown in which healthy xylem tissue is visible with expanding as well as dividing parenchyma cells. These growing parenchyma cells radiate from small
groups of conducting elements, xylem and phloem (on the right and on the left side of the figure, the latter not visible in the micrograph). The figure shows the harmonizing way of fitting of radiating parenchyma (from the right) with the radiating parenchyma around the group of conducting elements on the left. The tissue in between is comparable with Fig. 19. In Fig. 21 a strand of vascular elements is shown in a transverse section of a young tuber, 12 mm in diameter. This s.v.e., as all groups, consists of a few vessels and nearly always some phloem.

In Fig. 22 a StM is shown of a transverse section above cr of a living tuber cultivated 35 d in nutrient solution. This transection was 27 mm in diameter and contained healthy tissue. Groups of vascular elements can be seen that drift apart from one another by regularly expanding parenchyma cells, especially in radial direction. All intercellular spaces are filled up with gas, also in the cambial zone. In Fig. 23 a detail of a transverse section of the same tuber as in Fig. 22 is shown. Healthy tissue with three groups of conducting elements and smaller parenchyma cells, surrounded by vasicentric parenchyma (i.e. parenchyma forming a sheath or halo around a group of conducting elements and smaller parenchyma cells, and showing a deviating way of expansion and/or dividing) radiating from the centres with the smaller elements, can be seen. All intercellular spaces are visible as small white canals filled up with gas. In Fig. 24 a radial section of a living tuber, cultivated 40 d on nutrient solution is shown. Healthy tissue is visible in which all intercellular spaces are observed as small white canals filled up with gas, clearly showing the network of cells. In Fig. 25 a radial section of the same tuber as in Fig. 24 is shown. The figure shows that all intercellular spaces are filled up with gas and that they are continuous from phloem through the cambium into the xylem. In this part of the split tuber spongy tissue is beginning to develop.

In Fig. 26B a StM of a thin transverse section of a tuber is shown. The position of this section is indicated in Fig. 26A and is situated about halfway of the most elongating upper half of the hypocotyl. In this tuber it can be seen that the relation between the parenchymatic thickening growth or diffuse secondary growth (Tomlinson, 1961; Esau, 1964), the cambial secondary growth and the elongation growth of the tuber axis, has been a very harmonizing process in this case. The parenchyma cells in the section of Fig. 26B remain situated in rather regular radial rows in the tuber. In Fig. 20 the parenchyma cells grow much more actively and radiate from the small strands of vascular tissue. This difference in configuration of parenchyma cells may be explained by a relative difference in activity of the vascular cambium (Fig. 27). The incremental circumferential growth, \( AC = 2\pi x \Delta r \) (Mauseth, 1988), is the same in A and B, because \( \Delta r \), the increase of the radius, has the same value. Then the number of radial (anticlinal) divisions of the cambial initials is the same in A and B, but the number of tangential (periclinal) divisions is greater in A. This means that the cambial activity is higher in A, outweighed by a greater dividing activity of the xylem parenchyma cells in B. The greater expansion and division activities of the xylem parenchyma in indicated directions in B result in a greater distance between the strands of vascular elements in the tuber.
The radial and tangential dimensions of the xylem parenchyma cells in transverse section (Fig. 26B) are shown in Fig. 28 measured at 6 places along the radius of the tuber. At about 3.5 mm from the centre the most radial expansion of the cells is found and after calculating the surface area of the cells it is found that in a circular zone of about 1.5 mm to 6.0 mm from the centre the surface area of the xylem parenchyma cells is at its maximum. It is in this circular zone that the first cells die and spongy tissues arise.

Finally we may conclude that healthy tissue in a radish tuber consists mainly of living xylem parenchyma cells that have the potential to expand and divide. Between these living cells always straight narrow intercellular spaces occur, filled up with gas.

Expanding and dividing of the xylem parenchyma cells are rather different in various plants, i.e. the relation between the diffuse secondary growth and cambial secondary growth may vary in the plants. Great activity of the diffuse
secondary growth is especially found in tubers grown in nutrient solution leading to great and small fissures in the tuber. Smaller activity is found in tubers grown on clay soil mixture, leading to sound tubers with secondary xylem in which the strands of vascular elements are situated closer to each other and the xylem parenchyma cells longer stay in radial rows (Fig. 26B). In intermediate forms greater diffuse secondary growth brings about harmonious growth patterns in the secondary xylem (Fig. 20) without creating fissures.

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Figure 27. Diagrams explaining the result of relative different activities of the cambium in the tuber. The incremental circumferential growth, $\Delta c = 2 \pi \times \Delta r$ (Mauseth, 1988) is the same in A and B. The number of periclinal divisions of $ca$ is greater in A; consequently the cambial secondary growth is greater in A. In B the diffuse secondary growth is more important, leading to greater distances between the strands of vascular elements; sve 1, 2, comparable strands of vascular elements in A and B.

Figure 28. Cell dimensions of xylem parenchyma cells, measured at 6 places along the radius in the transverse section of the tuber shown in Fig. 26 B. ••••••, radial measurements; OOOOO, tangential measurements; □□□□□, estimates of cell surface area in transection (rad. $\times$ tang. $\times$ $\pi$) in $\mu m^2$/1000.
4.5. Histological consequences of strong local elongation of the tuber axis

In Figs 3 (plant 1), 26A and Table 1 is shown that the upper half of the hypocotyl elongated more than twice the length at 8 d during the following period of 19 d. Consequences of this elongation become especially evident in the rigid walls of the xylem vessels. In Fig. 29 a vessel in an elongating part of a tuber of 30 mm diameter is shown in a longitudinal section. The vessel members are torn apart along the end walls. In Fig. 30 part of the rim of the perforation as part of the end wall between vessel members is visible as a structure in isolated position within the rupture between the two vessel members. In Fig. 31 two vessel members are separated along their common end walls. The parenchyma cells show thickened wall parts adjoining to the newly formed cavity. On the left a sieve plate is visible in an intact, not torn, sieve tube; the living elements of the interxylary phloem which accompany the groups of vessels in the ground tissue of xylem parenchyma possess the capacity of elongating. In Fig. 32 three vessels were torn roughly along their end walls. Adjoining parenchyma cells grow into the newly formed cavity between the vessel members, and wall parts of these parenchyma cells thicken. In the right-hand vessel member below, three droplets of pectic substances are visible (cp. Figs 38, 40, 41). In Fig. 33 a torn vessel is also visible and parenchyma cells grew between the separated extremities, identifiable by the starch grains. Fig. 34 shows a vessel of which the longitudinal wall is broken to pieces; thicker parts of the wall between the bordered pits are visible as separate elongated, fibre-like fragments. A vessel with torn longitudinal walls is also visible in Fig. 35; thickened wall parts of adjoining parenchyma cells can be seen (arrow) and on the right the living parenchyma cells elongating in oblique longitudinal direction. Fig. 36 shows a broken small strand of vascular elements in older tissue of the tuber. On the left of the formed lacuna parts of the very strong elongated parenchyma cells are visible. A large lacuna formed in the old centre of the tuber after breakage of the primary xylem is shown in Fig. 37; on the right the strongly elongating parenchyma cells can be seen and on the left a newly differentiated strand of vascular elements is visible with one vessel and intact phloem. In Figs 36, 37 the older phloem strands are also interrupted.

A cross section of the radish tuber of Fig. 26, at about the level of section B (Fig. 26A) is shown in Fig. 38. The strong elongation of the upper half of the hypocotyl of this tuber (ΔH₁ = 113%) caused breakages of the vessel walls especially in the older tissues. The LM shows a transverse section of a strand of vascular elements near the centre of the tuber. All walls of the two bigger and four smaller xylem vessels (tr) are broken and interrupted. The wall parts of the parenchyma cells adjacent to the vessel walls are thickened. In one place the pectic substances in the thickened walls are pulled into the lumen of the vessel (arrow). The group of vessels is accompanied by phloem (ip). Very often the parenchyma walls adjacent to the vessels with broken walls thicken so intensely that the lumen of the parenchyma cell is nearly filled up with the pectic substance (Fig. 39). Some vessels are entirely filled up over some distance with
Figure 29. LM of longitudinal section of 67 d old tuber, 30 mm in diameter. The spongy tissue in the tuber shows vessel members separated along the end walls. Adjacent parenchyma cells expand and divide. Scale bar 50 μm.

Figure 30. As Fig. 29. Two vessel members separated along end walls; the rim of the perforation is torn off the lateral walls and is visible between the vessel members.

Figure 31. As Fig. 29. The longitudinal walls of the parenchyma cells adjacent to the fracture of the vessel thicken their walls (arrow). On the left a sieve plate can be seen in a living sieve tube.

Figure 32. As Fig. 29. Three vessels roughly torn along their end walls. Adjacent parenchyma cells grow into the newly formed cavity between the vessel members. Droplets of pectic compounds moved into the vessel through the wall (arrows). Figs 30-32 scale bar 20 μm.
Figure 33. As Fig. 29. Parenchyma cells grow between the separated extremities of a torn vessel, identifiable by the starch grains (arrows).

Figure 34. As Fig. 29. The lateral wall of the vessel is broken to pieces.

Figure 35. As Fig. 29. Adjacent to the broken lateral wall of the vessel the living parenchyma cells elongated very much. Arrow, thickened wall part of adjoining parenchyma cells. Figs 33-35 scale bar 20 μm.

Figure 36. As Fig. 29. Large lacuna arises in older tissues of the tuber after breaking of a sve. Scale bar 40 μm.
Figure 37. As Fig. 29. Lacuna arisen in the old centre of the tuber after breaking of the primary xylem. Part of vp of the halo is still intact. Scale bar is 200 μm.

Figure 38. As Fig. 26. Transverse section near the level indicated in A. Broken and interrupted vessel walls in a sve near the centre of the tuber. Parenchyma cells adjacent to the vessels show thickened walls and pectic compounds of the thickened parenchyma cell walls pulled into the vessel lumen (arrow, cp. Fig. 32).

Figure 39. As Fig. 38. The lumina of the parenchyma cells are nearly filled up with pectic compounds of the thickened parenchyma cell walls against the broken vessel walls. Fragments of the vessel walls that are perpendicularly cut, are coloured dark red and are black in the LM.

Figure 40. As Fig. 38. Some vessels are entirely filled up over some distance with the pectic compounds from the parenchyma cell walls (arrow).

Figure 41. As Fig. 38. Colouring of the pectic compounds in thick parenchyma cell walls against the vessel walls with the hydroxylamine-ferric chloride reaction (Reeve, 1959) after fixation with Nawaschin, containing chromic acid (Berlyn and Miksche, 1976). Figs 38-41 scale bar 20μm.
these pectic compounds from the parenchyma cell walls (Fig. 40). In Fig. 41 a cross section of two torn vessels and some phloem tissue on the right is shown. The thickened walls of the parenchyma cells adjoining the vessel walls are coloured red with the hydroxylamine-ferric chloride reaction (Reeve, 1959). This reaction is a specific reaction for histochemical localization of pectin. Thus the main component of the obviously soft, more or less liquid material of the thickened parenchyma cell walls consists of pectic substances.

Finally a survey is drawn in Fig. 26C of the outer half of the transverse section of the strongly elongated upper half of the hypocotyl of a 27 d old tuber grown in clay soil. It is shown that only the vessels near the cambium, indicated with number 1, have undamaged walls. The groups of vascular elements indicated with the numbers 2-4 have increasingly torn walls as the number becomes higher. However, in all strands of vascular elements in this part of the tuber the phloem part is entirely intact.

In conclusion we may say that as a result of elongation of the tuber axis the living elements in the secondary xylem expand and divide in longitudinal direction, including the phloem groups. However, the walls of dead elements, xylem vessels, are torn in different ways. The walls of the parenchyma cells adjacent to the torn vessels thicken and these parenchyma cells often grow into the broken vessels. The thickening walls become pectin-rich and this substance often streams into the lumina of the vessels. Later lacunae form, into which the surrounding parenchyma cells grow. When these lacunae become greater afterwards, the phloem strands are also interrupted.

4.6. Place of origin and characteristics of spongy tissue in the tuber

The first characteristics of sponginess in a living tuber can be observed as white spots in a section of the tuber tissue. Observing a transverse section under a stereomicroscope (Fig. 42) with a perfect black background, a white spot appears to be a number of gas bubbles in the apotracheal parenchyma. In Fig. 43 a longitudinal section is shown of apotracheal parenchyma in which sponginess originates. Some cells are almost filled up with a gas bubble; the outline of the gas bubbles fits well within the walls of the parenchyma cells. These cells with emboli are dead as are some of the neighbouring, oppressed cells, but not all cells in this tissue are dead. The viability test was carried out with fluorescein diacetate and with lissamin green, checked by Nomarski microscopy observing cytoplasmic streaming and the cell wall. The absence of a granular protoplasm layer against the walls indicates also a dead cell. No change in wall characteristics e.g. change in thickness or size of intercellular spaces, could be observed with Nomarski optics between living and adjacent dead cells (Figs 44, 45). The white spots of sponginess enlarge and increase in number (Fig. 46); this development takes place in the apotracheal parenchyma at some particular distance from the strands of vascular tissue. This development starts in the largest parenchyma cells between the centre of the tuber and the cambium (see Fig. 28). This means
that after some time the sponginess appears in the form of a ring with embran­chments directed outwards between the strands of vascular tissue. Takano (1966b) showed a weak reaction with triphenyl tetrazoliumchloride (TTC) of the same cells indicating a low intensity of the physiological activity. In the meantime the older spots of sponginess develop more cells with emboli (Fig. 47) and the surrounding apotracheal parenchyma cells (ap) lose the gas component out of their intercellular spaces resulting in a black image of the tissue around the spot of sponginess with intercellular spaces filled with liquid. Around the strands of vascular tissue the intercellular spaces between the smaller vasicentric parenchyma cells remain filled up with gas. In Fig. 48 a white spot of spongy tissue (on the right, below) and a strand of vascular tissue with vasicentric parenchyma (on the left) are visible. In the small, rather black zone of apotracheal parenchyma cells between the vasicentric parenchyma and the spongy tissue intercellular spaces are visible from which the gas component is partly removed and probably
transported to the area of emboli of the spongy tissue.

In Fig. 26B the first signs of sponginess in this tuber tissue appear after fixation as small dark spots (arrow) with dead cells in the zone with largest cells of apotracheal parenchyma (see Fig. 28). In Figs 49, 50 StM show the development of sponginess in a 27 d old tuber. The photographs show the same transection which is made at 43% of H₂ calculated from collum radicis and ΔH₂ = 192% (Fig. 3, plant 2; Table 1, plant 2; Table 6, plant 7). After the first sponginess in a ring around the centre (arrow) the next spongy tissue appears outwards between groups of conducting elements, thus forming a star-like pattern between halo's of vasicentric parenchyma. In Fig. 51 a StM of a longitudinal slice of the same tuber is shown. Dark spongy tissue with dead cells between cylinders of vasicentric parenchyma is visible.
Figures 49, 50. StM of a transverse slice of fixed tissue of a 27 d old tuber (Fig. 3 and Table 1: plant 2, and Table 6: plant 7). Section was made at $0.43 \times H_2$ from cr. Dead cells as black spots and bands between the halo’s of vp. The centre of the tuber is below. See text.

Figure 51. As Figs 49, 50; longitudinal slice. The black band to the right (arrow) indicates the level of cr, boundary between strongly elongated lower half of hypocotyl and root. In $H_2$ part of the tuber black bands of dead cells (arrow heads) alternate with halo’s of vp. Figs 49-51 scale bar 1 mm.

In Fig. 20 healthy tuber tissue is shown of which the parenchyma extends in a harmonizing way by expanding and dividing of cells. The parenchyma radiates around the groups of conducting elements. This situation is indicated in the first quadrant of Fig. 52; the direction of expanding parenchyma cells between the groups of conducting elements is represented as dashed lines. This situation occurs in healthy tuber development with greater dividing activity of the xylem parenchyma, i.e. greater diffuse secondary growth (Fig. 27B). The introduction to development of spongy tissue becomes visible as a distinction between the xylem parenchyma around the groups of conducting elements, the vasicentric parenchyma, and the other parenchyma, the apotracheal parenchyma, indicated in the second quadrant. The vasicentric parenchyma shows cells expanding in a radiating manner from their centres of conducting elements. Cell division may also occur with partition walls perpendicular to the radius of the
Table 6. Relation between occurrence of sponginess and elongation of H₁ and H₂ part of tuber. Clay soil culture.

<table>
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<tr>
<th>Plant no.</th>
<th>Plant age (d)</th>
<th>T₁ (mm)</th>
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Interval of time between moment of elongation in H₁ and H₂ ≥ 3 mm and moment of fixation (d)

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ring of vasicentric parenchyma. These 'halo's' originate with radii of a rather constant length. The apotracheal parenchyma cells, beyond the range of influence of a nearby group of vessels, show a random way of expansion, and when cells divide the new partition walls do not have a particular orientation (Fig. 19). The third quadrant shows the origin of the first spongy tissue within the tuber. The largest parenchyma cells (see Fig. 28) die and emboli arise in the dead cells (see Figs 42, 46). Many cells die soon and many emboli are formed; this phase will be called the cellular phase of sponginess.

In summary we may state that the development of sponginess in the radish tuber begins with the differentiation of the xylem parenchyma into two forms. Rings of vasicentric parenchyma ('halo's') originate with cells expanding in a
radiating manner from their centres of conducting elements (e.g. Fig. 23). The other form, apotracheal parenchyma, shows a random way of expansion and division. The next phase will soon be discernible when gas emboli (e.g. Fig. 42) in the largest apotracheal parenchyma cells occur. These largest cells are found in a circular zone around the centre of the tuber (see Fig. 28). After that emboli are found in outwards embranchments of apotracheal parenchyma, a star-like pattern of a mass of gas emboli between the halo’s of vasicentric parenchyma around the centre of the tuber forms the cellular phase of sponginess (Fig. 52-3).

4.7. Response of the living parenchyma after originating of sponginess and interval of time between beginning of elongation and origin of sponginess

In Fig. 20 the healthy development of xylem parenchyma is shown with expanding as well as dividing parenchyma cells. In Figs 53, 54 the difference between vasicentric parenchyma (left) and apotracheal parenchyma (right) just originates; in this case both types of parenchyma are expanding and dividing but the vasicentric form expands radiating from the centre and the apotracheal form expands in other directions. On the boundary line some parenchyma cells collapse. In Fig. 55 a SEM is shown in which the vasicentric parenchyma is rather sharply demarcated against the apotracheal parenchyma. The vasicentric parenchyma cells expanded, especially the outermost cells, and oppressed the adjacent apotracheal cells. The oppression of cells on the boundary line increases; 4-5 cell layers are oppressed at the circumference of the halo’s. These oppressed cells are dead and do isolate the cylindrical halo.

In Fig. 56 a longitudinal section is shown of a 44 d old tuber in which the hypocotyl elongated intensively ($\Delta H = 129\%$ during 21 d). The relatively little sponginess in this tuber, cultivated in nutrient solution, is visible as tissue with emboli and in addition the initiation of a lenticular lacuna by longitudinally tearing of the dead cells in the spongy tissue. In the fourth quadrant in Fig. 52 the transverse section shows a serious form of sponginess; this is also illustrated in Figs 57, 58 as StM pictures.

The more central, older, spongy apotracheal parenchyma died entirely and was torn up due to strong elongation of the tuber. The emboli disappeared and large lacunae originated between the older halo’s of still living vasicentric parenchyma cells. Besides the radii of the halo’s may enlarge (Fig. 57) and the dead apotracheal parenchyma cells become stretched along the circumference of the halo. This is the lacunar phase of sponginess (Fig. 58). The cellular phase is only found in the younger tissues in this quadrant because of later and less influence of the elongation of the tuber. In Fig. 52 the four quadrants are drawn with the same radius of the tuber because the suggestion that the development of sponginess is strictly related with the diameter of the tuber is not justified. A scheme of the origin and further spread of sponginess in relation to the elongation of parts of the tuber is shown in Fig. 59. The first sponginess appears in places of most elongation in young tubers near the centre of the tuber in the Wageningen Agric. Univ. Papers 91-1 (1991)
Figure 53. LM of transverse section of young tuber, 10 mm in diameter, showing introduction of sponginess near the centre of the tuber. The expanding and dividing vp (left) and ap (right) are separated by a layer of some oppressed, dead cells (arrow).

Figure 54. As Fig. 53. The vitality of the vp (left) is greater than that of ap (right).

Figure 55. As Fig. 16, but closer to the centre of the tuber. vp cells grow and oppress ap cells at the edge of h. Figs 53-55 scale bar 100 μm.

Figure 56. StM of longitudinal section of 44 d old living tuber grown in nutrient solution. Intensive elongation in this part of tuber and some sponginess is visible as tissue with gas emboli and the initiation of a lenticular lacuna (black) by longitudinally tearing of the dead cells. Scale bar 200 μm.

largest parenchyma cells. These places can be found between the cylindrical halo’s of living vasicentric parenchyma within which the vessels are broken. The vessels with most and largest fractures can be found in the oldest part of the tuber, i.e. the centre; the vessels without fractures can only be found near the cambium (Fig. 26C).

The surface area of the cambium layer enlarges strongly, both tangentially and longitudinally. Elongation of the tuber is effected by the cambial zone; finally, after some months, the tuber may only be supported by the cambial zone and some secondary phloem (Fig. 60).

The relation between moment of occurrence of sponginess and the number of days elongation in the original upper and/or lower half of the hypocotyl at 8 d, is indicated in Table 6 for 8 plants. It appears that cellular sponginess originates about 7-8 d after the beginning of elongation of that part of the tuber.
Figure 57. StM of transverse slice of tuber, 26 mm in diameter, after clearing. Halo’s of vp arise near ca; inwards the radii of h enlarge. lp in the central part. Scale bar 5 mm.

Figure 58. As Fig. 57, 57 d old tuber in greenhouse. cp (outwards) and lp (inwards, below). Living vp in halo’s around sve. Scale bar 2 mm.

Figure 59. Position and further spread of sponginess. Initiation of sponginess (dotted) can be found in the most elongated lower half of hypocotyl (H2). First appearance will be beyond the halo of living parenchyma cells around the central vascular strand with largest fractures. The area of sponginess spreads till the halo of the nearest vascular strand is reached (arrows). Average values of $\Delta H_1$, $\Delta H_2$ and $\Delta R$, see Fig. 8. Clay soil culture after 29 d. sve 1, oldest vascular strand with torn vessels; sve 2, younger vascular strand with torn vessels; sve 3, youngest, last differentiated vascular strand with uninterrupted vessels.

Figure 60. Old radish tuber in greenhouse. Elongation of tuber is effected by the cambial zone; after some months this tuber is only supported by a small rim of last formed sx, the cambial zone and sp. Scale bar is 10 mm.
in which the sponginess originates (see 8th column), i.e. 21-24 d after sowing. Some days later lacunar sponginess appears in the same elongating part of the tuber.

It is shown that no clear relation exists between the hypocotyl length of the seedling in clay soil at 8 d and the elongation of the tuber ($\Delta H_1$ and $\Delta H_2$ in Table 2). As the sponginess originates as a result of elongation of $H$, no relation between occurrence of sponginess and the hypocotyl length at 8 d is to be expected.

In conclusion it can be said that the response of the vasicentric parenchyma around a group of vascular elements to the cellular sponginess implies expanding of the cells in a radiating manner from the centre and sometimes also cell division, resulting in oppression of adjacent apotracheal cells along the circumference of the halo. After further elongation of the tuber lacunar sponginess occurs because of the fact that the dead apotracheal cells are torn longitudinally. Meanwhile the cellular sponginess extends outwards in the tuber.

Sponginess occurs only after 7-8 d after the beginning of the process of elongation of the tuber. No relation is found between the hypocotyl length at 8 d and the degree of elongation of the tuber (Table 2). Thus the appearance of sponginess is independent of the hypocotyl length of the seedling.

4.8. Some physiological data of tubers with spongy tissues

The plasmolysis studies of fresh radish tissues with sponginess show plasmolysis in both the vasicentric parenchyma and the living apotracheal parenchyma cells (Fig. 61). Plasmolysis is not visible in the small parenchyma cells in the centre of a halo, both near the vessels (Fig. 62) and near the phloem (Fig. 63). Only in these small cells amyloplasts are visible yet, as opposed to the larger cells of the vasicentric parenchyma.

In Table 7 some determinations are shown of the osmotic pressure values of tissues in tubers with sponginess. The average values of the whole secondary xylem, after removing the bark, are noted for plants 1 and 2. In plants 3, 5, 6, 7 the osmotic pressure values are determined for healthy (phase 1 and 2, Fig. 52) and spongy tissues (phase 3 and 4, Fig. 52) separately. The difference in osmotic pressure values of healthy and spongy tissues is not significant; the result of this test does not indicate an influence of the drying in. The osmotic pressure value of glassy tissue (plant 4) is low. Glassy tissue is characterized by intercellular spaces filled up with a liquid instead of gas.

During cultivation of the radish plants in clay soil but also in nutrient solution, attention was attracted to the prompt wilting of the leaves when the sun rises and the temperature goes up. The quick reaction of the entire leaves in the rosette, in spite of the long roots in the nutrient solution points to a great resistance of apoplastic transport in the plants, i.e. the tubers. Transpiration rate and absorption rate of three radish plants were measured (Fig. 64A). Under the experimental conditions the three points indicating the quantity of transpira-
Figure 61. Transverse section of 42 d old tuber with sponginess, 23 mm in diameter. LMNo shows immediate plasmolysis in both vp and ap cells under the influence of 12 bar mannitol solution. No amyloplasts present.

Figure 62. As Fig. 61. No plasmolysis of smaller and longer parenchyma cells near the vessels in a strand of vascular elements. Some amyloplasts present (arrows).

Figure 63. As Fig. 61. No plasmolysis of small and long parenchyma cells near the strands of phloem. Amyloplasts present (arrows). Figs 61-63 scale bar 20 μm.

Table 7. Determination of osmotic pressure values of secondary xylem in older tubers. No spongy tissue was present in tuber nos. 1, 2; in tuber nos. 3, 5, 6, 7 the osmotic pressure values in healthy secondary xylem near the cambium were compared with the values of the spongy secondary xylem in the centre of the tuber. Tuber no. 4 was entirely glassy.

<table>
<thead>
<tr>
<th>Plant Sponginess no.</th>
<th>Age of plant, (d)</th>
<th>Growth medium</th>
<th>Osmotic pressure value of all sx M. osmol</th>
<th>Osmotic pressure value of healthy tissue M. osmol</th>
<th>Osmotic pressure value of spongy tissue M. osmol</th>
<th>T_t (mm)</th>
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<td>1 -</td>
<td>27</td>
<td>clay soil</td>
<td>300 ± 4 Bar 6.7</td>
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<td></td>
<td>24</td>
</tr>
<tr>
<td>2 -</td>
<td>29</td>
<td>clay soil</td>
<td>256 Bar 5.7</td>
<td></td>
<td></td>
<td>23</td>
</tr>
<tr>
<td>3 +</td>
<td>43</td>
<td>clay soil</td>
<td>152 Bar 3.4</td>
<td>221.5 Bar 5.0</td>
<td>223 Bar 5.0</td>
<td>39</td>
</tr>
<tr>
<td>4 -</td>
<td>55</td>
<td>peat soil</td>
<td>153 Bar 3.4(r)</td>
<td></td>
<td></td>
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<td>56</td>
<td>peat soil</td>
<td>248 Bar 5.6</td>
<td>202.5 Bar 4.5</td>
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<td>35</td>
</tr>
<tr>
<td>6 +</td>
<td>66</td>
<td>nutrient solution</td>
<td>205 Bar 4.6</td>
<td>223 Bar 5.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 +</td>
<td>66</td>
<td>nutrient solution</td>
<td>235 Bar 5.3</td>
<td>189 Bar 4.2</td>
<td>192 Bar 4.3</td>
<td>45</td>
</tr>
</tbody>
</table>

(r): repeat of determination.
Figure 64. A. Transpiration rate (% fresh weight) and absorption rate (g eosin solution in % fresh weight) of three radish plants. The plants (1, 2 and 3) were sown September 28th and used for this experiment in November, resp. 12th, 16th and 17th; the diameters of the tubers were resp. 34, 34 and 31 mm. B. Transverse sections of the tubers of plants 1, 2 and 3 mentioned in A, drawn as identical transections. sve in the big mass of xylem parenchyma are shown as small black circles or spots. sve transporting eosin are indicated with black round marks (●). The inner line (arrow) indicates approximately the boundary line of spongy tissue in the centre.

Figure 64B shows an eccentric development of the tuber; one sector (left) elongated much less than the other two thirds of the tuber. In this small sector the vessels were found to be functional while in the other, larger sector which showed sponginess, only the youngest vessels near the cambium are able to transport the eosin solution. The transport of eosin becomes evident by colouring of the vessel walls, but the walls of libriform fibres and middle lamellae of small parenchyma cells near the vessels are also coloured. Besides small intercellular spaces in the parenchyma tissue adjacent to the vessels may be filled with eosin solution. The older vessels in the larger, elongated part of the tuber have obviously lost the capacity to transport water because of breakages of their walls (see Fig. 26C); no eosin solution could be observed in the torn vessels or near these vessels. In the healthy sector the parenchyma cells
show extension growth and the parenchyma was not divided in a vasicentric part (halo's) and an apotracheal part (see Fig. 52). The absorption rate of this plant was thus reduced, more than of the second plant. In plant 2 spongy tissue was found in the central part of the tuber and after growing 60 min. in an atmosphere of 80% r.h. the absorption rate was found to be less reduced than of plant 1. After checking the route of the transpiration stream it appeared that the vessels were functional in a rather broad ring around the central spongy tissue (Fig. 65). Between the central spongy tissue and the ring of tissue with functional vessels, only a small ring of tissue was found with uncoloured vessels and without sponginess. The absorption rate of this plant was less reduced than of the first plant. In plant 3 a large central area consisted of spongy tissue. After 120 min. in an atmosphere of 88% r.h. the transection of the tuber showed only red vessels near the cambium and the absorption rate was much reduced. When entering the growth cabinet the oldest 5 leaves, including the cotyledons, were removed from this plant, because these leaves were dead, dry and brown (the first 4 leaves) or yellowing and withered (the 5th leaf). The next two leaves, 18.5 and 21.0 cm long, were resp. strongly yellowing and beginning to become yellow. The higher 4 leaves, 20.0, 18.0, 16.0 and 12.5 cm long, were rather large and turgescent and the upper two leaves, 8 and 4 cm long, were small, dark green and turgescent. During the first 75 min. in the growth cabinet all leaves lost their turgescence in the order of age, except the youngest 4 cm long leaf. During the next 30 min. this situation did not alter, i.e. only the youngest 4 cm long leaf continued to be turgescent. Probably this youngest leaf communicates with the last differentiated functional vessels near the cambium.

In conclusion it may be said that the eosin solution is absorbed in proportion to the number of groups of vessels in the tuber that is still functional, i.e. is situated outside of the area of spongy tissues. At first the older vascular strands with torn vessels (Fig. 59) do still contain some nutrients out of the transpiration stream, but the contents of these vessels is not refreshed and after a period of dying of the apotracheal parenchyma and the forming of halo’s of living vasicentric parenchyma, the vasicentric parenchyma will also die at last. After a period of elongation of the tuber of about 7-8 d the sponginess becomes discernible in a transection of the tuber and the leaves connected with the torn vessels wither and die.

4.9. Comparison of appearance of spongy tissues in tubers cultivated in clay soil and in nutrient solution

In Table 8 the relation is shown between the occurrence of sponginess in tubers grown in nutrient solution and the percentage of elongation of upper and lower half of the hypocotyl in 12 plants. Generally the first sponginess originates about 40 d after sowing; but when taking into account the growth retarding of about 8 d of this group of plants because of the transplantation, the appearance of sponginess can be determined at about 32 d after sowing, i.e. 10 d later than
Table 8. Relation between occurrence of sponginess and elongation of H₁ and H₂ part of tuber. Culture in nutrient solution.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Plant</th>
<th>T₁</th>
<th>Elongation of H₁ and H₂ in % of H₁ and H₂ at 8 d and in mm</th>
<th>Interval of time between moment of elongation in H₁ and H₂ ≥ 3 mm and moment of fixation (d)</th>
<th>Interval of time between moment of elongation rate in H₁ and H₂ ≥ 0.5 mm/d and moment of fixation (d)</th>
<th>Sponginess (S) of tissues in H₁ and/or H₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>no.</td>
<td>age (d)</td>
<td>(mm)</td>
<td>%</td>
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<td>H₁</td>
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<td></td>
<td>ΔH₂</td>
<td>82</td>
<td>4.5</td>
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</tbody>
</table>

¹ only one small spot of sponginess; ± 2mm diam.
Figure 65. LM of transverse section of tuber 2 in Fig. 64. The route of the transpiration stream becomes visible after absorption of an eosin solution. After making freehand sections the walls of vessel primordia and differentiated vessels remained coloured and also the walls of libriform fibres.

Figure 66. LM of transverse section of 34 d old tuber grown in nutrient solution. $\Delta H_1 = 142\%$, $\Delta H_2 = -15\%$. Tuber without sponginess; section through $H_2$ shows old and torn vessels (v) and a newly differentiated vessel adjacent to phloem (vt).

Figure 67. As Fig. 66. Longitudinal section showing old vessels with many fractures (arrows) and an intact newly differentiated vessel next to a strand of phloem tissue.

Figure 68. As Fig. 66. Longitudinal section showing old interrupted vessels with fractures (arrows) and a newly differentiated, tertiary, vessel next to a strand of phloem tissue. Figs 65-68 scale bar 100 $\mu$m.
in plants cultivated in clay soil. The reason of this may be of two kinds. In the first place the availability of water and nutrients was unlimited while growing in nutrient solution, and therefore less chance existed for the apotracheal parenchyma cells to run short of these substances. In the second place the greater activity of the tertiary meristems, called secondary cambia (Hayward, 1938) around the small groups of secondary xylem vessels, also leading to the differentiation of one or two tertiary pitted vessel elements (Fig. 66) next to the interxylary phloem groups. These newly formed vessels differentiated later and are still intact and not torn as are the older vessels. In Figs 67 and 68 newly differentiated vessels can be observed on the right side of the LM next to a strand of interxylary phloem. The older vessels on the left side are torn by the tuber's elongation in this part of the axis. As well as in clay soil (Table 6) as in nutrient solution the occurrence of sponginess is related with elongation of (part of) the tuber. In clay soil sponginess appears 5-6 d after the moment that elongation attains more than 3 mm in upper or lower half of the hypocotyl (i.e. more than \( \pm 50\% \) of the average length at 8 d); in nutrient solution generally about 14-16 d go by before sponginess becomes visible. In plant 5 only 8 d passed before some sponginess became visible; possibly some intense and local elongation produced this earlier effect. In plant 8 sponginess is visible already after 6 d elongation in the lower half of the hypocotyl; in this case the sponginess may have been induced by the strong elongation of the upper half of the hypocotyl. When comparing the relation between occurrence of sponginess and the moment of the elongation rate in upper and/or lower half of the hypocotyl being higher than 0.5 mm/d, it seems that this relation is less clear than the relation between elongation becoming more than 3 mm and moment of origin of sponginess. This means that the percentage of elongation and the length of duration of this situation determine the occurrence of sponginess, more than the rapidity of the elongation process.

In summary it can be said that sponginess in tubers cultivated in nutrient solution occurs about 10 d later than in tubers cultivated in clay soil. New secondary cambia and the initiation of new, intact tertiary vessels probably play an important role in this delay. The sponginess originates also as a result of elongation of the tuber after cultivation in nutrient solution. The length of time in which a certain percentage of elongation is effected, is most determining for the moment of occurrence of sponginess thereafter.

4.10. Some more data about contraction in the plant axis

4.10.1. Moment and degree of maximum contraction in hypocotyl and root
From the figures illustrating the contraction of upper part and lower part of hypocotyl and upper part of root (e.g. Fig.4B, D, F) it is evident that this phenomenon can always be represented by a curve with a minimum. The maximum of contraction and the moment at which this maximum is reached is shown in Tables 9 and 10 for each plant. In Table 9 the growth of the plants in clay
Table 9. Maximum shortening in % of H₁, H₂ and R in the periods 11-16, 16-21 and 21-26 DAS, and thickness of tuber (T₁) 26 DAS. The 20 seedlings are arranged according to length of hypocotyl when 8 d old. Clay soil.

<table>
<thead>
<tr>
<th>Plant no.</th>
<th>H at 8 d (mm)</th>
<th>H₁ max. shortening in the period 11-16 d, etc. after sowing (%)</th>
<th>H₂ max. shortening in the period 11-16 d, etc. after sowing (%)</th>
<th>R max. shortening in the period 11-16 d, etc. after sowing (%)</th>
<th>T₁ at 26 d (mm)</th>
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<tbody>
<tr>
<td>1</td>
<td>10.5</td>
<td>11-16 16-21 21-26</td>
<td>11-16 16-21 21-26</td>
<td>11-16 16-21 21-26</td>
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<td>-12</td>
<td>-12</td>
<td>-12 28.7</td>
</tr>
<tr>
<td>13</td>
<td>13.0</td>
<td>-4</td>
<td>-4</td>
<td>-4</td>
<td>-4 28.7</td>
</tr>
<tr>
<td>14</td>
<td>13.5</td>
<td>-7</td>
<td>-7</td>
<td>-7</td>
<td>-7 26.0</td>
</tr>
<tr>
<td>15</td>
<td>13.5</td>
<td>-20</td>
<td>-20</td>
<td>-20</td>
<td>-20 24.3</td>
</tr>
<tr>
<td>17</td>
<td>14.5</td>
<td>-13</td>
<td>-13</td>
<td>-13</td>
<td>-13 30.6</td>
</tr>
<tr>
<td>18</td>
<td>14.5</td>
<td>-14</td>
<td>-14</td>
<td>-14</td>
<td>-14 21.3</td>
</tr>
<tr>
<td>19</td>
<td>15.0</td>
<td>-6</td>
<td>-6</td>
<td>-6</td>
<td>-6 24.2</td>
</tr>
<tr>
<td>20</td>
<td>16.5</td>
<td>-29</td>
<td>-29</td>
<td>-29</td>
<td>-29 24.5</td>
</tr>
<tr>
<td>Tot. no. (%) of plants with shortening</td>
<td>8 (40%)</td>
<td>14 (70%)</td>
<td>18 (90%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tot. of percentages of shortening (average)</td>
<td>108 (5.40)</td>
<td>180 (9.00)</td>
<td>493 (24.65)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 10. Maximum shortening in % of $H_1$, $H_2$ and $R$ in the periods 11-18, 18-24 and 24-30 DAS, and thickness of tuber ($T_t$) 30 DAS. The 12 seedlings are arranged according to length of hypocotyl when 8 d old. Nutrient solution.

<table>
<thead>
<tr>
<th>Plant no.</th>
<th>H at 8 d (mm)</th>
<th>$H_1$ max. shortening in the period 11-18 d, etc. after sowing (%)</th>
<th>$H_2$ max. shortening in the period 11-18 d, etc. after sowing (%)</th>
<th>R max. shortening in the period 11-18 d, etc. after sowing (%)</th>
<th>$T_t$ at 30 d (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.0</td>
<td>11-18 18-24 24-30</td>
<td>11-18 18-24 24-30</td>
<td>11-18 18-24 24-30</td>
<td>19.5</td>
</tr>
<tr>
<td>2</td>
<td>10.0</td>
<td>-5</td>
<td>-5</td>
<td>-5</td>
<td>24.2</td>
</tr>
<tr>
<td>3</td>
<td>10.5</td>
<td>-5</td>
<td>-5</td>
<td>-5</td>
<td>24.7</td>
</tr>
<tr>
<td>4</td>
<td>11.0</td>
<td>-9</td>
<td>-9</td>
<td>-9</td>
<td>15.5</td>
</tr>
<tr>
<td>5</td>
<td>11.0</td>
<td>-10</td>
<td>-21</td>
<td>-23</td>
<td>9.0</td>
</tr>
<tr>
<td>6</td>
<td>13.0</td>
<td>-3</td>
<td>-21</td>
<td>-20</td>
<td>9.8</td>
</tr>
<tr>
<td>7</td>
<td>14.0</td>
<td>-7</td>
<td>-3</td>
<td>-20</td>
<td>16.4</td>
</tr>
<tr>
<td>8</td>
<td>15.0</td>
<td>-7</td>
<td>-6</td>
<td>-10</td>
<td>21.1</td>
</tr>
<tr>
<td>9</td>
<td>15.0</td>
<td>-6</td>
<td>21</td>
<td>20</td>
<td>6.1</td>
</tr>
<tr>
<td>10</td>
<td>17.5</td>
<td>-21</td>
<td>-21</td>
<td>-20</td>
<td>13.4</td>
</tr>
<tr>
<td>11</td>
<td>18.0</td>
<td>-17</td>
<td>-17</td>
<td>-6</td>
<td>10.5</td>
</tr>
<tr>
<td>12</td>
<td>19.0</td>
<td>-16</td>
<td>-16</td>
<td>-22</td>
<td>12.2</td>
</tr>
</tbody>
</table>

Tot. no. (%): 5 (42%) 10 (83%) 12 (100%)

Tot. of percentages of shortening (average): 34 (2.83%) 128 (10.67%) 230 (19.17%)
soil from 11 till 26 d after sowing has been divided in three periods of 5 d each; in Table 10 the growth on nutrient solution from 11 till 30 d after sowing has been divided in three periods of 7, 6 and 6 d.

It is shown in the tables that contraction occurs in all 32 plants, but in some plants contraction only occurs in one of the three examined parts of hypocotyl and root. For example Table 9, plants nos. 1, 4, 14, 17, 18 show only root contraction, but plant nos. 6, 20 show only hypocotyl contraction. Hypocotyl contraction does not occur along the entire length normally; either contraction only takes place in upper or lower half of the hypocotyl or contraction occurs at different periods in these parts of the hypocotyl. Taken the data of Tables 9 and 10 together, contraction of the upper half of the hypocotyl occurs in 41% of all plants, contraction of the lower half of the hypocotyl in 75% of all plants and of the root in 94% of all plants. The moment of contraction in hypocotyl and root is different in clay soil; the maximum contraction in \( H_2 \) occurs mainly in the first period but in the root mainly during the last period of growth. In plant no. 4, Table 10, two different periods of contraction can be discerned in the upper half of the hypocotyl. When considering the intensity of contraction one can calculate the sum of percentages of maximum contraction in \( H_1 \), \( H_2 \) and \( R \). The intensity of contraction increases sharply from the upper half of the hypocotyl to the upper part of the root in both types of cultivation; taken together the averages of Tables 9 and 10 the total percentage of maximum contraction in \( H_1 \) amounts to 4.4%, in \( H_2 \) 9.6% and in \( R \) 22.6%. Applying Wilcoxon's test to the percentages of maximum contraction of the root the result does not clearly indicate an effect of the way of cultivation on the degree of contraction, although the percentages of -40% and -45% contraction of the root in Table 9 seem to point to more contraction in clay soil.

No relation can be observed between the length of hypocotyl and upper root part at 8 d and the contraction in \( H_1 \), \( H_2 \) and \( R \) in both types of culture. Perhaps less contraction occurs in the \( H_1 \) part of short hypocotyls in clay soil.

4.10.2. Comparison of plants with and without contraction in the hypocotyl; relation between maximum contraction and position of maximum thickness of tuber

In Fig. 69 a comparison is made between the growth of the tuber of 5 plants without contraction in both \( H_1 \) and \( H_2 \) (A) and the growth of the tuber of 5 plants with contraction in both \( H_1 \) and \( H_2 \) (B). These 10 plants show a normal development, but they also show more or less extremes in longitudinal growth of upper and lower half of the hypocotyl (Table 9). In both groups of 5 plants contraction of the upper root part takes place. The position of the largest diameter of the tuber \( (T_1) \), indicated with \( Z \), becomes lower in both groups of plants (cp. Fig. 7A), in absolute sense in A and in relative sense in B. The relative larger increase of \( H_2 \) is also illustrated (cp. Fig. 8), especially in plants with contraction in \( H_1 \) and the tubers in these plants with contraction in \( H \) seem to become thicker.

After calculation on 11 plants (Wilcoxon's test) it appears that the 6 tubers in which contraction has taken place (in \( H_1 \) and in \( H_2 \)) during their development become thicker than the 5 tubers in which no contraction occurred in both \( H_1 \)

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and H₂ with a critical level P ≤ 0.05. In Table 11 three plants with highest percentages of contraction in H₁ and four plants with highest percentages of contraction in H₂ are shown, and time and position of the maximum contraction are compared with the position of maximum thickness of the tuber. It is possible that contraction (negative longitudinal growth) would coincide with more increase of thickness of the tuber. However, from Table 11 it appears that in the 3 plants with strongest contraction in H₁ at 14-17 d the positions of maximum thickness of the tuber is always found in H₂. It is not a matter of delayed effect also: at 23-26 d the place of Tₜ still occurs in H₂ and has even shifted to lower positions yet. As for the lower part of the hypocotyl the situation is not clear as well. Maximum contraction in H₂ coincides with a position of Tₜ in H₁ in two plants at 17-23d; in the other plants maximum contraction in H₂ at 14-17 d coincides with a position of Tₜ exactly at or very near to the boundary between H₁ and H₂. In all 7 plants Tₜ becomes situated in lower positions afterwards corresponding to Fig. 7A, when the period of contraction has passed already. The results shown in Table 11 thus indicate that no direct or indirect relation exists between the phenomenon of contraction and increase of thickness of the tuber.

Returning to Fig. 69 the question arises about the increase of Tₜ (relative growth rate, ATₜ) in both groups of plants with and without contraction in both H₁ and H₂. After calculation on 11 plants again (Wilcoxon’s test) it appears that no statistical difference can be determined at all. This means that the increase of the maximum diameter of the tubers in both groups of plants is the same in terms of percentage. Then one may conclude that the point of departure is not equal in both groups of plants. After calculation on 11 plants again (Wilcoxon’s test) it appears that the values of Tₜ (Fig. 2) of the seedlings at 8 d are statistically different in both groups of plants: Tₜ (with contraction) > Tₜ (without contraction) with a critical level P < 0.05 (Table 9, plant no. 6 has been fixed too early; only 6 plants were available with contraction in both H₁ and
Table 11. Three plants with highest percentage of contraction in H₁ (Table 9, nos. 6, 7, 16) and 4 plants in which most contraction occurs in H₂ (Table 9, nos. 2, 8, 10, 19). Time and position of maximum contraction are compared with the position of maximum thickness in the hypocotyl. This position is indicated as a fraction of the total length of the concerning part of the hypocotyl (H₁ or H₂) counting from cr. Clay soil culture.

<table>
<thead>
<tr>
<th>Time and position</th>
<th>H₁</th>
<th></th>
<th>H₂</th>
<th></th>
<th>H₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Most contraction in H₁</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T₀, mm</td>
<td>30%</td>
<td>0.67</td>
<td>0.71</td>
<td>0.92</td>
<td>0.70</td>
</tr>
<tr>
<td>ΔT₀, %</td>
<td>12.1</td>
<td>537</td>
<td>17.0</td>
<td>710</td>
<td>9.2</td>
</tr>
<tr>
<td>Position of T₀ from cr.</td>
<td>H₂</td>
<td>H₂</td>
<td>H₂</td>
<td>H₂</td>
<td>H₂</td>
</tr>
<tr>
<td>Most contraction in H₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T₀, mm</td>
<td>29%</td>
<td>0.53</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>ΔT₀, %</td>
<td>8.7</td>
<td>383</td>
<td>5.8</td>
<td>190</td>
<td>6.3</td>
</tr>
<tr>
<td>Position of T₀ from cr.</td>
<td>H₁</td>
<td>H₂</td>
<td>H₂</td>
<td>H₂</td>
<td>H₂</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time and position</th>
<th>H₁</th>
<th></th>
<th>H₂</th>
<th></th>
<th>H₂</th>
</tr>
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<tbody>
<tr>
<td>Most contraction in H₁</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T₀, mm</td>
<td>37</td>
<td>106</td>
<td>70</td>
<td>147</td>
<td>17</td>
</tr>
<tr>
<td>ΔT₀, %</td>
<td>3.7</td>
<td>106</td>
<td>3.4</td>
<td>70</td>
<td>3.7</td>
</tr>
<tr>
<td>Position of T₀ from cr.</td>
<td>H₁</td>
<td>H₂</td>
<td>H₂</td>
<td>H₂</td>
<td>H₂</td>
</tr>
</tbody>
</table>

Table 12. This means that seedlings with greater diameters of the collum radicis will have larger tubers afterwards, and show contraction of the hypocotyl. Finally the question arises about the length of the tubers. The total length

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of the tuber at 26 d is not equal to the value of L (Fig. 2), because the higher part of \( H \) may at first not increase in thickness and may not really belong to the tuber at 26 d and the contribution of a root part to the tuber varies enormously after 26 d. So the total length of the tuber has been estimated at sight. After calculation on the available data of 5 plants in each group (Fig. 69A, B) it appears (Wilcoxon’s test) that no significant difference can be found. Thus the contraction in \( H_1 \), \( H_2 \) or \( R \) does probably not have any demonstrable influence on the diameter and length of the tubers.
5. Discussion

5.1. Growth analysis

The contribution of hypocotyl and root to the origin of the radish tuber may vary extremely (Golinska, 1928; Hayward, 1938). Even within the variety Saxa Nova the growth of the tuber appears to be more complicated than the models of the varieties Cherry Belle and Long White Icicle (Ting and Wren, 1980) show. The tuber development in 'Saxa Nova' cannot really be recapitulated in one series of drawings as a model, due to the large individual diversity regarding relative position and time of measured values (Fig. 2 and e.g. Table 1, Figs 4, 5, 8).

The lateral expansion of the tuber of 'Saxa Nova' is always positive during more than 35 d after sowing and may be achieved by a combination of the activity of the vascular cambium and cell enlargement in the differentiating xylem and phloem as in the tubers of Cherry Belle as in Long White Icicle (Ting and Wren, 1980). That the growth activity within the xylem must play a role of importance in the lateral expansion of the tuber can be deduced from the very often occurring fissuring of the developing tuber, especially when growing on nutrient solution. The degree of lateral expansion of the tubers appears to be independent of the medium in which the roots grow (Fig. 6A, B) when considering the 7.5 d growth retarding due to transplanting of the seedlings in the nutrient solution after 8 d. Probably the nutrient conditions were practically optimal in both cases. In clay soil, the maximum diameter of the tuber does shift to lower positions along the hypocotyl during the first 29 d of culture (Fig. 7A). On nutrient solution this position shows a conspicuous shifting from positions above the collum radicis to below this point; after 40 d of culture the maximum diameter moves upwards again (Fig. 7B). The position of the maximum diameter appears to be strongly dependent on the moment of measurement.

The longitudinal growth of the upper half of the hypocotyl, the lower half of the hypocotyl and the upper 5 mm of the root during development of the tuber may be both positive and negative. The extent of longitudinal growth in these parts is independent of each other (Tables 1, 2, 3) and the same differences in longitudinal growth occur in both culture media of the roots.

The negative longitudinal growth, contraction, always shows a curve with a minimum (e.g. Fig. 4B, D, F). The lower this shortening is measured along the plant axis, the more contraction is found (Tables 9, 10); the least contraction is found in the upper part of the hypocotyl, the most in the root. The moment at which this process of shortening occurs differs very much among the plants (Table 10). Contraction of a certain part of hypocotyl or root does not mean that this part remains shorter afterwards; the following longitudinal elongation
may compensate for the contraction completely (cp. Fig. 4C and D). No direct relation appears to exist between the contraction and the lateral expansion of the hypocotyl; the position of most contraction in the hypocotyl does not coincide with the position of maximum thickening of the hypocotyl (Table 11). Calculated on the total number of plants (see Table 4) the medium in which the roots grew appears to influence the longitudinal growth (Fig. 8); in clay soil the lower half of the hypocotyl shows a strong longitudinal increase after 29 d and in nutrient solution the upper half of the hypocotyl becomes longer after a comparable period of development. This difference in longitudinal growth appears strongly dependent on the moment of measurement (Fig. 8).

The positive longitudinal growth of the hypocotyl continues a long time. Suzuki (1978) found a relationship between hypocotyl elongation and thickening; thickening was insufficient in radishes with long hypocotyls and stimulation of hypocotyl elongation during 8 d after germination is considered to counteract the xylem differentiation and the thickening. A relation between hypocotyl elongation and thickening during such an early stage of growth has not been investigated in this work but this relation has not been found during later stages of development (Tables 2, 3).

In conclusion it can be determined that the shape of the tuber 30 d after sowing is still changing. The radish tuber must be interpreted morphologically as a mass of tissue that transformed in all directions during development; this remodelling of the tuber continues a long time. The end of the remodelling of the tuber in longitudinal direction coincides with the moment when the flowering stem reaches about 10 cm after about 30 d in clay soil culture and 53 d in nutrient solution and when a closed xylem cylinder originates in the tuber. Simultaneously libriform fibres are formed again by the same initials that made the xylem parenchyma temporarily, and rays originate. Up to this moment it is not possible to construct a model of the development of the radish tuber, not even of a particular variety.

5.2. Sponginess of radish tuber tissue

It is shown that the upper and lower half of the hypocotyl and also the upper part of the root elongated very much (Figs 4, 5: $\Delta H_1$, $\Delta H_2$, $\Delta R$). Consequences of this elongation become especially evident in the rigid walls of the xylem vessels. Soon after the beginning of elongation (Fig. 26C) the longitudinal vessel walls show fractures or the vessel members are separated. All the living elements of the tuber, including the interxylary phloem, possess the capacity of elongating, and remain temporarily intact. The proportion of functional vessels in the tuber is shown by way of the transport of an eosin solution (Gaff, Chambers and Markus, 1964). It appeared that only the last differentiated vessels in a strongly elongating tuber were intact and were able to transport the transpiration stream (Fig. 64). The coming to a standstill of the transpiration stream through the central and older part of the tuber must lead to deficiency of water
and nutrients in the mass of parenchyma and lead to stress in these actively expanding and usually dividing (diffuse secondary growth, Tomlinson, 1961; Esau, 1964) cells. Joyce, Aspinall and Edwards (1983) investigated the influence of water deficit on growth and anatomy of the radish fleshy axis and found a reduction of the expansion of the 'ray' parenchyma cells in the tuber by even a comparatively mild stress regime, and the expansion was completely inhibited by severe stress. Hagiya (1952), investigating the physiological circumstances under which spongy tissue arises in the radish tuber, found the disappearance of sugar in the large types of cells of the xylem parenchyma which were far apart from the conducting tissue. He also found that the content of soluble matter in those large cells around the centre of the tuber that showed sponginess, was less than in the parenchyma cells in the centre and in the more circumferential part of the tuber. Hagiya concluded that the rapid growth of these large cells and the difficulty in supply of nutrition from the conducting tissue causes a sort of starving condition. Krug and Liebig (1979) found that sponginess in radish tubers var. sativus, cv. Hilmar was stimulated by low light intensity and high temperatures, i.e. circumstances that will stimulate the elongation of the seedlings and therefore the breakage of the vessels. Park and Fritz (1983) and Takano (1966c) found that after optimum fertilization the effect of surplus nutrient supply on sponginess was absent. They concluded that this result might also be attributed to the poor effect that fertilization has on this characteristic. Hey and Kobryn (1988) however, found that a higher nutrient concentration at somewhat lower daytime temperatures was promising to improve radish tuber quality, diminishing the occurrence of sponginess.

In the tubers of ‘Saxa Nova’ three stages of development to sponginess were distinguished (Fig. 52). Sponginess becomes evident when the largest cells around the centre (cp. Fig. 28) die and will contain gas emboli (e.g. Figs 42, 43; cp. Hagiya, 1952); this stage has been called cellular sponginess. Lacunar sponginess as the next stage originates when lacunae form in the older parts of the tuber as a result of tearing of tissues after elongation in that part of the tuber. Elongation proved to be the cause of breakage of vessels, and the subsequent development of sponginess after 7-8 d when growing in clay soil and about 10 d later when growing in nutrient solution (Figs 26, 38, 59; Tables 6, 8). The conclusion of above mentioned authors that fertilization has no direct influence on the occurrence of sponginess is therefore correct, although large cells at great distances from the vessels seem to suffer from starvation. Low light intensity will probably have more influence, when stimulating elongation. The starving conditions in the large cells around the centre cannot be caused by some interruption of the phloem transport as these tissues do not break by elongation of the tuber initially (e.g. see Fig. 31). Therefore, the main cause of the occurrence of sponginess is the interruption of the apoplastic transport. Temporarily the broken vessels will contain some xylem sap yet (root pressure), but these closed canals (Fig. 33) will not refresh their contents any more. When new xylem vessels differentiate some recovery of the elongation damage can occur as happened in older tubers cultivated in nutrient solution (Figs 66-68).
In conclusion it may be said that prevention of sponginess in radish tubers is only possible by avoiding or retarding the strong local elongation of the tuber. As the impulse for elongation is to be found in the longitudinal activity of the vascular cambium, probably (synergistically) activated by auxin and cytokinin (see Webster and Radin, 1972; Vreugdenhil, Oerlemans and Steeghs, 1984), retarding of elongation might be effected by inhibiting these growth regulators at a certain moment in development. Another way of escape can be the selecting of tubers with vascular cambia that do not have the property of longitudinal activity, a property that might be characterized by a particular type of initials or by especially directed types of initials, in the vascular cambium. Finally tubers with a relative early origin of tertiary vessels may resist the interruption of the apoplastic transpiration stream.

6. Acknowledgements

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7. References


