Stellingen

1. Een chrysant met wijde en lange vaten is gevoeliger voor vroegtijdige bladverwelking op de vaas dan een chrysant met nauwe en korte vaten. (dit proefschrift)

2. De vatopbouw van een snijbloem is *a priori* niet optimaal voor het huiskamergenot. (dit proefschrift)

3. De vatlengteverdeling in een dwarsdoorsnede van de stengel is niet gelijk aan de vatlengteverdeling in een stengelvolume. (M.H. Zimmermann and D. Potter. 1982. *IAWA Bulletin n.s.* 3: 103-109; dit proefschrift)

4. Een xyleemvat heeft niet een vooraf vastgestelde lengte. De lengte van een xyleemvat is stochastisch bepaald volgens een proces wat beschreven kan worden met een instelbare halfwaardelengte. (dit proefschrift)

5. Een veredelaar kan niet elke vatopbouw verkrijgen ingeval van het mechanisme van een instelbare halfwaardelengte. (dit proefschrift)

6. Een fenotype is niet alleen het resultaat van het genotype en omgevingscondities, maar is ook onderhavig aan stochastische variatie. (dit proefschrift)

7. De tracheïde is een xyleemvat dat uit één vatelement bestaat.

8. Het feit dat wij stochastiek gebruiken om de werkelijkheid te beschrijven duidt op het menselijk onvermogen de werkelijkheid ten diepste te bevatten.
   "God dobbelt niet" (Albert Einstein)

9. NWO wil met de Vernieuwingsimpuls het wetenschappelijk onderzoek opfrissen met meer creativiteit en onorthodoxe ideeën. Echter, wanneer de onderzoeksscholen de voorselectie bepalen, wordt dit streven in de kiem gesmoord.

10. Wetenschap wordt gekenmerkt door twijfel; het christelijk geloof wordt gekenmerkt door zekerheid.

11. Veel oudere werknemers verdienen meer ondersteuning en waardering dan ze krijgen.

12. De analogie tussen de behandeling van het vluchtelingenprobleem en het mond- en klauwzeerprobleem binnen het fort Europa geeft aan dat globalisering een holle frase is, zelfs voor beleidsmakers op het hoogste niveau.

13. Veiligheidshalve moeten biologische batchproducten (bijvoorbeeld soja-olie, diermeel of bloed) samengesteld worden uit een zo klein mogelijk aantal traceerbare oorspronkelijke partijen.

14. Vermaatschappelijking van zorg moet niet primair gedreven worden door economische motieven, maar door gezonde naastenliefde.

Stellingen behorend bij het proefschrift:
Functional anatomy of the water transport system in cut chrysanthemum
Jaap Nijsse
Wageningen, 22 juni 2001

Functional anatomy of the water transport system in cut chrysanthemum

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Functional anatomy of the water transport system in cut chrysanthemum

Jaap Nijsse

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
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Voorwoord

Het gras verdort, de bloem valt af; maar het Woord onzes Gods bestaat in der eeuwigheid (Jesaja 40:8)

De natuur is indrukwekkend. Je hoeft niet ingewikkeld te doen om dat te kunnen beamen. Toch ging er een nieuwe wereld voor me open toen ik op mijn verjaardag een verrekijker van mijn ouders kreeg. Daarmee kun je de wilde vogels goed bekijken. Wat is een middelste zaagbek mooi! Later, op de middelbare school en in Wageningen leerde ik de microscoop gebruiken. Alweer ging er een wereld open. Ik zag een trichoombos op een *Arabidopsis* blaadje, de hofstippels in een houtvat: wondermooi! Ik dank God dat Hij dit alles zo mooi geschapen heeft en dat wij hiervan genieten mogen. Ik ben Hem dankbaar voor de jaren dat ik aan dit proefschrift werken mocht. Ik hoop dat ik met dit proefschrift een goede bijdrage geleverd heb aan de plantenanatomie en dat de snijbloemenwereld er voordeel van heeft. Daarnaast hoop ik hiermee een stukje verwondering uit de microwereld over te brengen.

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Jaap

Abbreviations

γ	surface tension
η	viscosity
Θ	contact angle
λ	exponential factor
ρ	density
τ	half-length value
ψ_{xp}	xylem pressure
a	acceleration
$a_<$	semiminor axis of an ellipse
$a_>$	semimajor axis of an ellipse
A	(cross-sectional conduit) area
c	constant / normalisation factor
C_a	air concentration
C	hydraulic conductance
CS	hydraulic conductivity
d	diameter
D	distribution
D_a	diffusion constant of air in water
F	force
g	gravity constant
h	height
k_a	solubility of air in water
K_h	percentage of initial hydraulic conductance
L	length of a xylem vessel
n	number of repetitions
N	number of conduits or vessel members
N_a	number of molecules
P	pressure
P	fraction of vessels (chapter 2.3)
P_{air}	air pressure
P_{atm}	atmospheric pressure
P_c	perimeter of a cross-sectional conduit area
q	flow rate
r	radius
Re	Reynolds number
R_{fp}	percent of refilling
R_h	hydraulic resistance
RH	relative humidity
t	time
T_{half}	time to reach half K_h
v	velocity
x	distance/length
Y	value / number of red pixels

Chapter 1

General Introduction

J. Nijsse

The problem of early leaf wilting

Early leaf wilting, defined as 'leaf wilting within the first days of vase life', is a serious problem in the cut chrysanthemum production chain. Symptoms, shown in figure 1, are wilted leaves while the flowers still appear fully turgid. Early leaf wilting occurs rather unpredictable (Van Meeteren, 1989). Therefore, the vase life of cut flowers that suffer from early leaf wilting is difficult to guarantee. But, flower retailers and consumers ask more and more for a guaranteed vase life. Different cultivars (varieties) can show a different sensitivity to early leaf wilting (Van Doorn, 1997; Van Doorn and Reid, 1987; Van Meeteren, 1989; Van Meeteren, 1992). Indications exist that growing conditions can influence the problem (Marousky, 1973), as well as post-harvest handling (Van Meeteren, 1992; Van Meeteren and Van Gelder, 1999). Different batches, grown in different greenhouses or with a different harvest date, often show remarkable differences in occurrence of the problem of early leaf wilting (Van Meeteren and Van Gelder, 1999). It is conceivable that early leaf wilting originates from a negative water balance. In other words, the output of water (transpiration) exceeds the input of water (vase water uptake) in the cut stalk. The leaf cells loose their turgor and, as a consequence, the leaves loose their 'stiffness'. Van Meeteren (1989) hypothesised that early leaf wilting is caused by the presence of air that has been introduced at the cut surface in xylem vessels. In intact plants air cannot easily enter xylem vessels

Fig. 1. Chrysanthemum cut flower with early leaf wilting symptoms. Leaves have lost their turgescence, but flowers look intact. (Photograph: Uulke van Meeteren)

(Zimmermann, 1983). In cut flowers all xylem vessels that cross the plane of the cut are opened and air may enter these cut open vessels. Air bubbles in xylem vessels (air-emboli) will cause a considerable decrease of the water conductivity of these vessels. Consequently, the resulting decrease in water uptake may cause a negative water balance, and in turn, wilting of leaves.

Is vascular blockage by air embolism the cause of early leaf wilting?

The following experimental results indirectly point out that early leaf wilting is indeed a consequence of air-blockage of xylem vessels:

- *Recutting under water reduces the problem (Van Meeteren and Van Gelder, 1999).*

The idea is that the new cut surface crosses vessels that were not crossed by the former cut surface. Consequently, the new cut surface will contain fewer vessels with air blockage. This experimental result points to vascular blockage in general, it does not exclude vascular blockage by particles, e.g. micro-organisms or macromolecules. However, in the same experiment, recutting in air did not result in a good water balance, which indicates new air embolisms rather than blockage by particles. Furthermore, early blockage by micro-organisms is not to be expected, for micro-organisms need some time (a few days; Van Doorn *et al.*, 1991) to multiply to 'clogs' that block the vessels.

- *Degassed vase water reduces the problem (Van Meeteren, unpublished).*

Air-saturated water cannot dissolve air bubbles. In contrast, degassed vase water will dissolve as much air as needed to ultimately reach a saturating air-concentration. Degassed water, therefore, will dissolve the air-blockages (at least to some extent) and restore the xylem water transport.

- *Ice-mixed vase water reduces the problem (Van Meeteren, 1992).*

If ice is added to vase water, no early leaf wilting occurs. If water is cooled by adding ice, it can dissolve considerable amounts of air because of two reasons. First, the lower the water temperature, the more air can be dissolved (Van Meeteren, 1992). Second, ice crystals contain no air. Thus, after melting of the ice crystals, the vase water has a high capacity to dissolve air. Like degassed water, ice-mixed vase water is hypothesised to dissolve partly or completely the air blockages in the xylem vessels.

- *Vacuuming the stem in the vase reduces the problem (Van Meeteren, 1989).*

If the vase and the stem part that is in the vase are vacuumed, leaf wilting is prevented or, if already present, the leaf turgidity is restored.

The main project

In 1997, three groups of Wageningen University and the Research Station for Floriculture and Glasshouse Vegetables started a joint project "Predicting differences in water balance during vaselife of cut flowers as caused by growing conditions and genotype, using an explanatory quantitative model of water uptake and -transport in cut flower-stalks". This project focussed mainly on early leaf wilting in cut

chrysanthemum. The aim of the project was to come to a fundamental biophysical understanding of the mechanisms underlying the dynamics of the water balance in order to enable a better prediction of vase life. A fundamental approach was needed for several reasons. First, there exists a very restricted knowledge of the influence of growing conditions on the plant development, and on vase life. Second, physiology of cut flowers differs in several aspects from intact plants. Therefore, cut flowers need their own fundamental research. Third, many years of rather applied or empirical investigations on cut flower quality yielded no satisfying tools to predict vase life. This, added to the pressure of the market to provide a strong guarantee of quality, raised the question: 'What are the biological/physical processes behind the problems with the water balance in cut flowers?'. Or, with the focus of this project: 'What are the processes behind early leaf wilting?'.

Five researchers from different disciplines were directly involved. Five sub-projects were formulated, roughly one sub-project for each researcher. Figure 2 shows the main research project divided into the five sub-projects. Thus, knowledge was bundled from the fields of water transport physiology, anatomy, cut flower quality research, and cultivation research. This thesis deals with the anatomical aspects of the main project, carried out in a four-year PhD-project from January 1997 till January 2001. The strong co-operation within the main project resulted in several joint publications. Some of these joint publications have been included in this thesis.

The anatomical aspects of early leaf wilting: a PhD-project.

The anatomical project had the general aim to provide the anatomical knowledge and methods within the above-mentioned main project. More specific, several aims were formulated:

1. To quantify the xylem hydraulic architecture in chrysanthemum stems.
2. To visualise xylem contents, in order to enable embolism studies.
3. To compare the stem hydraulic architecture of different stems in relation to their water balance.

Cut stalks of chrysanthemum were investigated to reveal the three-dimensional structure of the water transport system. Especially the cut surface and the (xylem) pathways starting from the cut surface were studied. Several conventional and modern methods of plant microscopy were used. Anatomical characteristics of thin stem sections were studied using digital image analysis. Cryo Scanning Electron Microscopy (cryo-SEM) was used to provide accurate information concerning the presence or absence of water in the stem tissues. Therefore a method was developed to obtain flat cross-sectional planes through frozen stems in order to visualise the xylem contents. Finally, different batches of chrysanthemum stems were compared by their anatomical properties: e.g. vessel dimensions, xylem thickness and calculated conductivity.

The research was conducted at the Horticultural Production Chains Group and at the Laboratory of Plant Cell Biology of Wageningen University.

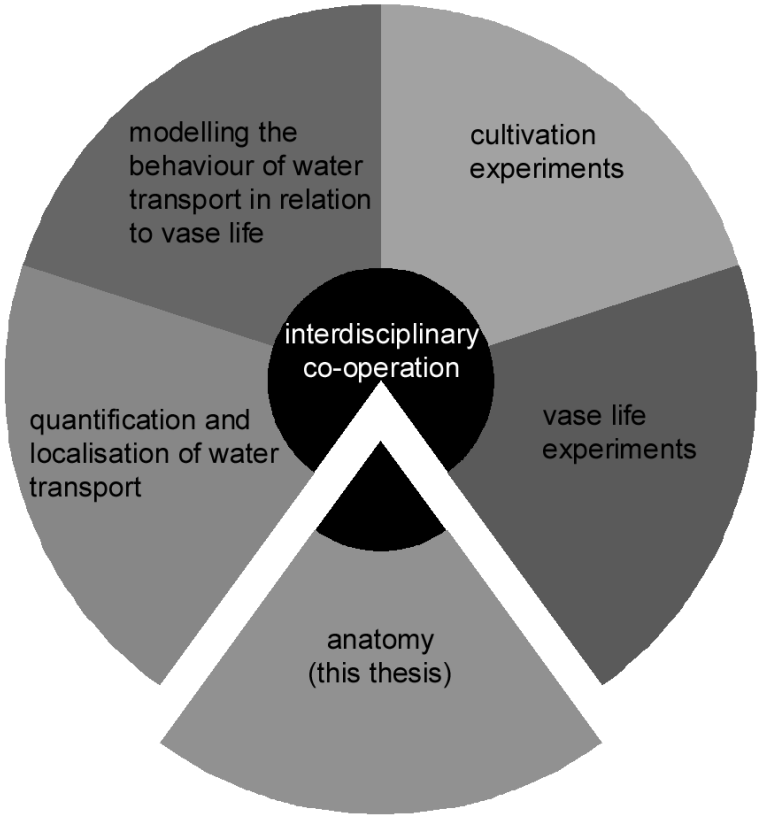

Fig. 2. Five research projects bundled in the project "Predicting differences in water balance during vaselife of cut flowers as caused by growing conditions and genotype, using an explanatory quantitative model of water uptake and -transport in cut flower-stalks". This thesis deals with the anatomy-part.

The plant material

The chrysanthemum (*Dendranthema x grandiflorum* Tzvelev) originates from China and Japan, where it has been cultivated probably long before the Christian era (Sander, 1931). The name chrysanthemum comes from the Greek words *chrysos* (gold) and *anthos* (flower), referring to the yellow colour of the original species (Rupprecht, 1833). In Europe, the first chrysanthemums were introduced in The Netherlands in 1688 (Rupprecht, 1833). These first introduced plants disappeared, but in 1764 chrysanthemums were introduced in England and in 1789 in France (Rupprecht, 1833) and from that time on the chrysanthemums were established in Europe and also in America (White, 1930). The further history of the cultivated chrysanthemum is a success story, not at least since the flowers quickly produce variations, both after crossing and by mutations (Morrison, 1931). Since 1969 the chrysanthemum is the second most important cut flower in the Netherlands, after the rose (Spaargaren, 1996). The present chrysanthemum is a complex hybrid of (most probable) *Chrysanthemum indicum* and *Chrysanthemum morifolium* (White, 1930).

Mostly the plant is hexaploid, with 54 chromosomes (Scopes and Langton, 1976). The chrysanthemum belongs to the Asteracea (Compositae). The Asteraceae form a large family of dicotyledonous plants, having their flowers arranged in dense heads of many small florets with anthers united in a tube (Raven *et al.*, 1992). There are many types of chrysanthemums. The variation exists mainly in the number, colour and shape of the flowers. A common chrysanthemum type is the spray type, where the terminal flower bud is removed, to obtain a cluster of several more or less equal flowers. Santini's are a special group of spray chrysanthemums, having small flowers with a diameter of less than 4 centimetre. Stalks with single blooms, which can grow very large, are obtained by removing all buds except the terminal one (Spaargaren, 1996).

Chrysanthemums are short-day plants, which means that under natural conditions they flower in the autumn. There exist numerous ways of cultivating chrysanthemums both outside and in the greenhouse. Recently, Carvalho and Heuvelink (2001) have reviewed the influence of greenhouse climate and plant density on external quality of cut chrysanthemums. We present here the year-round cultivation method as used for spray-type cut chrysanthemums in the greenhouses of Wageningen University. This cultivation method must be regarded as just an example, but it is used to produce the plant material used in the experiments of this thesis.

Cuttings are obtained from stock-plants that are kept in the vegetative stage by a long-day (LD) treatment at relatively high temperature (19°C). After rooting the cuttings are transplanted into 14 cm diameter plastic pots with commercial potting soil. The plants are watered every day. The average temperature is kept at 18°C, and the photoperiod is artificially kept at 16 hours (LD), either by use of a black screen at summer days or by use of artificial light at winter days. After three to four weeks, when 15 to 17 leaves have formed, the photoperiod is shortened to 8 hours (short day: SD) to induce flowering. The photoperiod of 8 hours is maintained until harvest, 12 or 13 weeks after potting. The terminal bud is removed by hand four weeks after transition to the short-day treatment. Plants are harvested and used for experiments at commercial maturity, which is reached at the opening of the outer two rings of disk florets in the flowers.

This cultivation method is applied to our experiments and differs from commercial cultivation methods since the plants are grown in pots. The plants are placed on a platform in a square arrangement (distances are equal to the pot diameter). In contrast, commercial cuttings are rooted in peat blocks and placed on the soil in the greenhouse at a density of about 55 plants per square meter, dependent on the cultivar and the season (high light allows a high planting density). The reason for growing plants in pots is that intact plants can be transported from a long-day greenhouse compartment to a short-day compartment. This enables frequent supply of mature chrysanthemums from just two greenhouse compartments. Furthermore, plants grown in pots can be transported as a whole to the laboratory.

Structure of this thesis

- **Chapter 1. General introduction**, containing a description of the problem of early leaf wilting, the outline of the (PhD-) project, a general description of the chrysanthemum, and an outline of this thesis.
- **Chapter 2. Hydraulic architecture of the cut chrysanthemum.** First, a general anatomical and morphological description of the cut chrysanthemum is provided, with special reference to the water transport system. The second part is a mathematical description of the hydraulic architecture of the chrysanthemum stem, related to the measured hydraulic conductivity. In the third part of this chapter, a theory is postulated that explains the exponential vessel length distributions as commonly found in chrysanthemums and other vascular plants.
- **Chapter 3. Visualisation of xylem contents.** The use of several methods to acquire planes in frozen materials are analysed, discussed and contrasted. This method of cryo-planing, and subsequent cryo-scanning electron microscopy, is a unique method to visualise the contents of xylem vessels in cut chrysanthemums at high resolution.
- **Chapter 4. Dynamics of embolism induced at the cut surface.** Partly with use of the proposed cryo-planing techniques in chapter 4, several studies are carried out to study air entrance and removal in xylem vessels after cutting and subsequent treatments.
- **Chapter 5. Embolism repair related to stem hydraulic architecture.** This chapter starts with an interdisciplinary discussion concerning induction and removal of air blockage in cut flowers. In the second part is tried to get a simple description of the vessel dimensions, and to relate the thus described vessel characteristics to water balance behaviour.
- **Chapter 6. General discussion.** In this chapter the state of the art in the investigated matter is evaluated. Several points that need attention, both for further research and 'just to think about' are highlighted.

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Chapter 2

Hydraulic architecture of the cut chrysanthemum

Overview

To understand the influence of the vascular anatomy on water uptake and related problems in cut flowers, a proper knowledge of the hydraulic architecture of the plant is needed. The morphology and anatomy of the cut chrysanthemum is described, with an emphasis on the water transport system (2.1). To enable calculation of the hydraulic conductivity of the chrysanthemum stem, the stem xylem vessel anatomy of one batch of flowers was thoroughly analysed using digital image analysis (2.2). Dimensions and number of vessels along the stem decrease higher up the stem. Several anatomical parameters were quantified and expressed in mathematical descriptions as function of height up the stem. Measured and calculated hydraulic conductivity are compared and discussed. The results of the vessel length analyses inspired to a new theory on how plants determine the length of xylem vessels (2.3). The vessel length theory is based on steered stochastics and provides the most simple explanation of vessel length regulation in plants.

2.1

Morphology and anatomy of the cut chrysanthemum

J. Nijsse

Introduction

To come to a detailed knowledge of the anatomical aspects of water transport in cut chrysanthemums, first a general description of cut chrysanthemums is needed. This chapter presents an overview of the morphology and anatomy of cut chrysanthemums, with an emphasis to the water transport system.

The plant

Chrysanthemums grown for a cut flower are propagated by cuttings of terminal shoot parts. The cuttings grow out to an erect stem with about thirty internodes. The typical internode length is 2-4 cm. Significant branching occurs in the highest (about seven) nodes after flower induction and removal of the terminal 'crown' bud. Flowers are located at the end of these branches (Fig. 1). The next paragraphs describe the main organs of the chrysanthemum plant. Unless stated otherwise, the information was obtained by observations by the author. Most information was acquired in the more specific experiments of the next chapters. If useful, the method to obtain the information is mentioned or referred to.

Fig. 1. Chrysanthemum plant (cultivar 'Cassa') grown in a pot at Wageningen University (Photograph: Koos Keijzer)

The shoot

The vegetative shoot is erect with a helical arrangement of the leaves. This leaf arrangement is regular, and corresponds to the fraction of 5/13 in the series of Fibonacci. This means that subsequent leaves have an angle of divergence of (5/13)*360°=138.5°. Thus, starting from a certain leaf along a

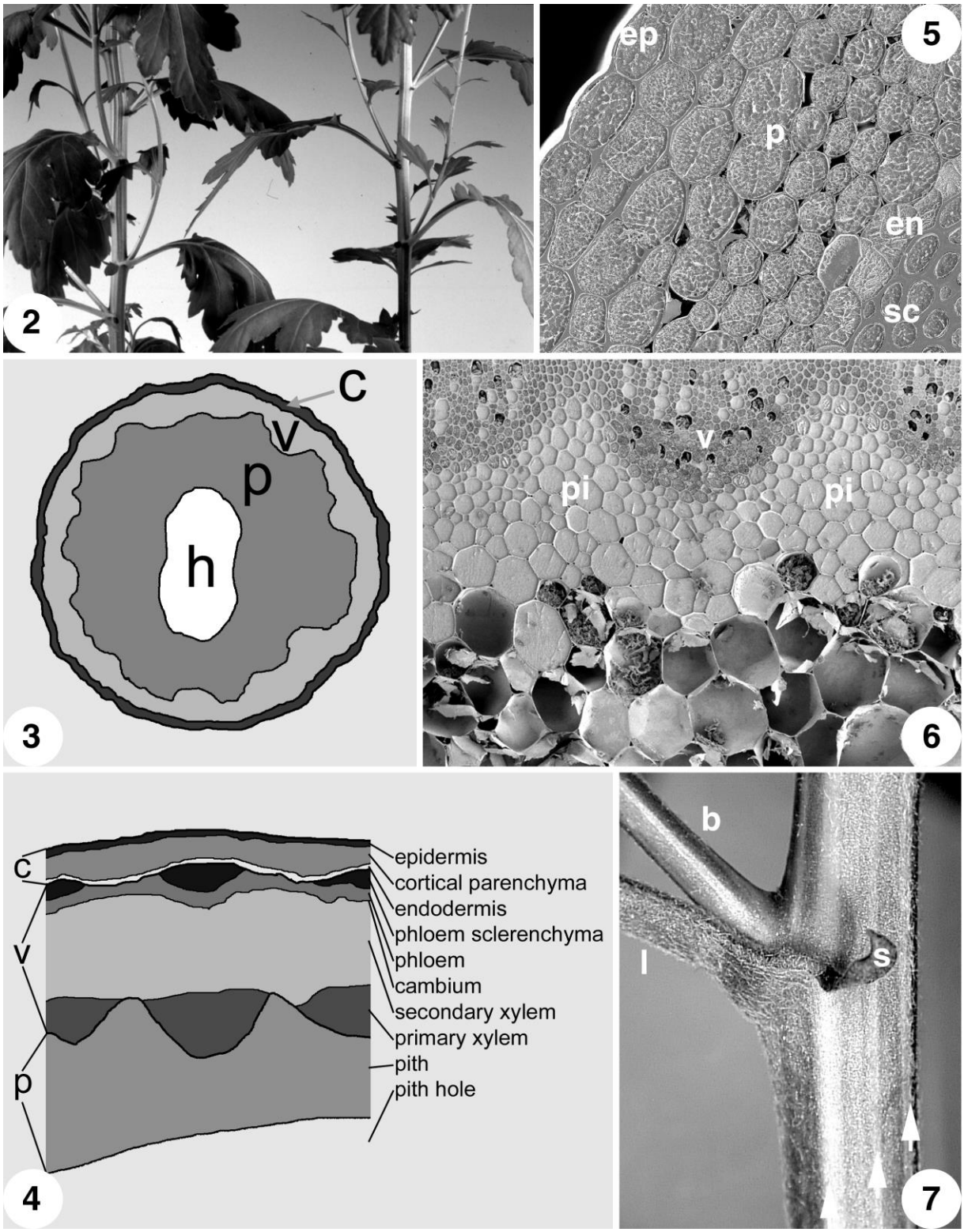

Fig. 2. Two chrysanthemum stems. Chrysanthemums have a helical leaf arrangement. The left stem has a right-handed leaf orientation (leaves wind from down-left to up-right). The right stem has a left-handed leaf orientation. The two branches of the right stem have both a right-handed leaf orientation. **Fig. 3.** Schematic drawing of a chrysanthemum stem transverse section. Roughly the stem consists of three

concentric tissue layers: the cortex (c), the vascular tissue (v), and the pith (p). Often a pith hole (h) occurs. **Fig. 4.** Schematic drawing of a detail of the three tissue-layers shown in Fig. 3. **Fig. 5.** Cryo-planed transverse section through the cortex of a chrysanthemum stem. The cortex consists of the epidermis (ep) of one layer, several layers of cortex parenchyma (p), and the endodermis (en; one layer). Some fibres of the phloem sclerenchyma (sc) are visible in the lower right corner. Horizontal field width = 203 µm. **Fig. 6.** Cryo-planed transverse section through the pith (pi) of a chrysanthemum stem. Closer to the vascular tissue (v), pith cells tend to be smaller and smaller. The larger pith cells more near to the centre of the stem are often empty. Horizontal field width = 1220 µm. **Fig. 7.** Chrysanthemum stem node with a leaf (l) and a flower branch (b). The stem bears longitudinal ribs (arrows), caused by the sclerenchyma bundles of the underlying vessel bundles. s: stipula of the leaf.

chrysanthemum stem, the first leaf that is located straight above it is 13 leaves and 5 helical windings higher. Interestingly, the windings can be either left- or right-handed. (A stem has a right-handed leaf orientation if the helical leaf arrangement winds from down-left to up-right along the facing stem side.) Even within one chrysanthemum plant the branches can have differently oriented windings. Figure 2 shows two chrysanthemum stems. The left stem in this figure has a right-handed leaf arrangement, and the right stem has a left-handed leaf arrangement but both branches of the latter stem have a right-handed leaf arrangement.

Figure 3 provides a schematic overview of a transverse stem section. In main lines, the stem can be divided into three concentric tissues: cortex, vascular tissue, and pith. Figure 4 shows the three tissues in more detail. The cortex (Fig. 5), covered by the epidermis, consists of some layers of parenchymatic cells and the endodermis (Fig. 4). The epidermis has few stomata and trichomes. The cuticle is thin and exhibits no particular wax structures. The parenchymatic cells are comparable to the mesophyl in leaves: they possess chloroplasts and are loosely packed with air spaces in between. The next tissue layer, the vascular tissue, is discussed below in detail. The central tissue, the pith, is shown in Fig. 6. Pith cells die at maturity, which can be concluded from the poor solute content in the cells as appears from the smooth ice crystallisation structure in the cryo-SEM image. The more eccentric the pith cells are positioned, the smaller they tend to be. The centre of the pith mostly lacks water and often a pith hole occurs. In cut chrysanthemum stems the pith is often large, which provides the stem to have the mechanical properties of a pipe, rather than a bar. As widely known, a pipe is more rigid compared to a bar with the same constituents per length unit.

More than the cortex and the pith, the vascular tissue needs a thorough analysis in this thesis, because of its important role in water transport from the roots (or cut plane) to the transpiring tissues. In the primary stem, the vascular tissue is arranged in bundles, the vascular bundles. In plants in general, vascular bundles can be more or less interconnected. At the leaf and branch insertions certain bundles leave the stem as leaf or branch traces. The gaps above the places where bundles left the stem are in a regular way filled by branches of continuing bundles. The total number of bundles in the stem is therefore stable and not dependent on the amount of bundles that leave the stem. We studied the vascular bundle network in chrysanthemum stems in order to understand the primary water transport ways. At

the outside of a stem ribs can be felt and seen (Fig. 7). They represent large vascular bundles since they are the thick sclerenchymatous fibres at the outside of the phloem. We tried to construct a rough scheme of the bundle network, just by studying the stem outside. However, even after removal of the cortex the exact branching pattern of the bundles was not always visible. We therefore made serial sections through a stem section that included a node and the two adjacent internodes. From these sections we reconstructed the detailed vessel bundle network. It may be noted that we could limit such a reconstruction to only one internode-node-internode segment, because of the repetitive organisation of the vascular network. Figures 8a-f show six representatives of the serial sections from one internode to the next. As already appeared from the arrangement of the leaves, the stem contains 13 possible positions for leaves (13 orthostiches; Reinders *et al.*, 1943). In Fig. 8f the numbers indicate the position of the main leaf trace of the corresponding leaf. The bundle at zero is the main trace of the leaf that is positioned at the node directly above the section. The bundle at '1' leads to the next leaf, etc. Additionally, the leaf below the place of section is positioned at '12'. The section in this figure is oriented upside up, and indicates that the leaf arrangement must be right-handed. The result of the reconstruction of the vessel bundle network in this right-handed stem is shown in Fig. 9 (method of graphic representation derived from Benzing, 1967). In this figure, the height is compressed 25 times compared to the width, to enable proper visualisation of the network. In reality therefore, the network is much more stretched. The relative importance of the individual bundles (e.g. the hydraulic conductivity) cannot be concluded from this network-scheme. In Fig. 8f, the bundles marked with a low number (closer to 'their' leaf) are larger than the bundles with high numbers. But this also does not provide clarity about the relative conductivity. A closer view (not shown) reveals that bundles with low-number-marks have narrower vessels and more sclerenchymatous tissue. The high abundance of sclerenchyma mainly causes the large appearance of the bundles to the nearest leaves. Besides its main bundle, each leaf has eight smaller bundles connected to the stem vascular bundles: four at each side of the main bundle. From the scheme of the network in Fig. 9, it appears that neighbouring 'main' bundles serve leaves that are positioned 5 leaves higher at the left and 8 leaves higher at the right. Because of that, main bundles branch 'earlier' to the left than to the right. This means that the upward tangential spread of sap flow is larger to the left than to the right. With this background it is nice to find out that the outer leaf traces make a sort of correction for this unequal tangential spread: they originate one bundle further to the left than to the right. A scheme of the vascular bundle network of a left-handed chrysanthemum stem can be obtained by mirroring Fig. 9.

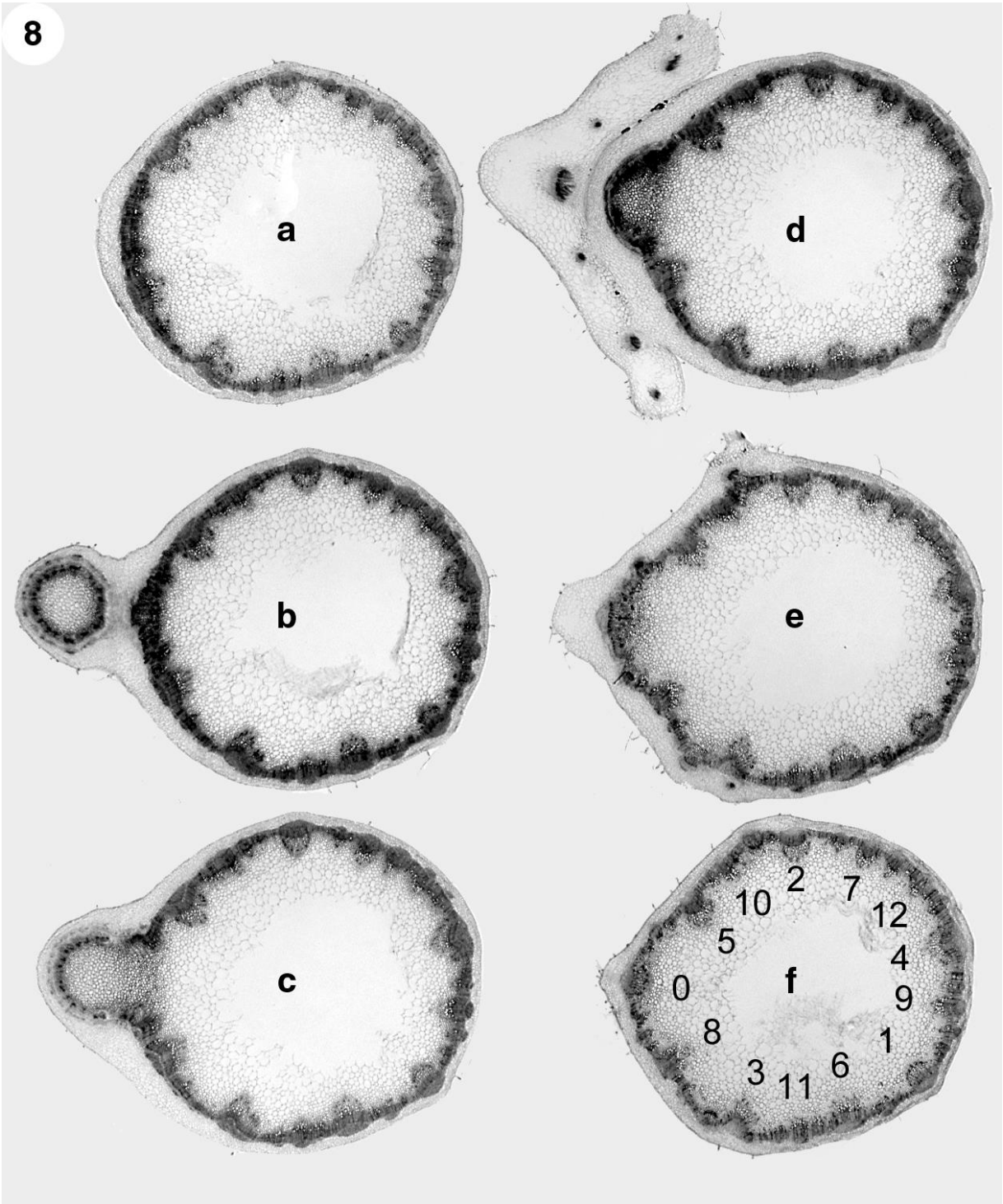

Fig. 8. Transverse light microscopical sections down through a chrysanthemum stem node. a: close above the node. b: just above the branch attachment. c: through the branch attachment. d: just above the leaf attachment. e: through the leaf attachment. f: close below the node. The numbers indicate the positions of the main leaf traces of subsequent leaves. Leaf 0 originates from this node, leaf 1 from the next higher node, etc.

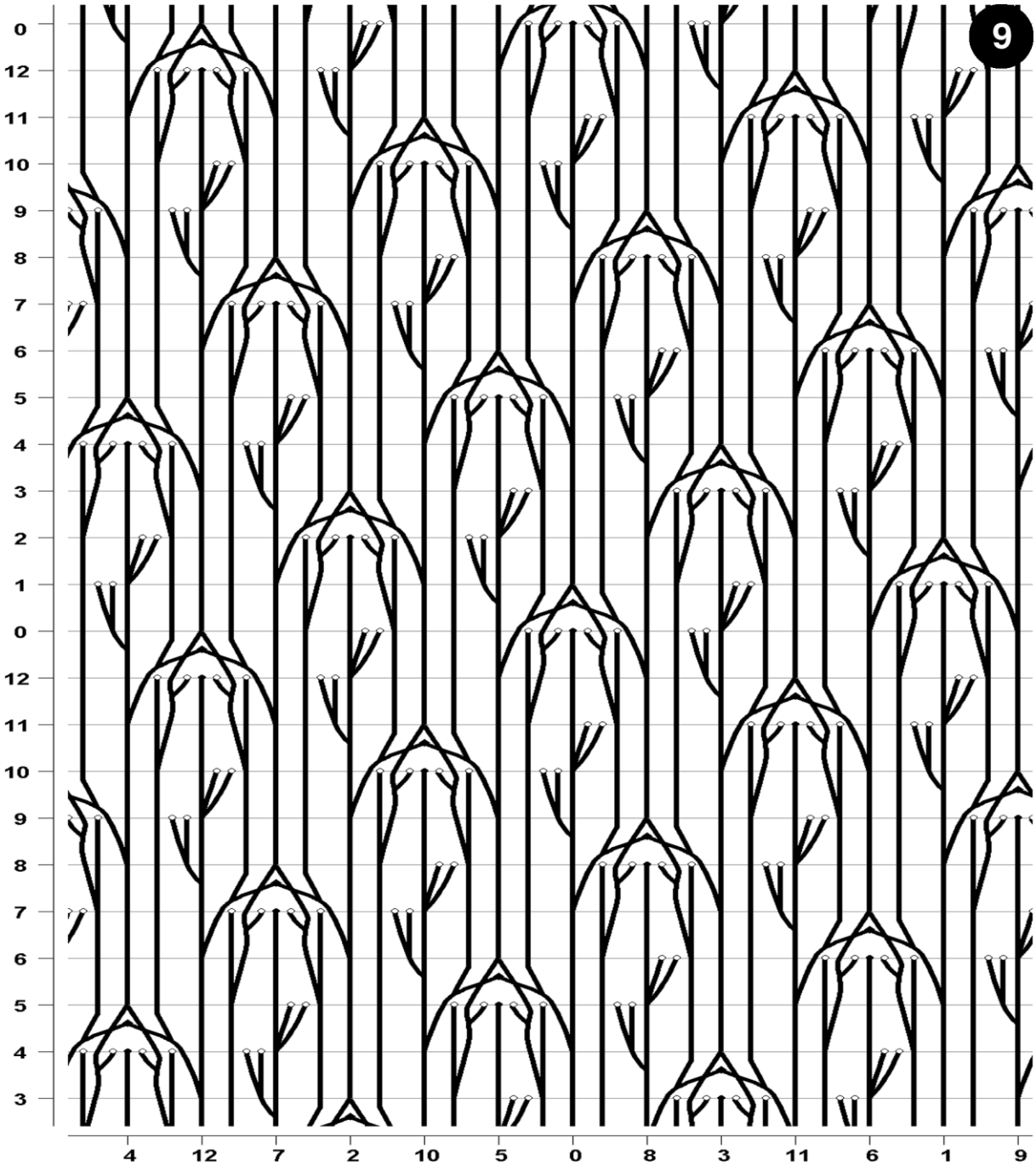

Fig. 9. Schematic 2-dimensional reconstruction of the vessel bundle network in chrysanthemum. The numbers at the baseline of this map indicate the numbers of the main leaf traces, as explained with Fig. 8f. The map represents exactly the circumference of a stem, i.e. the bundles at the left side of the map are in reality connected to the bundles at the right side. The numbers at the left of the map represent relative node numbers. The numbers at the base of the map indicate the relative positions of the main leaf traces of subsequent leaves. Each main leaf trace exits the stem at its corresponding node. Each leaf has in total 9 leaf traces: the main trace, and 4 lateral traces at both sides. Above each leaf is a potential branch connection. In this map the height has been compressed about 25 times compared to the width, to enable a proper visualisation of the network.

The vascular bundles in chrysanthemum are of the open collateral type, with the phloem at the outside and xylem at the inside (Fig. 4). Figures 10a-c show vascular bundles at three heights in the stem. The lower in the stem, the more developed the tissues are. Secondary growth (thickness growth after the stretching of the internode) is present at all three heights. Of course the lower parts of the stem have originated earlier. However, the secondary growth causes continuously newly developed cells at the cambium. Therefore, the lowest stem parts hold cells of all ages of the plant, whereas the higher parts hold only younger cells. To be clearer: the cells just formed by the cambium in Figs. 10a-c all have the same age, irrespective of the height in the stem, but the oldest cells (protoxylem and protophloem) have a different age, dependent on the height in the stem. Figure 11 shows a detail of several tissues within the vascular bundle. Sclerenchyma can be distinguished by its thick cell walls. The phloem has much thinner cell walls and a less ordered structure. The xylem is discernible by its mostly thick walled cells, which are neatly ordered in radial rows that stretch away from the cambial zone. The xylem has several cell types: vessels, parenchymatic cells, and libriform fibres, all shown in Fig. 12. The vessels are in most cases larger than the surrounding non-vessel cells. The diameter of vessels can vary from a few micrometers to about 60 micrometer (Nijsse *et al.*, 2001). They possess bordered pits, which do not occur in the other cell types. In cryo-SEM images the vessels are often clearly distinguishable by their contents: vessels are either empty (in case of embolism) or have a dilute solution, which has a smooth appearance caused by the lack of a fine ice crystallisation pattern (Nijsse *et al.*, 2000). In Fig. 12 the vessels are filled (they show an ice bottom). Parenchymatic cells have thinner walls and the organelles are visible. Parenchymatic cells stay in contact with vessels by ‘half-bordered pits’: at the vessel side a bordered pit, and at the parenchyma side the absence of a secondary cell wall. The xylem fibres have thick walls. They have simple pits to all three cell-types. Using cryo-SEM, the content of fibres can appear from densely crystallised to smooth, which points to different solute contents. Although not further studied, the fibre contents show that some fibres are dead and some still alive. Dead fibres occur mainly in regions without vessels. Figure 13 shows three types of xylem vessels in a longitudinal section. The helical-type (see also Fig. 14) is formed during the length growth of the internode. The circular bordered pitting-type is typical for vessels that are formed after the stretching of the internode. The scalariform-type is an in-between form. All vessels in the secondary xylem are of the circular bordered pitting-type. The primary xylem exhibits a gradient from ring-type to the circular bordered pitting-type. In chrysanthemum most protoxylem is still intact, as can be concluded from their normal appearance (as other vessels) in cryo-SEM images. A high magnification of a bordered pit in a secondary vessel is shown in Fig. 15. Xylem sap flowing from one vessel to another always has to pass a bordered pit. Note the large surface over which the sap can pass the pit membrane. The pit channel (the narrow entrance from the vessel to the pit cavity) is slit-shaped.

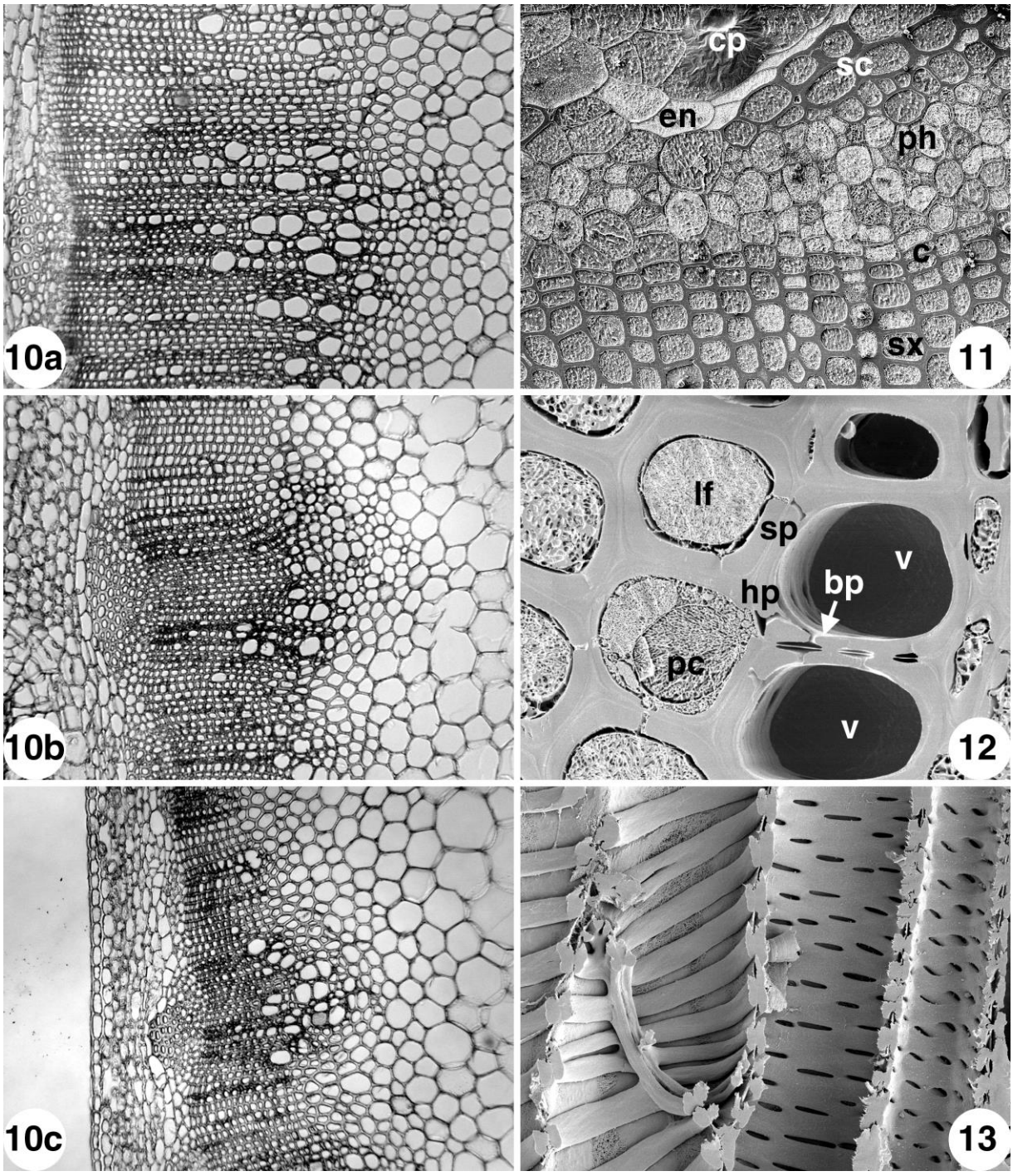

Fig. 10. Light microscopical transverse sections of vessel bundles at different heights in a mature chrysanthemum plant. a: at 10 cm, b: at 30 cm, c: at 60 cm above the soil surface. Horizontal field width = 960 µm. **Fig. 11.** Cryo-planed transverse section through the cambial area of a chrysanthemum stem. cp: cortical parenchyma, en: endodermis, sc: sclerenchyma, ph: phloem, c: cambium, sx: secondary xylem. Horizontal field width = 220 µm. **Fig. 12.** Cryo-planed transverse section through the secondary xylem of a chrysanthemum stem. Vessels (v) are interconnected by bordered pit pairs (bp). Parenchyma cells (pc) show several organelles as visible by different ice crystallisation patterns. They have relatively thin walls. Parenchyma cells are connected with vessels by 'half-bordered pits' (hp). Libriform

fibers (lf) have thick walls and simple pits (sp). Horizontal field width = 37 µm. **Fig. 13.** Longtitudinal section through neighbouring vessels. The vessel at left has helical wall thickenings, typical for primary vessels. The vessel in the middle is of the scalariform type. The vessel at the right has individual circular bordered pits, as typical for secondary vessels. (critical point dried, ambient SEM). Horizontal field width = 57 µm.

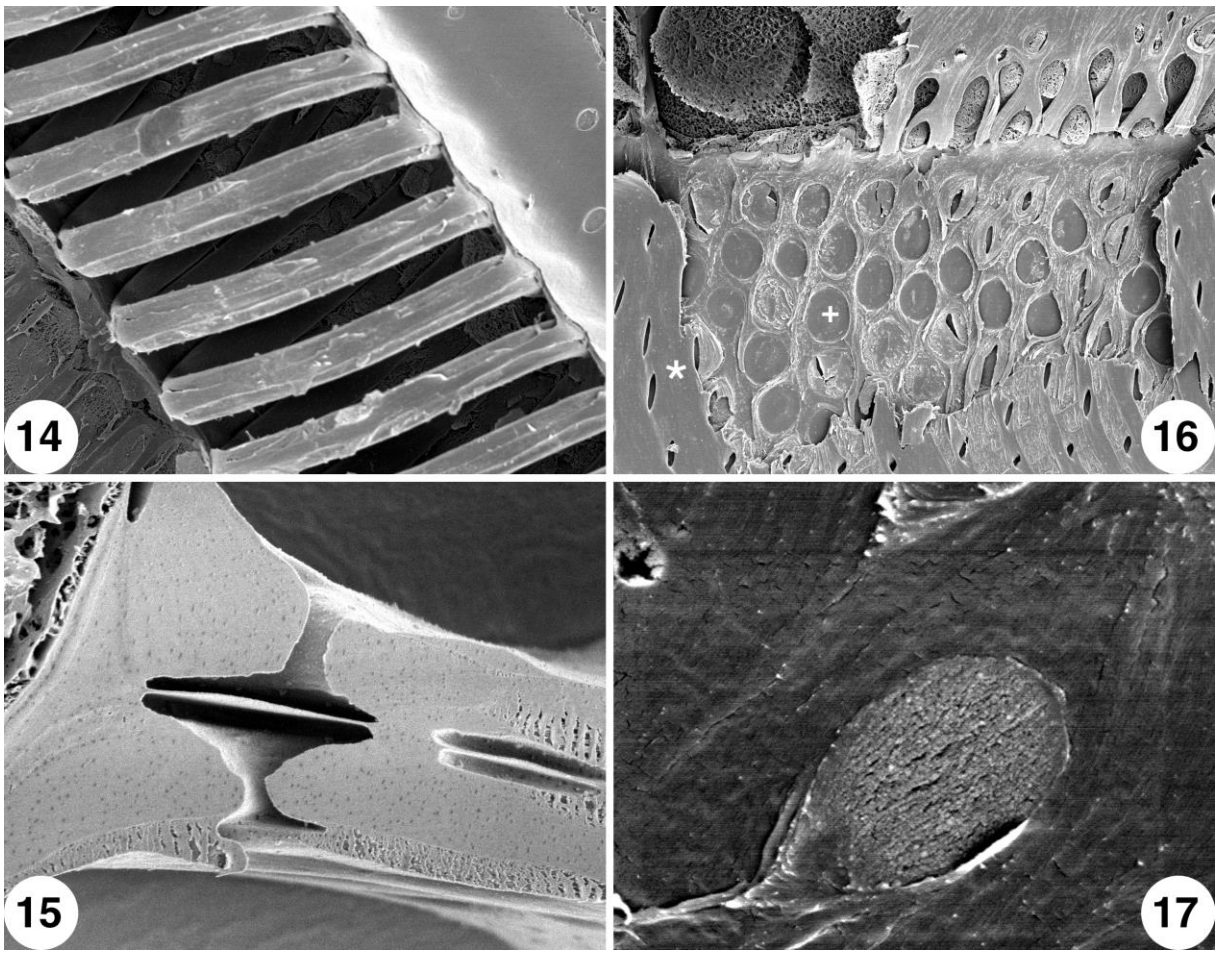

Fig. 14. Outside longitudinal view on an empty primary vessel with helical wall thickenings (Cryo-fractured). Horizontal field width = 57 µm. **Fig. 15.** High magnification of a cryo-planed bordered pit pair. Horizontal field width = 9.7 µm. **Fig. 16.** Longitudinally cryo-fractured vessel wall, showing bordered pits at different depths of the fracture: from the slit-shaped entrance (*) to the circular pit cavity (+). Horizontal field width = 57 µm. **Fig. 17.** Pit membrane in a longitudinally cryo-fractured vessel wall. The holes in the membrane could be artefacts of the freezing procedure. Pores in the membrane are at least far below the micrometer scale. Horizontal field width = 7.3 µm.

The two slits at both sides of a bordered pit are always in a more or less crossed orientation towards each other. Figure 16 shows a longitudinal fracture through a vessel wall, exhibiting bordered pits that have fractured at different depths. The pit membrane consists of the middle lamella and the two primary walls of the two adjoining vessels. Note that the pit membrane of a bordered pit is not bordered by a lipid membrane; it consists mainly of a network of pectic polysaccharides (Raven *et al.*, 1992). In principle xylem sap can move freely through the pit membrane. The pores in the membrane are difficult to visualise (Fig. 17). The width of the pores is certainly far below the micrometer scale.

The leaves

The leaves of cut chrysanthemums in general are pinnately lobed (Fig. 18), but the shape of the lobes can vary for different cultivars. The leaf bears stipules (Fig. 7) at the attachment to the stem. As depicted in Fig. 9, a leaf has nine leaf traces, of which the mid-trace or main trace is the largest by far. The leaf appears to be connected to nearly half of the shoot vascular bundles. These multiple connections will provide a safe defence against sectorial blockage of the water transport in the shoot. Figure 19 shows the upper surface of the leaf, and Fig. 20 the lower surface at the same magnification. Compared to the upper surface, the lower surface has much higher numbers of stomata and trichomes (Detail: Fig. 21). When ruptured, the trichomes spread the typical chrysanthemum scent. The ‘non-specialised’ epidermal cells have an amoeboid shape. Stomata slightly emerge from the surrounding epidermal tissue (Fig. 22). Figure 23 shows a cryo-fractured leaf halfway the lamina. From the upper surface down to the lower surface, the following tissues are present: upper epidermis, palisade parenchyma, spongy parenchyma with leaf veins, carrying vascular bundles, and the lower epidermis. As usual, the upper and the lower epidermis both consist of one cell layer.

The flowers

The flower is the most decorative organ of the ornamental chrysanthemum. There exist numerous variations in flower colour, shape, and size. Chrysanthemum flower heads possess both disk florets, making up the central portion of the aggregate, and ray florets, which are arranged on the outer periphery (Fig. 24; a detail of the daisy-type ‘Cassa’ chrysanthemum flower). Some cultivars have flower heads with only ray florets (e.g. the spider types, as ‘Super Yellow’). Below the ray florets are a few rings of green ‘leaflets’, the bracteae, which initially surround the young flower bud (Fig. 25). The florets open sequentially from the periphery to the centre. Figure 26 shows the upper surface of a ray flower leaflet. Each cell exhibits a cuticular wrinkled microstructure (Fig. 27). Cells at the lower surface of a ray flower leaflet (Fig. 28) are more stretched and they lack this wrinkling.

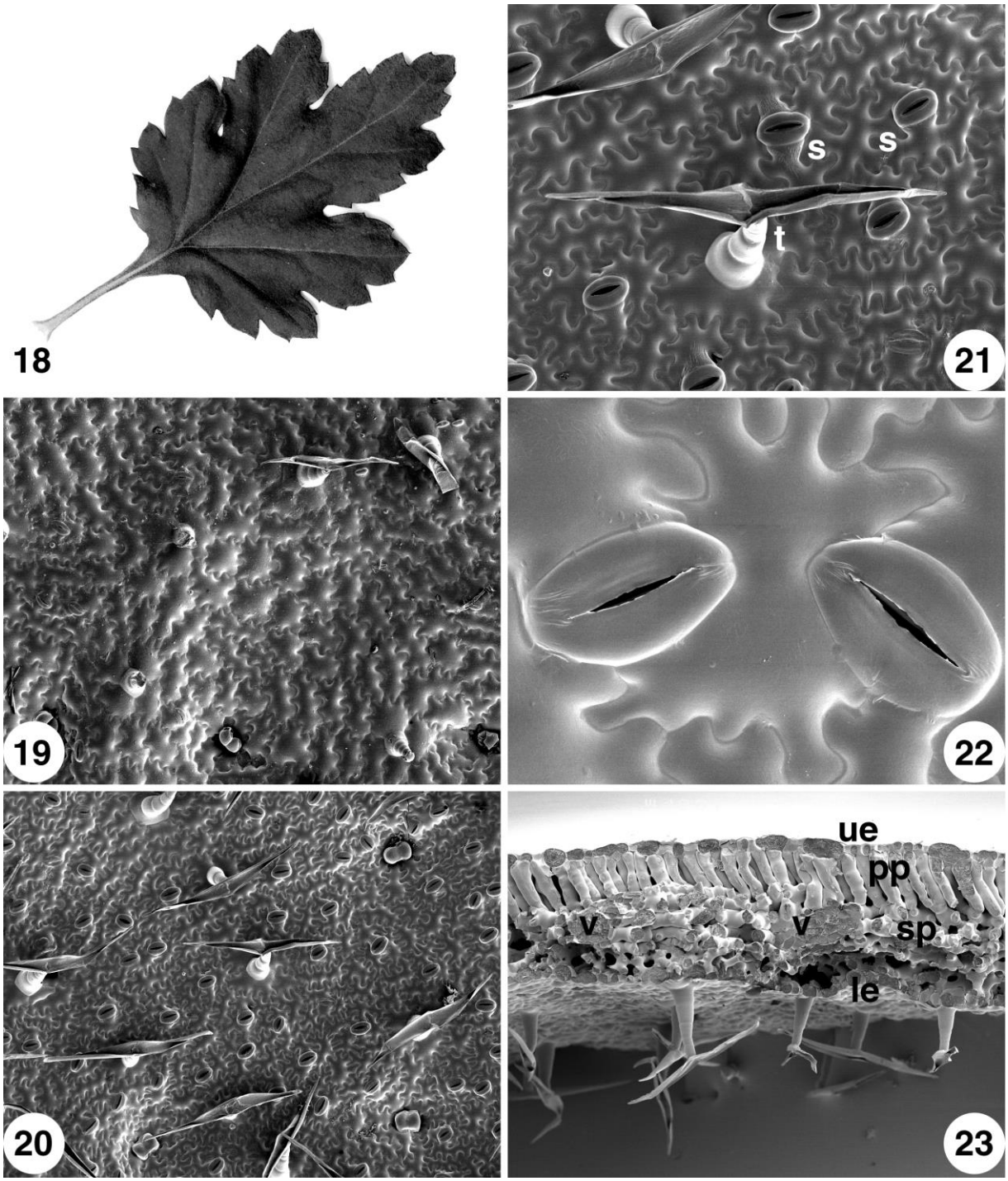

Fig. 18. Chrysanthemum leaf taken from a mature stalk, halfway its height. (cultivar Cassa). **Fig. 19.** Cryo-SEM image of the upper surface of a mature leaf, halfway the blade. Horizontal field width = 1140 µm. **Fig. 20.** Cryo-SEM image of the lower surface of a mature leaf, halfway the blade. Horizontal field width = 1140 µm. **Fig. 21.** Detail of Fig. 20. (t: trichome, s: stomata) Horizontal field width = 460 µm. **Fig. 22.** Cryo-SEM image of two stomata at the lower side of the leaf. Horizontal field width = 114 µm. **Fig. 23.** Cryo-fracture through a mature leaf, halfway the blade. From the upper to the lower surface the tissue layers are: upper epidermis (ue), palisade parenchyma (pp), spongy parenchyma (sp) with leaf veins (v), and the lower epidermis (le). Horizontal field width = 1140 µm.

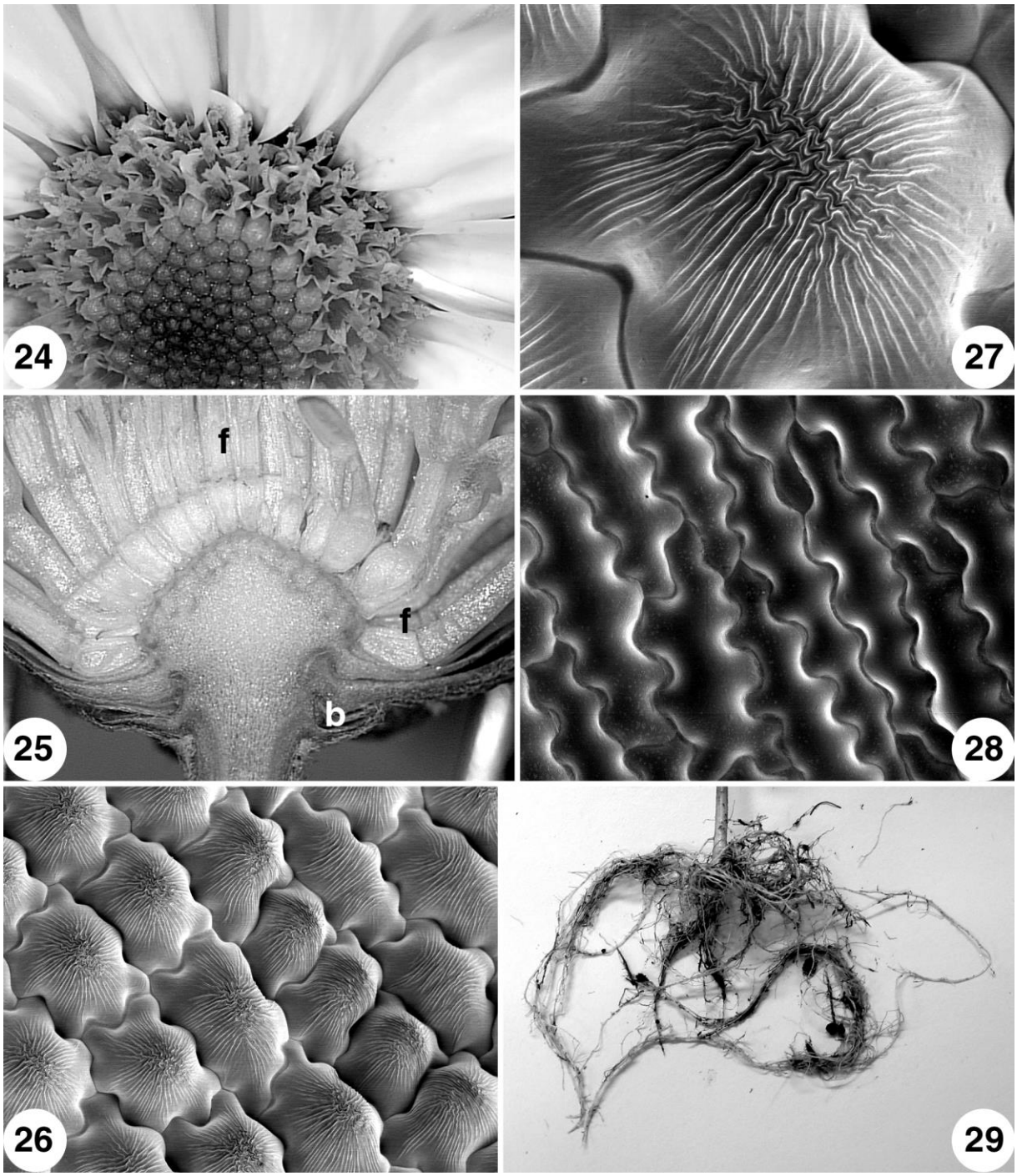

Fig. 24. Detail of a chrysanthemum flower (cultivar Cassa) at commercial maturity. **Fig. 25.** Longitudinal section through a chrysanthemum flower (b: bracteae; f: florets). **Fig. 26.** Cryo-SEM image of the upper surface of the blade of a chrysanthemum ray floret. (cultivar Cassa) Horizontal field width = 220 µm. **Fig. 27.** Higher magnification of the upper surface of the ray floret blade of Fig. 26. Horizontal field width = 57 µm. **Fig. 28.** Cryo-SEM image of the lower surface of the blade of a chrysanthemum ray floret. Horizontal field width = 220 µm. **Fig. 29.** The roots of a pot grown 'cut' chrysanthemum at maturity (cultivar Cassa). Horizontal field width is about 15 cm.

The roots

Cut flowers lack roots! However, obviously, during cultivation a good root system is indispensable. Because cut-chrysanthemums are propagated as cuttings, they do not have a taproot, but possess an adventitious root system (Fig. 29). Unlike in monocotyledonous adventitious root systems (Raven *et al.*, 1992), in cuttings one root can be more prominent than another root.

Concluding remark

Cut chrysanthemums are quite heterogeneous. At first glance, they sometimes seem to have more differences than similarities to each other. The descriptions that are provided in this chapter could serve to provide a general overview of some aspects of cut chrysanthemums. It can be stated that at a decreasing scale (e.g. from macroscopic to microscopic) chrysanthemums (and all organisms in general) tend to show fewer differences. However, we have not studied the heterogeneity of the anatomical and morphological data that are presented here.

Acknowledgement

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2.2

Xylem hydraulic conductivity related to conduit dimensions along chrysanthemum stems.

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Abstract

The stem xylem conduit dimensions and hydraulic conductivity of chrysanthemum plants (*Dendranthema x grandiflorum* Tzvelev cv. Cassa) were analysed and quantified. Simple exponential relations describe conduit length distribution, height dependency of conduit length distribution, and height dependency of stem hydraulic conductivity. These mathematical descriptions can be used to model the xylem water transport system. Within a chrysanthemum stem of 1.0 m, the conduit half-length (the length within which 50% of the conduits have their end) was 0.029 m at soil surface and decreased by half at a height of 0.6 m. With each 0.34 m increase in height up the stem, the hydraulic conductivity decreased by 50%. The resistance calculated from conduit lumen characteristics was 70% of the measured resistance. The remaining unexplained part of the hydraulic resistance is at least partly caused by inter-conduit connections.

Introduction

There are several quantitative studies of anatomical features of the xylem water transport system in plants (e.g. Fisher and Ewers, 1995; Villar-Salvador *et al*., 1997; Ewers *et al*., 1997). In higher plants tracheary elements are the main conduits for water transport from roots to leaves. Dicotyledonous and monocotyledonous plants possess both tracheids and vessels. Tracheids are single tracheary cells while vessels are the product of longitudinally fused tracheary elements. Here both tracheids and vessels will be referred to as conduits. Bordered pit pairs connect neighbouring conduits. Conduits are of finite length and to pass from one conduit to another water must pass through bordered pit pairs.

The hydraulic conductivity of the xylem water transport system depends on the hydraulic conductivity within the conduit lumina and the hydraulic conductivity of the inter-conduit connections. Assuming the conduits as elliptic pipes arranged in parallel, it is possible to calculate the conductivity of the xylem conduit lumina in a stem segment from their cross-sectional dimensions (see appendix). This calculated

conductivity is higher than the measured conductivity since it does not include the inter conduit resistance. Pickard (1981) stated that the resistance of conduit ends (i.e. bordered pits) may contribute significantly to the total resistance, but that *a priori* no accurate estimation of this resistance is possible due to the complex geometry of the system (see also Chiu and Ewers, 1993; Van Ieperen *et al*., 2000). However, it can be stated that bordered pits affect the resistance of stems with long conduits to a lesser extent than stems with short conduits. Thus diameter and length of the conduits and how they are interconnected determine the hydraulic properties of a vascular system.

The aim of the present study was to acquire a detailed but simple description of the hydraulic architecture in chrysanthemum stems in order to relate hydraulic conductivity to anatomical properties of xylem conduits, and to get a means to compare the hydraulic properties of different locations in one plant or phenotype, different cultivars or plants grown under different conditions. To understand dynamics of water flow in plant stems (e.g. in case of induction and removal of embolisms), a proper knowledge of the hydraulic architecture of the stem is indispensable. In this study the anatomical basis of height dependency of hydraulic conductivity in chrysanthemum stems was investigated to test the method at various conductivities within one phenotype. Using digital image analysis, xylem conduit lengths and cross-sectional dimensions were quantified as a function of the height in the stem. Calculated cross-sectional hydraulic conductivities were integrated over the length of a stem segment and compared with the actually measured hydraulic conductivity.

Materials and Methods

Plant material

Dendranthema x grandiflorum Tzvelev (chrysanthemum) is a herbaceous perennial, originating from East Asia. It has pinnately lobed leaves spirally arranged around an erect stem. For the experiments (October 1998) chrysanthemum plants of the cultivar Cassa were propagated via stem cuttings. Rooted, 0.05 m long cuttings were transplanted into pots and grown in a greenhouse of Wageningen University (Van Meeteren and Van Gelder, 1999). Total growth period from transplanting the cutting to harvest was about 12 weeks. Forty four uniform flowering plants were collected early in the morning in the dark period (when they were fully turgid) and transported in their pots to the laboratory. A stem segment of 0.3 m in length was cut under water from each plant, using a new razor blade for each cut. The stem segments were cut off at eleven heights in four replicates. The 0.3 m stem segments began at the following heights above the soil surface: 0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.50, and 0.60 m. The cuts were always made in internodal regions of stems. Leaves were removed from the stem segments with a razor blade, leaving 0.01 m of the petioles on the stem. No significant branches (and flowers) were present at the sampled heights. Hydraulic conductivity measurements and determination of the xylem conduit length distribution as well as light microscopic analysis on cross sections of the upper and lower end were carried out on the segments. The stem internode lengths were measured in all plants.

Hydraulic conductivity measurements

The upper end of each stem segment was connected to a silicone rubber tube with a pulling pressure difference of 25 kPa, created by a hanging water column. The lower end of the stem segment was placed in a container on a balance (Sartorius LC3200D), which contained an aqueous solution of sodium bicarbonate (1.5 mM), calcium chloride (0.7 mM) and copper sulphate (5 µM) at room temperature (20±2°C; for details see also Van Meeteren *et al*., 2000). Water flow through the stem segment was calculated from weight changes measured with the balance, and recorded by a personal computer at 1 s^{-1} sampling rate and averaged over 30 seconds. Measured flow rates were corrected for evaporation at ambient pressure. Once the flow rate had remained stable for 10 minutes the hydraulic conductivity (*CS*) was calculated using the measured length of the stem segment (*x*), the applied pressure difference (ΔP) and the flow rate (*q*):

$$CS = \frac{q * x}{\Delta P}, \text{ (in µmol } s^{-1} \text{ kPa}^{-1} \text{ m)} \qquad 1$$

Xylem conduit length distribution

Direct xylem conduit length measurements are hardly possible due to the enormous macroscopic lengths in combination with the microscopic diameters. A solution to this problem is to add a dye that cannot pass inter-vessel connections to a cut stem segment and to count how much vessels still continue at increasing distances from the plane of the cut. After the hydraulic conductivity measurements, and within 50 minutes after harvest, the stem segments were connected to a pumping system using the silicone rubber tubing attached for the conductivity measurement. With a pulling pressure difference of 50 kPa applied to the upper end of the segments, the lower end was placed in an aqueous 1% (w/w) suspension of red latex particles suspension (modification of the method of Zimmermann and Jeje, 1981). The latex suspension was prepared one day in advance to allow aggregates of particles to precipitate. Latex particles (< 2 µm diameter) can easily enter an open conduit, but are too large to pass bordered pit pairs (Zimmermann, 1983). The particles are carried into the open conduits by the mass flow of water, eventually blocking them. Once water flow through the stem segments ceased, it was assumed that all cut open conduits were stained. The segments were removed, placed in plastic bags and stored in a refrigerator (4°C) for at most 2 weeks before being subjected to further processing to determine xylem conduit length distribution. After storage, thick (2 mm) transversal sections were made with a razor blade. To distinguish the effect of nodes on conduit endings, two or more sections were cut out of every internode in order to have both nodal and inter-nodal intervals. Images of the sections were made using a Sony 3CCD (DXC-950P) colour camera attached to a binocular microscope (Leica MZ8), giving a resolution of 13 µm per pixel. Earlier analyses of xylem conduit length distribution were based on counting individual filled conduits (e.g. Zimmermann and Jeje, 1981; Darlington and Dixon, 1991; Fisher and Ewers, 1995). In this study we adapted a technique based on measuring the total red-stained area of the stem using an image analysis programme (SCIL_Image 1.3, University of Amsterdam, Faculty of Mathematics and Computer Science, Amsterdam, The Netherlands). This method enabled the analysis of hundreds of

sections instead of just a few. The measured red area (amount of red pixels) of a section was divided by the average area of each conduit (see cross-sectional area analysis) to calculate the number of stained conduits in that section. For each stem segment the length distribution was described by its half-length (τ_c) as explained in the Appendix.

Cross-sectional area analysis

Portions of stem from just above and below the sampled stem segments were stored in 75% ethanol. Thin transversal sections were made with a slide microtome from the transversal planes that had faced to the upper and lower ends of the stem segments. The sections were embedded in Kayser's glycerol gelatine (Merck 9242, Darmstadt, Germany) on microscope slides. Digital images were made with the Sony 3CCD camera attached to a light microscope (Leitz Dialux) giving a resolution of 1.26 µm per pixel. More than 30 images were needed to acquire a detailed record of the entire xylem area of one transverse section. The number of conduits, (average) conduit cross-sectional area and the longest and shortest axis of an ellipse superimposed on the conduits, were measured using the image analysis system. Conduits were distinguished from fibres by eye and marked with mouse clicks, using the presence of bordered pits as a criterion. Four sections (both ends of two 0.30 m segments sampled from heights of 0.10 m and 0.30 m above soil level) were analysed. Hydraulic conductivity of the sections was calculated assuming conduits as infinite, uniform elliptic, parallel arranged pipes. See appendix for details on conductivity calculations.

Results

Description of the plants

From 0.10 to 0.90 m along the stem, the internode length was more or less constant at between 34 and 41 mm (Fig. 1). Below 0.10 m and above 0.90 m the internodes were shorter. The lowest 0.10 m of the stem developed before and during propagation and the part higher than 0.90 m developed during the generative phase; this may explain the shorter internode lengths at both ends of the stem. The plants had about 7 flowers, originating from nodes higher on the stem than 0.60 m. The stems had one leave at each node and apart from the flower branches the stems had no side shoots.

Hydraulic conductivity

Figure 2 shows the results of the hydraulic conductivity measurements on the 0.30 m stem segments. Hydraulic conductivity decreased with height up the stem and this conductivity/height relationship could be described by an exponential function:

$$CS_{h,0.30m} = CS_{0,0.30m} e^{\lambda h} \qquad 2$$

with h in meters and CS in µmol s^{-1} kPa^{-1} m and with $CS_{0,0.30m}$ = 0.65 $\pm$ 0.03 and λ = -2.09 $\pm$ 0.16. In other experiments (not shown) it was found that conductivity was independent of the applied pressure.

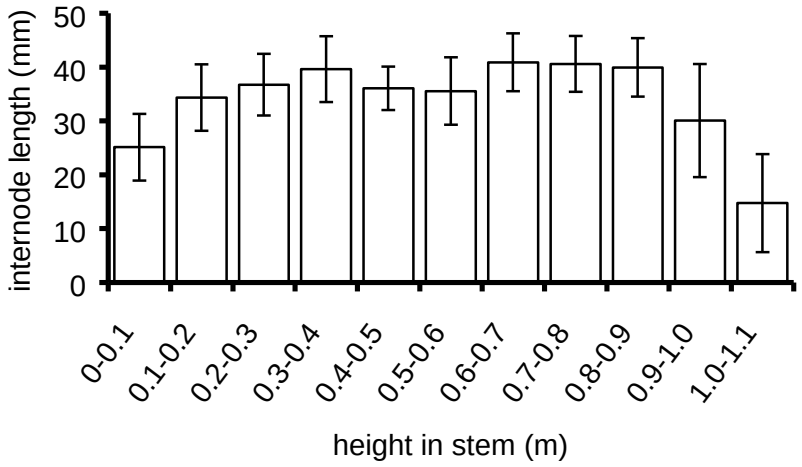

Fig. 1. Average internode length per 0.1 m height in the stem of 44 chrysanthemum stems. Vertical bars represent standard deviations of the means.

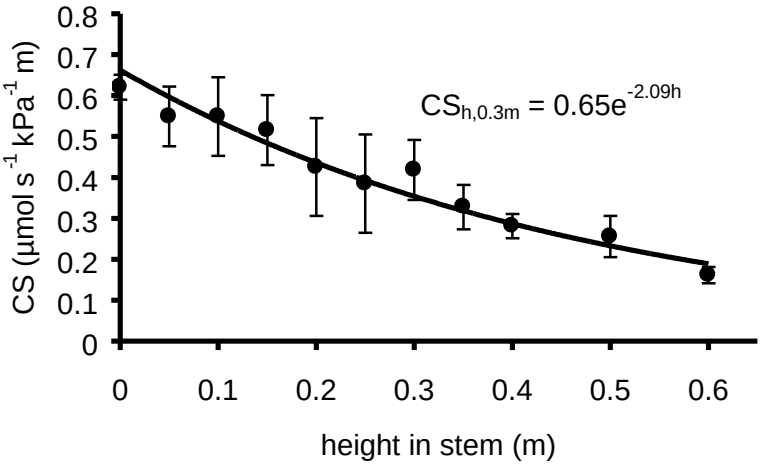

Fig. 2. Measured hydraulic conductivity ($CS_{h,0.3m}$) along the stem for 0.30 m long stem segments (*h* to *h*+0.30 m). Vertical bars represent standard deviations of the means. The curve is the fitted exponential function. (R^2 = 0.81, n = 44).

Conduit length distribution

An example of the red pixel counts (*Y*) on sections with increasing distances from the lower cut surface of the stem segment is shown in Fig. 3 and it is evident that there are many more short conduits than long ones. The log-scale plot shows the same data with a logarithmic *Y*-axis, revealing a decreasing exponential relation. Nodes (locations indicated with bars at the top of the graph) seem to have no effect on the distribution of conduit ends, because no slope differences are visible between adjacent pixel counts over nodal and inter-nodal intervals. By calculating the value of the conduit half-length (τ_c), the length distribution of the conduit parts above the cut surface is completely described. For all 44 stem segments this half-length was determined.

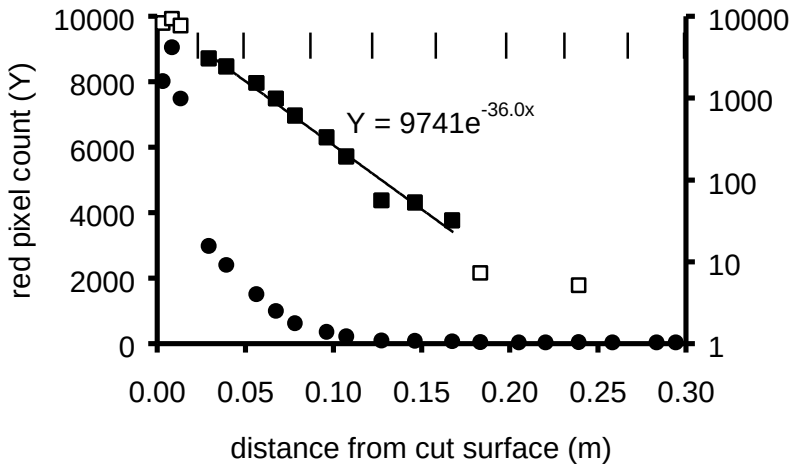

Fig. 3. The number of red pixels (*Y*) counted at increasing distances from the cut surface for a stem segment starting at 0.50 m above soil surface. Results are both plotted on a normal scale (circles, left *Y*-axis) and on a logarithmic scale (squares, right *Y*-axis). The line shows the fitted exponential function for determination of the half-length (τ_y). The open squares were not included in the exponential fit. The vertical bars on top of the graph indicate the location of nodes in the stem segment.

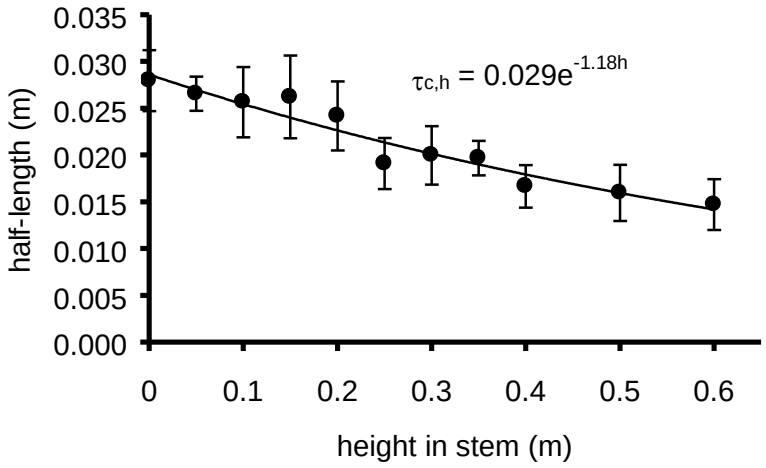

Fig. 4. The relation of conduit half-length (τ_c) and height (*h*) in the stem. Vertical bars represent standard deviations of the means. The line is the fitted exponential $\tau_{c,h}$ function. ($R^2 = 0.70$, n = 43).

Red pixel counts lower than 15 were not taken into account for the analysis to prevent a rise of the signal to noise ratio close to zero. During storage the latex suspension filling the conduits occasionally showed some shrinkage close to the end of the stem segment. Red pixel counts from that regions were discarded. The mean values of τ_c for the different heights in the stem are shown in Fig. 4. The value of τ_c as function of the height in the stem was fitted with an exponential function:

$$\tau_{c,h} = \tau_{c,0} e^{\lambda h}, \text{ (}h\text{ and }\tau\text{ in meters)} \qquad 3$$

where $\tau_{c,0}$ = 0.029 ± 0.001 and λ = -1.18 ± 0.12.

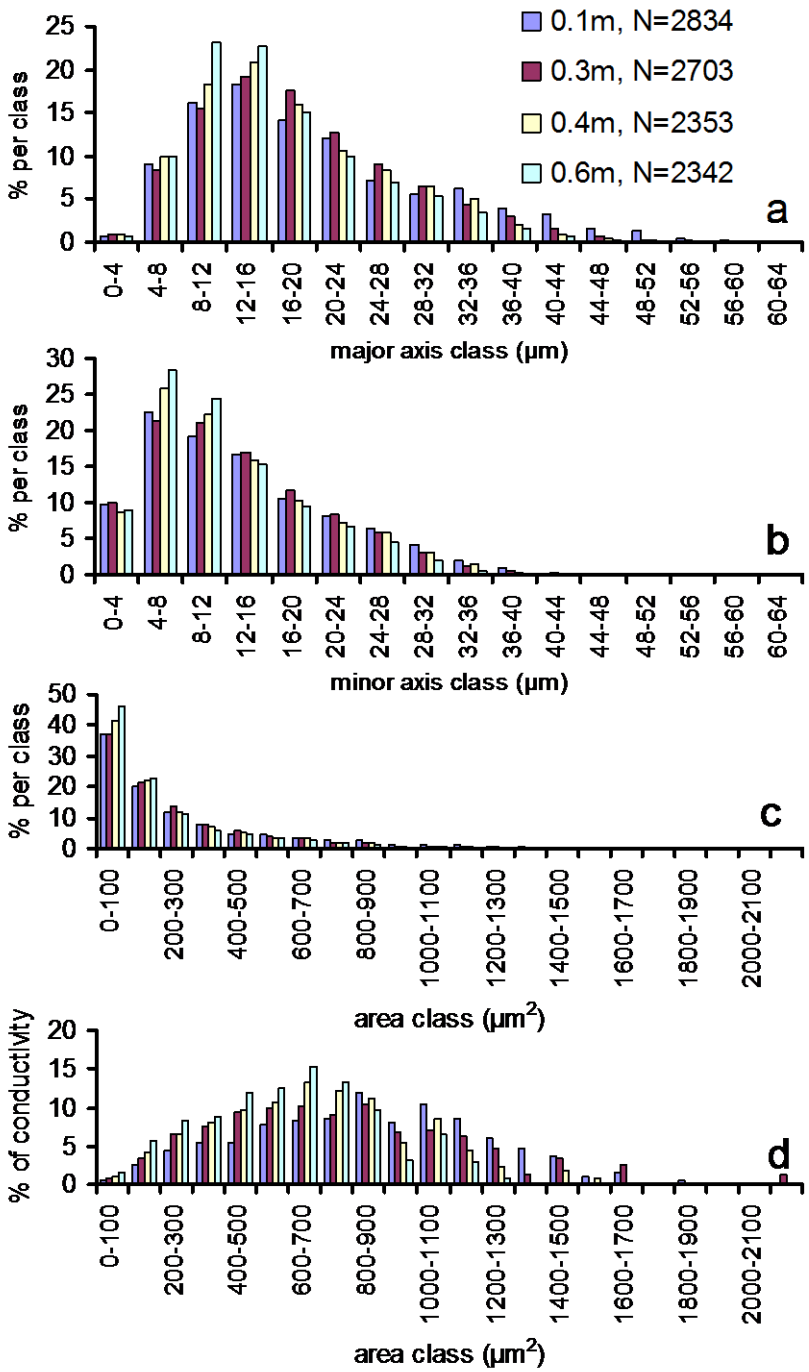

Fig. 5. Histograms of cross-sectional conduit dimensions at heights of 0.10, 0.30, 0.40, and 0.60 m in the stem. (**a**) Histogram of 4-µm classes of the major axes. (**b**) Histogram of 4-µm classes of the minor axes. (**c**) Histogram of 100-µm^2 area classes. (**d**) Histogram of the conductivity per 100-µm^2 area class.

According to this function the estimated value of τ_c is 0.029 m at soil level, and halves with every 0.59 m higher up the stem. The correction of the pixel counts for average conduit area caused only a small change in the $\tau_{c,h}$ function.

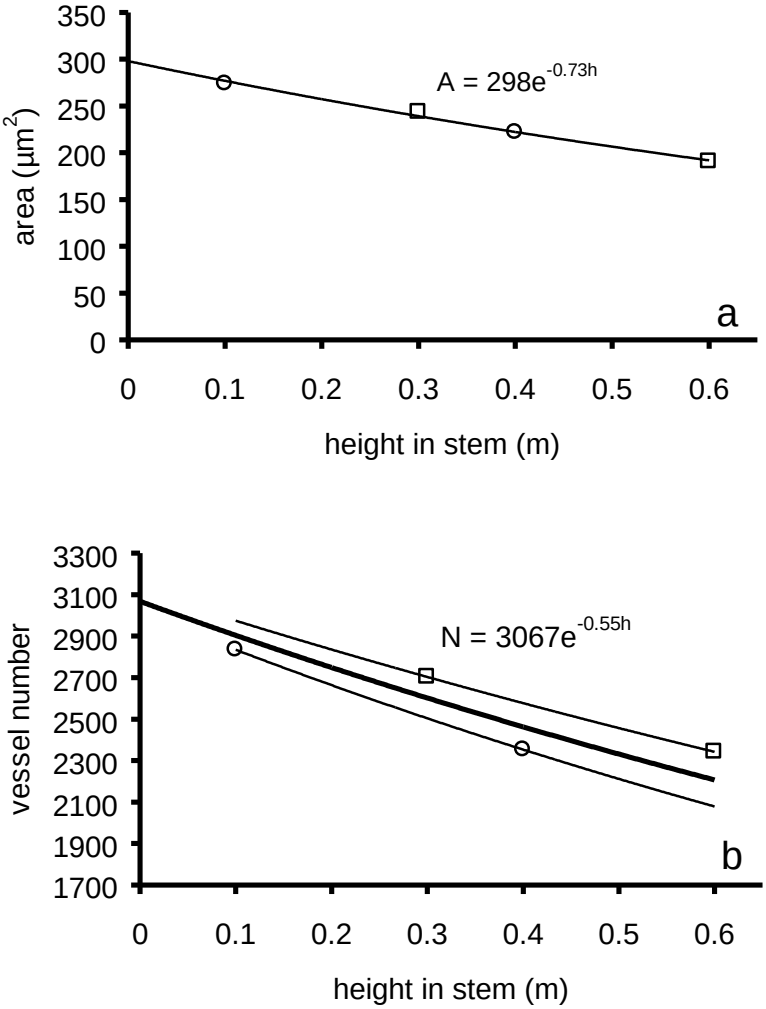

Fig. 6. (**a**) The average cross-sectional area of individual conduits (*A*) at heights of 0.10, 0.30, 0.40 and 0.60 m in the stem. The line is the fitted relation of average area and height in the stem. (**b**) The number of conduits (*N*) in a cross-section at four heights. The bold line represents the estimated cross-sectional number of conduits as function of height in the stem. Circles and squares: both ends of the two segments starting at height of 0.10 and 0.30 m in the stem.

Cross-sectional area

Within the four analysed sections the number of conduits (more than 2000) was sufficient to determine the histograms of cross-sectional conduit dimensions. Estimations of whole stem conductivity based on only four sections need to be treated with caution. Figures 5a-c show the histograms for the major and minor axes and the areas of the conduits at 0.10, 0.30, 0.40, and 0.60 m height in the stem. The

distributions of the major and minor axes have their highest frequency at respectively 14 and 8 µm. The areas vary from very small (less than 100 µm^2) up to more than 1500 µm^2. The frequency of occurrence of conduits within different area classes decreases with increasing area. More than 35% of all conduits lie within the smallest area class (0-100 µm^2) used. The influence of the height in the stem on the distribution of conduit areas seems to be small. However, there are relatively more large conduits in the lower parts of the stem.

The average cross-sectional conduit area (*A*) in the four sections is shown in Fig. 6a, and its dependency on height up the stem is given by:

$$A_h = 298e^{-0.73h}, \text{ (}A\text{ in µm}^2\text{, }h\text{ in meters)} \qquad 4$$

This A_h-function was used in the conduit length determinations.

The height dependency of total cross-sectional conduit number is given by (Fig. 6b):

$$N_h = 3067e^{-0.55h}, \text{ (}h\text{ in meters)} \qquad 5$$

In the lower area size classes the absolute number of conduits is quite similar for all heights in the stem.

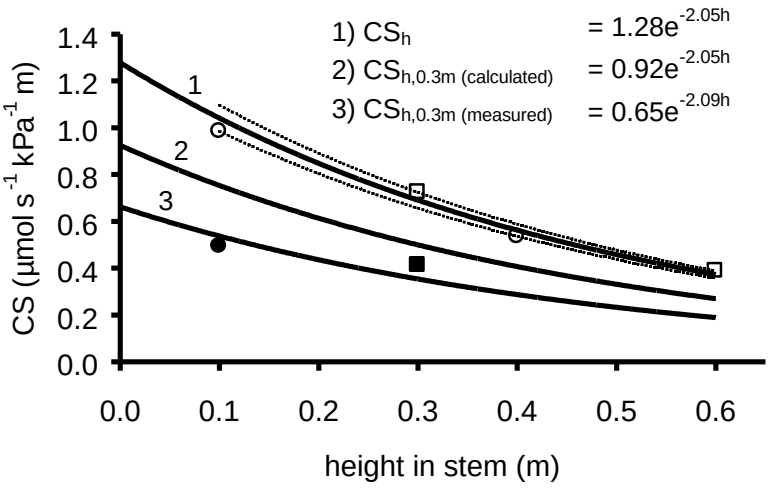

Fig. 7. Relation of hydraulic conductivity (*CS*) and height in the stem (*h*). First a CS_h-function was fitted for the *CS* as calculated from the cross-sections (curve 1). Circles and squares: calculated *CS*-values of both ends of the two analysed segments starting at height of 0.10 m and 0.30 m in the stem. This CS_h-function was integrated over 0.30 m (*h* to *h*+0.30 m) to calculate $CS_{h,0.3m}$ for 0.30 m long segments (curve 2), see appendix for details. Curve 3 represents the fitted relation for the measured $CS_{h,0.3m}$, as determined in Fig. 2. The filled symbols show the measured *CS* values for the two stem segments that were used for cross-sectional analysis.

The hydraulic conductivities calculated from cross-sectional dimensions of all conduits of the four sections are shown in Fig. 7. The hydraulic conductivity as a function of height in the plant (CS_h) was estimated by (Fig. 7 curve 1):

$$CS_h = 1.28e^{-2.05h} \qquad 6$$

with *CS* in µmol s^{-1} kPa^{-1} m and *h* in meters. The calculated hydraulic conductivity as a function of the height in the plant decreased more with increasing height than did the number of conduits. According to these functions the estimated conductivity value halves every 0.34 m, but the number of conduits halves every 1.26 m. If the conduit dimensions were not height dependent, the half-lengths for conduit number and for CS_h should have had the same value. To compare the calculated conductivity with the measured conductivity, the calculated conductivity of the sections was integrated over a length of 0.3 m (Fig. 7 curve 2, see appendix for calculation):

$$CS_{h,0.3m} = 0.92e^{-2.05h}, \text{ (units see Eq. 6)} \qquad 7$$

Curve 3 in Fig. 7 shows the measured conductivity as determined in Fig 2. Closed symbols indicate the measured conductivity in the segments that were used for anatomical analysis. The measured conductivity is for all heights in the stem about 30% lower than the calculated conductivity. This means that the calculated resistivity (reciprocal of the conductivity, see appendix) in the stem segments is 30% lower than the measured resistivity of the stem segments. The data used to calculate hydraulic conductivity from sections yielded also the conductivity of the individual conduits. In Fig. 5d a histogram of the conductivity of the four sections per area class is shown. From Fig. 5c and Fig. 5d can be derived that the largest 5% of the conduits cause 50% of the total conductivity.

Discussion

In this study a mathematical description has been developed for both hydraulic conductivity and conduit length distribution as functions of height in a chrysanthemum stem. We preferred to describe the anatomical relations as exponential functions, even in cases where linear functions gave as good a fit. This preference for exponential functions was for several reasons. First, exponential functions can represent first order stochastic underlying processes. Secondly, exponential functions are independent upon a starting value (e.g. where you choose the *h* = 0 point). Lastly, simple exponential functions do not generate negative values as results (e.g. negative conduit lengths).

With increasing distance from the cut end where the red latex was introduced, a greater percentage of red latex containing conduits come close to their upper end. Zimmermann (1983) suggested that conduits would show some tapering at the ends, so it is not sure whether the ends of long conduits have the same average area as the average of all conduits in the cross-section. End tapering of the conduits makes the red pixel count per conduit less, and thus the estimated conduit half-length becomes lower than the real conduit half-length. On the other hand, the wide conduits have greater impact on pixel counts than the small conduits. If the wide conduits are longer than the small ones, the conduit half-length is overestimated. However, if there was a difference between the half-length determined by counts of red pixels and the real conduit half-length, this would be rather consistent throughout the stem. Thus the absolute half-length could have been under- or overestimated, but the estimation of half-length changes as a function of height in the stem is not altered. The alternative, counting the number of red conduits, can also cause

uncertainties. Neighbouring conduits can be counted as one conduit, and the further from the cut surface, the fewer conduits are coloured red and relatively more individual conduits will be distinguished. This effect would result in an overestimation of the half-length.

Assuming that conduits are randomly located in the longitudinal direction, the length distribution of the entire conduits can be calculated using the double-difference algorithm (DD-algorithm; Zimmermann and Jeje, 1981; Zimmermann, 1983). This DD-algorithm has serious problems with non-random conduit ends (Tyree, 1993) and with stochastic fluctuations of the conduit counts. Therefore, in this paper an alternative way to describe conduit lengths, as described in Darlington and Dixon (1991), was used. In this method the part of the conduits below the plane of the cut is not taken into account and the length distribution of the parts of the conduits above the cut is directly analysed. In case of an exponential length distribution, as in our results, the entire conduit length distribution is simply described by the exponential length distribution function multiplied by the height above the cut surface. Description of the conduit length by one number (the half-length) provides an opportunity to compare different conduit length distributions. However, plants growing under (seasonal) changing conditions may show non-exponential conduit length distributions. In oak xylem very large conduits are formed in spring, differing in all dimensions from conduits formed in summer. Consequently, the conduit length distribution for oak therefore appears to be irregular (Zimmermann and Jeje, 1981). Thus, comparing single half-lengths is not applicable for all plants. We found a decrease of conduit length when going higher up the stem, which is also found in other plant species (Zimmermann and Potter, 1982). In contrast to the findings in some other diffuse-porous species (Salleo *et al*., 1984), conduit length in chrysanthemum stems decreases gradually and there are no special regions where conduits end. No relation was found between internode length and conduit length.

The number of conduits in the small-area classes were nearly the same throughout the stem, but the presence of a relatively small number of wide conduits caused the considerably higher conductivity in the lower stem. The presence of wider conduits in the lower stem is frequently encountered (Zimmermann and Potter, 1982; Zimmermann, 1978; Altus *et al*., 1985) and may be explained by lower auxin gradients further from the leaves (the six-point hypothesis from Aloni and Zimmermann, 1983).

The calculated resistivity in the studied chrysanthemum stems was about 70% of the measured resistivity. It is tempting to attribute the extra 30% of resistivity to that of the bordered pits, whose resistance is not included in our analysis. This step, however reasonable, needs to be considered critically. As in most estimations of conductivity based on anatomical studies so far undertaken (e.g. Ewers *et al*., 1997; Lovisolo and Schubert, 1998) the hydraulic conductivity was calculated from cross-sectional diameters of the conduits. However, slight errors in the measured diameter are amplified by the fourth power when the diameter is used to estimate the conduit hydraulic conductivity (Mapfumo, 1994). Additionally, the calculation of the hydraulic conductivity is based on the assumption that the conduits are all pipes arranged in parallel, whereas in reality the conduit system is a complex bundled network of conduits with finite length. In the studied stem segments, the leaf traces extended for four internodes before the connection with the other vascular bundles. We calculate

that probably 10% of the calculated conductivity was not effective during the conductivity measurements due to isolated bundles (unpublished data). Other factors that can cause a lower conductivity than calculated are: non-laminar flow, changing of diameter within conduits, and the perforation plates of the conduit members (Chiu and Ewers, 1992). In chrysanthemum the perforation plates are wide and simple and therefore not thought to cause any significant additional hydraulic resistance. We cannot, therefore, say what exact percentage of the measured resistivity is caused by resistance of the conduit lumina, but if our cross-sectional area measurements were correct, the resistivity due to the properties of the vessel lumina is higher than 70%. Interestingly, this fraction is rather constant throughout the stem and seems, therefore, to be independent of conduit dimensions. In other words: higher in the stem the resistivity of conduit lumina is larger, but the resistivity due to inter-conduit passages is also larger. The increase of the inter-conduit resistivity is explained by the finding that higher in the stem the conduits are shorter. Thus higher in the stem the water flux has to cross more inter-conduit passages per unit of stem length. The dimensions of bordered pit pairs can also vary within one plant (Van Alfen *et al*., 1983; Tyree and Sperry, 1989), resulting in different resistances.

Although not all anatomical properties of the xylem conduit system have been revealed here, this study shows how a simple and accurate description of hydraulic-anatomical properties of plant stems can be achieved. The approach used in this study allows the comparison of conduit lumen characteristics and hydraulic properties at different locations in one plant, or between cultivars or between different species. Three-dimensional imaging techniques (Lewis, 1995) will be needed to explore the detailed shape of individual conduits (diameter and roundness fluctuations) and the conduit connection patterns. In the near future the mathematical description of anatomical properties will enable proper 3D-modelling of the hydraulic architecture of plant stems (for a recent general model see West *et al*., 1999). Besides providing a better (mechanistic) understanding of plant vascular development and functioning, 3D-models can predict the consequences of disruptions of parts of the system caused by, for example, cavitation, mechanical damage or infections.

Acknowledgements

We thank Annie van Gelder, Joke Oosterkamp and Belén Uriz for their practical assistance. We thank Jeremy Harbinson, Olaf van Kooten, Rob den Outer, Rob Schouten and Michiel Willemse for helpful advice and critical reading. This research is financially supported in part by the Technology Foundation, STW, applied science division of NWO.

APPENDIX

Conduit length distribution

The number of red pixels (Y) as a function of the distance from the plane of the cut in chrysanthemum stems can be described by an exponential function as follows:

$$Y_x = Y_0 * e^{-\lambda_y x} \qquad 8$$

where Y_0 is the (extrapolated) number of red pixels at the plane of the cut, x is the height above the plane of the cut and λ_y is the exponential factor from which the half-length (τ_y; analogous to half-time of radio-active isotopes) can be calculated:

$$\tau_y = \frac{ln\,2}{\lambda_y} \qquad 9$$

This half-length τ_y is of particular interest, because for every value of x the following is true:

$$Y_{x+\tau} = ½ Y_x \qquad 10$$

in other words: τ_y is the distance along the stem over which the number of red pixels halves or doubles.

The number of red conduits as a function of distance from the plane of the cut (N_x) can be calculated from the red area divided by the average cross-sectional conduit area (A_x).

$$N_x = \frac{Y_x}{A_x} = \frac{Y_0 * e^{-\lambda_y x}}{A_0 * e^{-\lambda_A x}} = N_0 * e^{-(\lambda_y - \lambda_A)x} \qquad 11$$

A_x is calculated from the cross-sectional area measurements (see Fig. 6b), and N_0 is the number of all conduits at the plane of the cut. From this equation, the conduit half-length (τ_c), can be derived:

$$\tau_c = \frac{ln\,2}{\lambda_y - \lambda_A} \qquad 12$$

or:

$$\frac{1}{\tau_c} = \frac{1}{\tau_y} - \frac{1}{\tau_A} \qquad 13$$

Hydraulic conductivity

The hydraulic conductance (C) of a water transport system can be described as:

$$C = \frac{q}{\Delta P} \quad \text{(e.g. in µmol s}^{-1}\text{ kPa}^{-1}\text{)} \qquad 14$$

where q is the flow rate and ΔP is the pressure difference. The hydraulic conductance is dependent on the length of the transport system. The longer the system (e.g. stem segment), the lower the conductance of the system. The hydraulic conductivity (CS) is the hydraulic conductance per unit of length (x):

$$CS = C * x \quad \text{(e.g. in µmol s}^{-1}\text{ kPa}^{-1}\text{ m)} \qquad 15$$

The reciprocal of *conductance* is *resistance* and the reciprocal of *conductivity* is *resistivity*.

In his review on the ascent of sap in plants, Pickard (1981) gave a theoretical, mathematical description of the water transport within xylem conduits. Assuming the conduits as uniform, infinite and elliptic pipes, the conductivity per conduit (CS_c) is given by (Pickard, 1981):

$$CS_c = \frac{\pi}{4\eta} \frac{a_>^3 a_<^3}{\left(a_>^2 + a_<^2\right)} \qquad 16$$

where $a_>$ and $a_<$ are the semimajor and the semiminor axes of the ellipse and η is the viscosity of the liquid ($1.00*10^{-3}$ Pa s for water at 20°C). This formula is a more general form of the Hagen-Poiseuille formula for circular capillaries where $a_> = a_<$ = radius. Thus, the conductivity of a conduit lumen can be calculated from the major and the minor axes of an ellipse superimposed on its cross-sectional area.

The conductivity in a cross-section (CS_h) of the combination of all conduits (j) at a certain height above soil surface (h) in the stem is calculated by:

$$CS_h = \sum_{i_h=0}^{j_h} CS_{i_h} \qquad 17$$

assuming the conduits are pipes arranged in parallel. The conductance of such a stem segment starting at height h and with a length of x is given by:

$$\frac{1}{C_{h,x}} = \int_{h=h}^{h+x} \frac{1}{CS_h} \delta h \qquad 18$$

In the results of this study the relation of calculated conductivity and height in the stem is described using an exponential function:

$$CS_h = CS_0 * e^{-\lambda_{CS} h} \qquad 19$$

To compare the calculated hydraulic conductivity in cross-sections with the measured hydraulic conductivity in stem segments with a length x, the conductivity of the sections was integrated to give the conductivity of segments from height h over a distance of x as a function of h as follows:

$$\frac{1}{CS_{h,x}} = \frac{1}{x} * \frac{1}{C_{h,x}} = \frac{1}{x} * \int_{h=h}^{h+x} \frac{1}{CS_h} \delta h = \frac{1}{x} * \int_{h=h}^{h+x} \frac{1}{CS_0 * e^{-\lambda_{CS} h}} \delta h \Leftrightarrow \qquad 20$$

$$\frac{1}{CS_{h,x}} = \frac{1}{x} * \frac{1}{\lambda_{CS} * CS_0} * \left[e^{\lambda_{CS} h}\right]_{h=h}^{h+x}$$

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2.3

Non-determined determination of xylem vessel length in plants

J. Nijsse

Plant xylem vessels are made of linearly fused vessel elements. The length of a xylem vessel depends on the number of fused vessel elements and their individual lengths. The mechanism by which the plant regulates the length of xylem vessels has not been explained before. Here we show a general and most simple explanation how plants determine the length of xylem vessels. The mechanism is based on steered stochastics by which the plant creates a wide range of vessel lengths with mostly short and fewer long vessels, as commonly found in plants.

Most angiosperm plants have xylem vessels, which are responsible for long distance transport of water and nutrients. During vascular development, single cells fuse into linear strings. After fusion and formation of a secondary cell wall these cells (vessel elements) lose their nuclei and cell contents, leaving a hollow, dead, finite capillary (the vessel). Long vessels transport water more efficiently than short vessels because intervessel transport (i.e. transport through bordered pit pairs) is considered to contribute largely to water flow resistance (Pickard, 1981). On the other hand, short vessels are safer in case of extreme drought, frost or mechanical damage since embolisms usually cannot spread further than the vessel in which they occur (Zimmermann, 1983). It is generally hypothesised that plants growing under moderate environmental conditions have longer xylem vessels than plants growing under more severe conditions (Zimmermann, 1983).

It is difficult to measure vessel length, mainly because of the enormous length compared to the microscopic diameter (Zimmermann, 1983). Until now the most useful method is the (coloured latex) particle uptake method (Zimmermann and Jeje, 1981). With this method, a dilute aqueous particle suspension is infused or taken up into the vessel system at a transverse cut plane. Water moves freely from vessel to vessel, but the particles (±1µm) cannot pass the pit membranes. Eventually particles fill the whole cut open vessels and water transport is stopped. By counting the clogged vessels at several distance intervals from the cut surface, the length of the vessels above the plane of the cut surface can be determined. Often an exponential relationship is found for the number of clogged vessels as a function of the distance to the cut surface (Zimmermann and Potter, 1982; Zimmermann, 1983; Darlington and Dixon, 1991). This has led us to a theory that provides the simplest explanation for xylem vessel length regulation by plants.

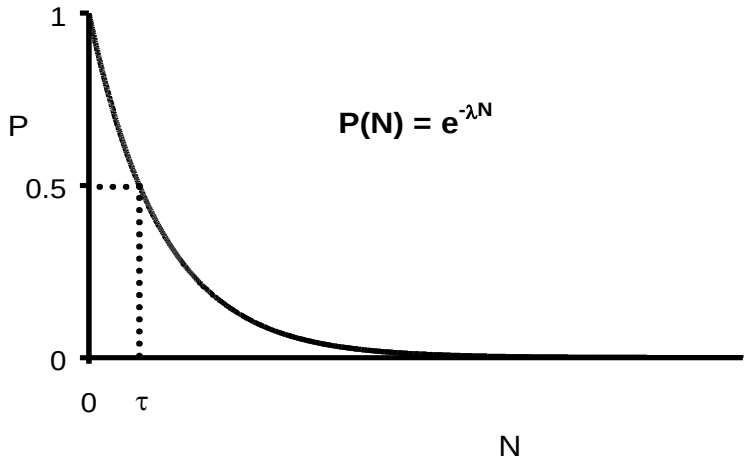

Fig 1. Fractions of vessels (*P*) that have more than *N* elements in case of a similar chance for every additional vessel element to be the terminal element. Note the analogy with the life-time of radio-active atoms. τ is the 'half-length value'.

Assume that during formation of a vessel every additional vessel element has the same chance to be the final element. In that case, the length of a single vessel cannot be predicted, but the length distribution of a population of vessels can be estimated. Analogous to the lifetime distribution of radioactive atoms, the length description of a population of vessels is then described by:

$$P(N) = e^{-\lambda N}$$ (figure 1)

where *N* is the number of vessel elements (i.e. vessel length) and *P(N)* is the fraction of the vessels that have more than *N* elements. λ Is the exponential factor from which the half-length value (τ; analogous to radio-active half-time value) can be calculated by:

$$\lambda = \frac{\ln 2}{\tau}$$

The length distribution (fraction of vessels with a length of *N* as function of *N*) is:

$$D(N) = -\partial P(N) / \partial N = \lambda e^{-\lambda N}$$ (figure 2)

In plants, special regions can exist where vessels end or start, e.g. in branch junctions or in abscission layers. Often however, vessels are randomly located in the longitudinal direction (Skene and Balodis, 1968; Zimmermann, 1983). The vessel population crossing a (virtual) cross-sectional plane through a stem is not representative for the entire vessel population. This is easily explained by imagining a second cross-sectional plane close to the first plane. Most long vessels crossing the first plane also cross the second plane, but the shorter vessels have less or no chance to cross both planes. Thus in a cross-sectional plane, the distribution of vessel lengths changes in direct correlation with their length:

$$D_{cross}(N) = cND(N) = \lambda^2 N e^{-\lambda N}$$ (figure 3)

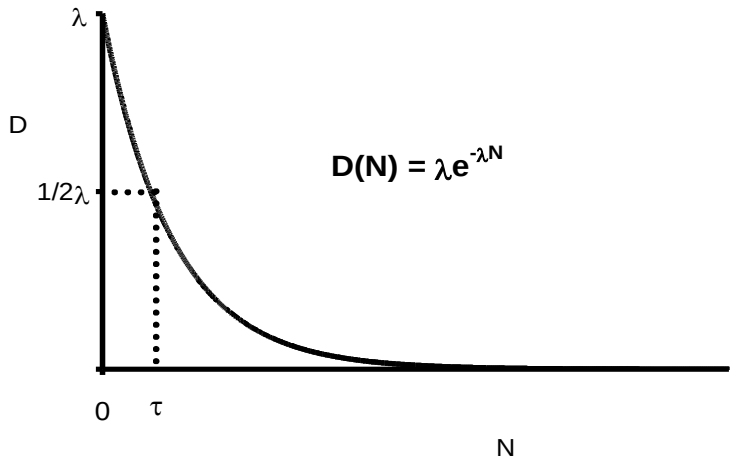

Fig 2. Xylem vessel length distribution (*D* = fraction of vessels with a length of *N* as function of *N*) for vessels with a half-length value of τ.

The constant *c* is a normalisation factor, which appears to be equal to λ. Long vessels contribute more to the xylem sap transport than short vessels (during transport a unit of sap stays longer in long vessels). If the length distribution is corrected for the impact on water transport, a 'hydraulic vessel length distribution' is made:

$$D_{hydr}(N) = cND(N) = \lambda^2 Ne^{-\lambda N} = D_{cross}(N)$$

Hence, the hydraulic vessel length distribution is equivalent to the cross-sectional vessel length distribution.

If the particle uptake method is used to determine vessel lengths, a cross-sectional cut is made and the cut open vessels take up the particles. Of these vessels, the part below the cut plane is not measured. Normally the fraction of the vessels that is below the cut surface is randomly distributed. However long vessels can have a longer part cut off than short vessels. Therefore the length distribution of the upper (or lower) parts of the cut open vessels is:

$$D_{cut}(N) = \frac{c}{N} D_{cross}(N) = \lambda e^{-\lambda N} = D(N)$$

which is nicely the same distribution as the real vessel length distribution.

The presented vessel length determination by plants needs only one assumption: the existence of a same chance for each added vessel element to be the end of the vessel (or complementary: a same chance to fuse). In terms of regulatory mechanisms in the plant, this is a most simple alternative. As shown in the graphs, (hydraulic) vessel length distribution consists of a range of vessel lengths, depending on the half-length value. It is known that the plant can regulate vessel length distributions. More lateral branches mostly have shorter vessels and some trees have much longer vessels in spring than in summer (Zimmermann and Potter, 1982).

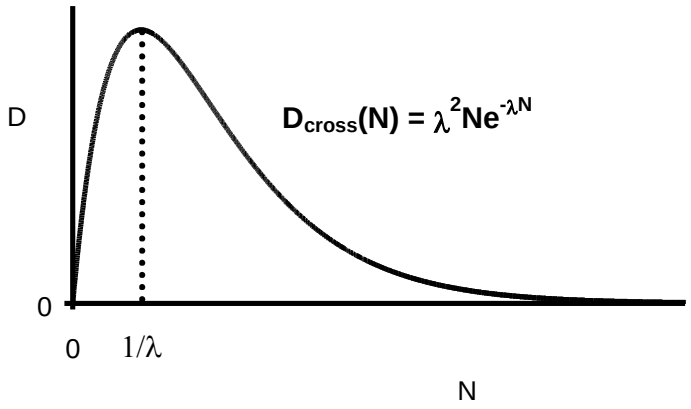

Fig 3. Cross-sectional xylem vessel length distribution for vessels with a half-length value of τ.

Plants can easily regulate vessel lengths by just changing the element-fusion chance. Obviously stems that have more half-length constants in one plane do not show this simple exponential $D_{cut}(N)$ function. In the presented functions, vessel length was defined by the number of fused elements. If vessel elements have a stable average length, the vessel length distribution functions can also be written in length units. Vessel element length can vary (Baas et al., 1983), but is not thought to be of influence on total vessel length (Zimmermann, 1983). Our theory does not precisely predict where the chance mechanism is located. The arrangement and fusion of vessel elements is the result of a complex process. Auxins, cytokinins and ethylene play roles in vessel element differentiation (Fukuda, 1997). The fusion chance itself might be a result of one hormone flux or gradient, but more likely the fusion chance results from the output of several regulatory chains. This theory provides a useful insight in plant structure, both in explaining simply ruled ingenious structures and in enabling to model plant (vascular) architecture. Our findings fit to the idea of the non-determined fractal-like nature of the architecture of plants (West et al., 1999).

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Chapter 3

Visualisation of xylem contents

Overview

This chapter presents a method to visualise xylem vessel contents. The contents of intact xylem cannot be visualised after making a simple (cross-)section through the xylem. Cutting results in a rapid and non-controlled redistribution of xylem vessel contents. Xylem contents can be fixed and visualised by freezing and subsequent investigation in a cryo scanning electron microscope (cryo-SEM). One of the most used techniques to visualise internal structures of frozen biological specimens is freeze fracturing, however, not all specimens (including xylem) can be fractured in all desired planes. Existing methods were reviewed and combined with our own results to improve the method of cryo-planing. Cryo-planing and subsequent visualisation in a cryo-SEM enables to investigate internal structures of frozen hydrated specimens in any desired two-dimensional plane.

3.1

Cryo-planing for cryo-SEM

J. Nijsse and A.C. van Aelst

Review article, also published in Scanning 21: 372-378 (1999).

Abstract

In the past decade, investigators of cryo-planing for low temperature scanning electron microscopy (cryo-SEM) have developed techniques that enable observations of flat sample surfaces. This study, reviews these sample preparation techniques, compares and contrasts their results and introduces modifications that improve results from cryo-planing. A prerequisite for all successful cryo-planing required a stable attachment of the specimen to a holder. In most cases, clamping with a screw mechanism and using indium as space-filler sufficed. Once this problem was solved, any of three existing cryo-planing methods could be used to provide successful results: cryo-milling, microtomy in a cold room and cryo-ultramicrotomy. This study introduces modifications to the cryo-planing technique that produces flat surfaces of any desired plane through a specimen. These flat surfaces of frozen, fully hydrated samples can be used to improve observations from cryo-SEM as well as to enhance results from X-ray microanalysis and (digital) image analysis. Cryo-planing results of chrysanthemum (*Dendranthema x grandiflorum* Tzvelev) stems, hazel (*Corylus avellana* L.) stems and rapeseed (*Brassica napus* L.) pistils are presented to illustrate the use of the planing method on respectively fibrous, hard, and delicate materials.

Introduction

Low temperature scanning electron microscopy (cryo-SEM) has been used to visualise surfaces of fully or partly hydrated frozen biological specimens (Read and Jeffree 1991, Wergin and Erbe 1991, Leprince *et al.*, 1998, Nijsse *et al.*, 1998a). To observe the internal features of a sample, a ‘fractured’ surface must be created. Freeze fracturing is the conventional method used to acquire fractured surfaces. With this method the sample is frozen and the specimen is broken or cracked to expose the fractured faces. Freeze fracturing is a simple and rapid technique; however, the fracture generally follows the path of the least resistance, which is often along the inter-membranes (Willison and Rowe 1980). Although this technique has been successfully used for numerous biological investigations, Huang *et al.* (1994) summarised five disadvantages of freeze fracturing: 1) the plane of fracture is

uncontrolled; 2) some planes of fracture are virtually impossible to achieve; 3) the fractured face is generally not a flat plane; 4) irregular fractured surfaces yield unreliable X-ray signals for microanalysis; and 5) multiple fractures at successive levels within the same specimen are usually impossible to perform. To avoid these disadvantages of freeze fracturing, a technique known as cryo-planing was developed. This technique, which has also been referred to as cryo-shaving (Sano *et al.*, 1995) and cryo-polishing (Nijsse *et al.*, 1998b) is used to produce flat surfaces (planes) that reveal the internal structures of frozen specimens.

Cryo-planing has been accomplished by several different investigators using various protocols. The general procedure is as follows: 1) freeze the (intact) tissue, reduce its size and mount on a holder; 2) plane the tissue at the desired level; and 3) observe and further process, if needed. The following sections discuss the details of these procedures.

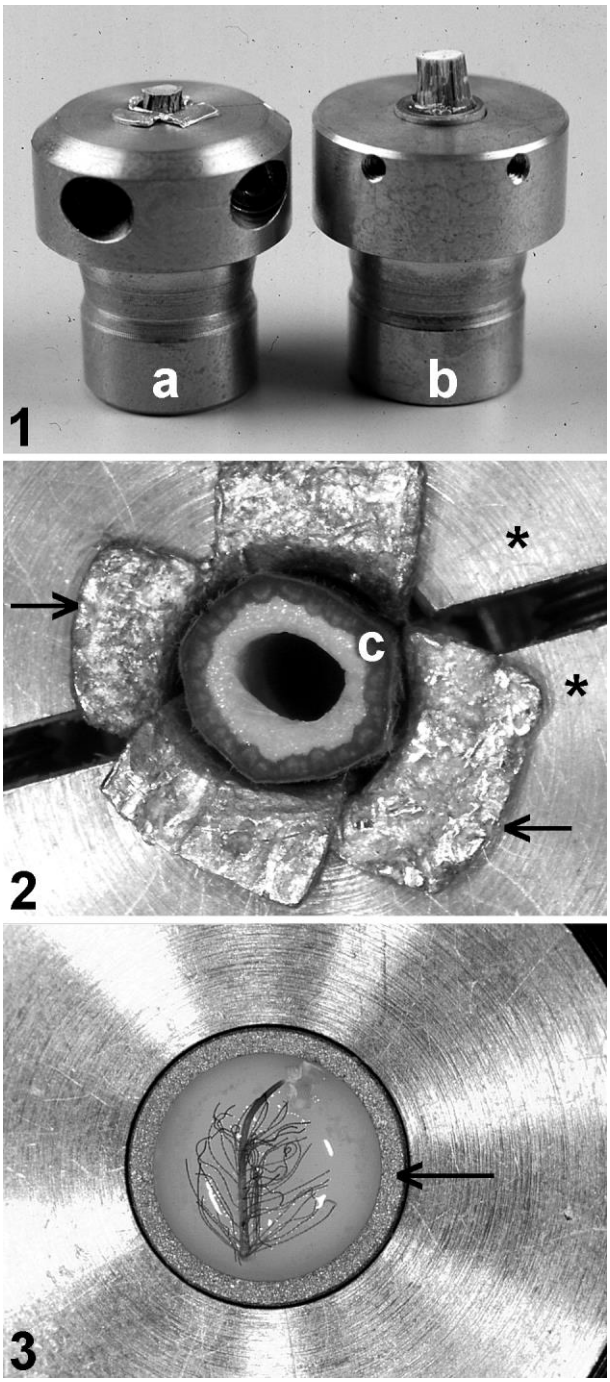


Fig. 1. Two different types of screw-clamp holders specially manufactured for the Reichert Ultracut E/FC4D. The holder **a** is designed for clamping stem samples. The holder **b** is designed for clamping 'cup shaped' sample holders. Horizontal field width = 6.4 cm. **Fig. 2.** Detail of the centre of clamping holder **a**. The frozen chrysanthemum (c) stem is surrounded by indium foil (arrows) and clamped in a hole between two halves of the holder (*). Horizontal field width = 1.7 cm. **Fig. 3.** Detail of the centre of clamping holder **b**. In the 'cup shaped' sample holder (arrow) a stamen with stamen hairs of *Tradescantia* is frozen on Tissue Tek. The 'cup shaped' sample holder is clamped with two screws into a just fitting hole of the clamping holder. Horizontal field width = 2.2 cm.

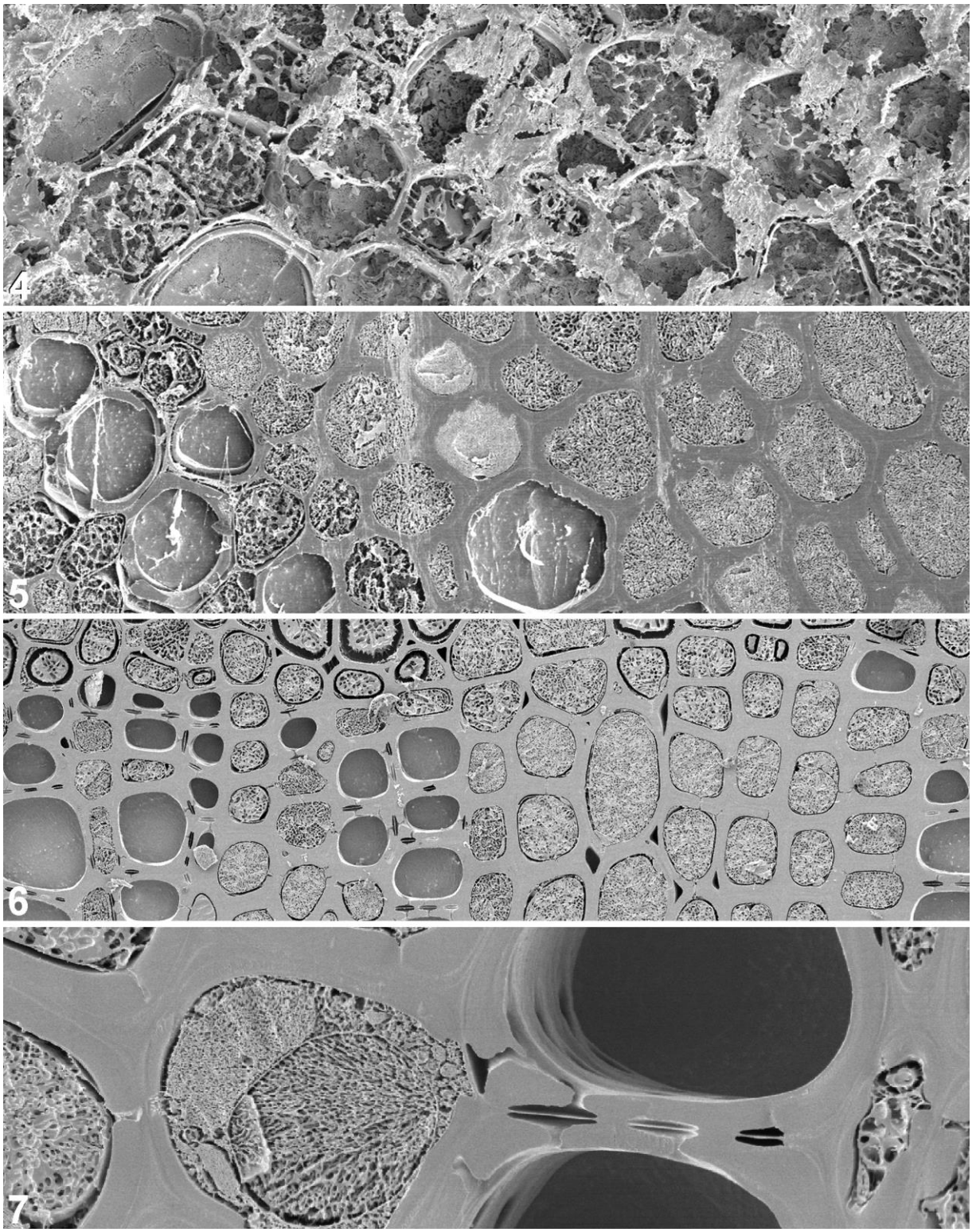
Sample preparation

Choosing the proper method for freezing depends upon the type of information that is ultimately desired (Willison and Rowe 1980). Most biological materials are frozen intact, to prevent artefacts such as redistribution of fluids or mechanical damage. Biological samples are ordinarily frozen using liquid nitrogen (LN_2) or melting nitrogen. To avoid freezing tension cracks in large specimens, slow freezing can be useful (Carvalho *et al.*, 1999)

Restrictions of the applied planing technique and the amount of available space on a specimen holder and on the cold stage in the SEM will dictate the final size of the frozen sample. Several methods have been used to reduce the sizes of frozen samples. Bastacky (*pers. comm.*) initially used a carbide tipped circular saw to reduce the size of frozen lung tissues and then a fine diamond circular dental saw for the final specimens (Bastacky *et al.*, 1987, Wu *et al.*, 1996). Investigators working with plant tissues have used fracturing (Tyree *et al.*, 1999), cutting (Dong *et al.*, 1997), trimming (Canny 1997), splitting (Canny 1997), and probably also sawing (Usumi *et al.*, 1996) to reduce sample sizes. However, the details of these procedures were not presented. In our laboratory, we successfully used a circular diamond saw driven by a hobby drill (22 mm diameter, Minicraft, Black and Decker; result in Fig. 4), to obtain transversal surfaces of frozen stems of chrysanthemum (*Dendranthema x grandiflorum* Tzvelev) and hazel (*Corylus avellana* L.).

Before the planing procedure the sample has to be mounted in or on a holder that fits in the planing device (Fig. 1) and subsequently in the microscope. Mounting frozen specimens onto a holder is frequently difficult. To avoid problems, investigators (e.g. Van Cauwenberghe *et al.*, 1993) have used water based glues (Tissue Tek, O.C.T. compound; or tissue freezing medium, TBS) to mount tissue prior to freezing. Very small specimens, such as root hairs or fungal mycelia can be glued together prior to freezing (Huang *et al.*, 1994). Aggregating specimens in this manner allows cryo-planing and observation of several specimens on a single holder. We used ‘cup-shaped’ stubs filled with tissue freezing medium to embed stamens and pistils (Fig. 3, 10 and 11)**,** thus providing a stable and firm fastening of these delicate structures. Sample mounting prior to freezing works well when no further size reduction of the frozen sample is needed.

In many cases, specimens must be mounted after they are frozen. Theoretically, this procedure could be accomplished by using a cryo-adhesive or a mechanical device. Unfortunately, no cryo-adhesives appear to be available for this purpose. However, investigators have successfully mounted frozen specimens by placing Tissue Tek on or in a holder at ambient temperature and than quickly plunging the holder and specimen in a cryogen (Canny 1997, Nijsse *et al.*, 1998b). When this procedure is used, care must be taken to avoid thawing and redistribution of structures in the specimen. In chrysanthemum, we found that mounting frozen stem segments at ambient temperature was not associated with any apparent thawing artefacts. With this method of mounting, poor attachment or failure to attach was frequently encountered, resulting in a poor mechanical stability.



Figs. 4-7. Transversally planed surfaces of the central cylinder of a frozen chrysanthemum stem. **Fig. 4.** Surface after 'trimming' at liquid nitrogen temperature with a circular diamond saw. Smearing and damaging effect at the surface is clearly visible. The cell walls are visible at some places. Horizontal field width = 170 µm. **Fig. 5.** Surface after applying a cryo-mill (Reichert-Jung Polycut E, equipped with a cryo-

stage). The cell walls are visible in the surface, some rupture and smearing of the walls, especially in vertical direction, is visible. Horizontal field width = 170 µm. **Fig. 6.** Surfaces of a sample that was screw-clamped using indium and planed in a cryo-ultramicrotome (Reichert Ultracut E/FC4D) with a glass knife and finally with a diamond knife (Histo no trough, 8 mm 45°, Drukker International, The Netherlands). No damage or smearing is visible. Horizontal field width = 170 µm. **Fig. 7.** Detail of same sample as Fig 6, revealing different pit types, cell wall layers of tracheal and parenchyma elements and organelles in parenchyma cells. Horizontal field width = 36 µm.

Suitable fitting of the sample to the holder is essential for optimal cryo-planing results. Huang *et al.* (1994) suggested modifying the holder to match the shape of the specimen.

Using a mechanical device to mount a frozen specimen also has difficulties. In most cases (e.g. in Bastacky *et al.*, 1990), the frozen sample is merely attached by clamping into a vice-like device using screws. Unlike adhesives, this procedure is not temperature dependent. However, one disadvantage of clamping is that an irregularly shaped sample may contact the holder at only a few points and thereby be subject to charging. In addition, small or delicate specimens may fracture due to the pressure exhorted during the mounting procedure or insufficient pressure may not result in a mount that prevents the specimen from moving during the cryo-planing step. To help avoid these problems, Utsumi *et al.* (1998) added a drop of glycerol to the mounted specimen. This procedure helped strengthen the attachment of specimen to the holder. Erbe (*pers. comm.*) strengthens the mount by wrapping or inserting a layer of indium metal between the specimen and the holder. Indium is a very pliable metal even at LN_2 temperatures (Fig. 2) and tends to mould and fill the spaces between the specimen and vice as the screws are tightened. Successful mechanical attachment of specimens by using indium has been achieved with a wide range of biological samples.

Cryo-planing

Cryo-planing, which is aimed to produce a flat internal surface that is free of artefacts, is performed by at least two methods: ultra-milling (van Doorn *et al.*, 1991, Nijsse *et al.*, 1998b, Carvalho *et al.*, 1999), or microtomy (Huang *et al.*, 1994, Sano *et al.*, 1995). To perform ultra-milling, commercial equipment such as the Reichert-Jung Polycut E (Leica, Germany), equipped with a cryostage can be used. This instrument consists of a cold stage that accommodates the specimen holder and a rotating mill whose face contains diamond chisels. As the rotating diamond face is gradually moved toward the specimen, the frozen tissue is milled to produce a flat surface of internal tissues of the specimen. Using this method, relatively large areas, measuring up to several square centimetres, can be planed. However, due to the high speed of the rotating diamonds, tissues that are not adequately supported (e.g. walls of empty xylem vessels) frequently break or fracture at levels below the planed surface.

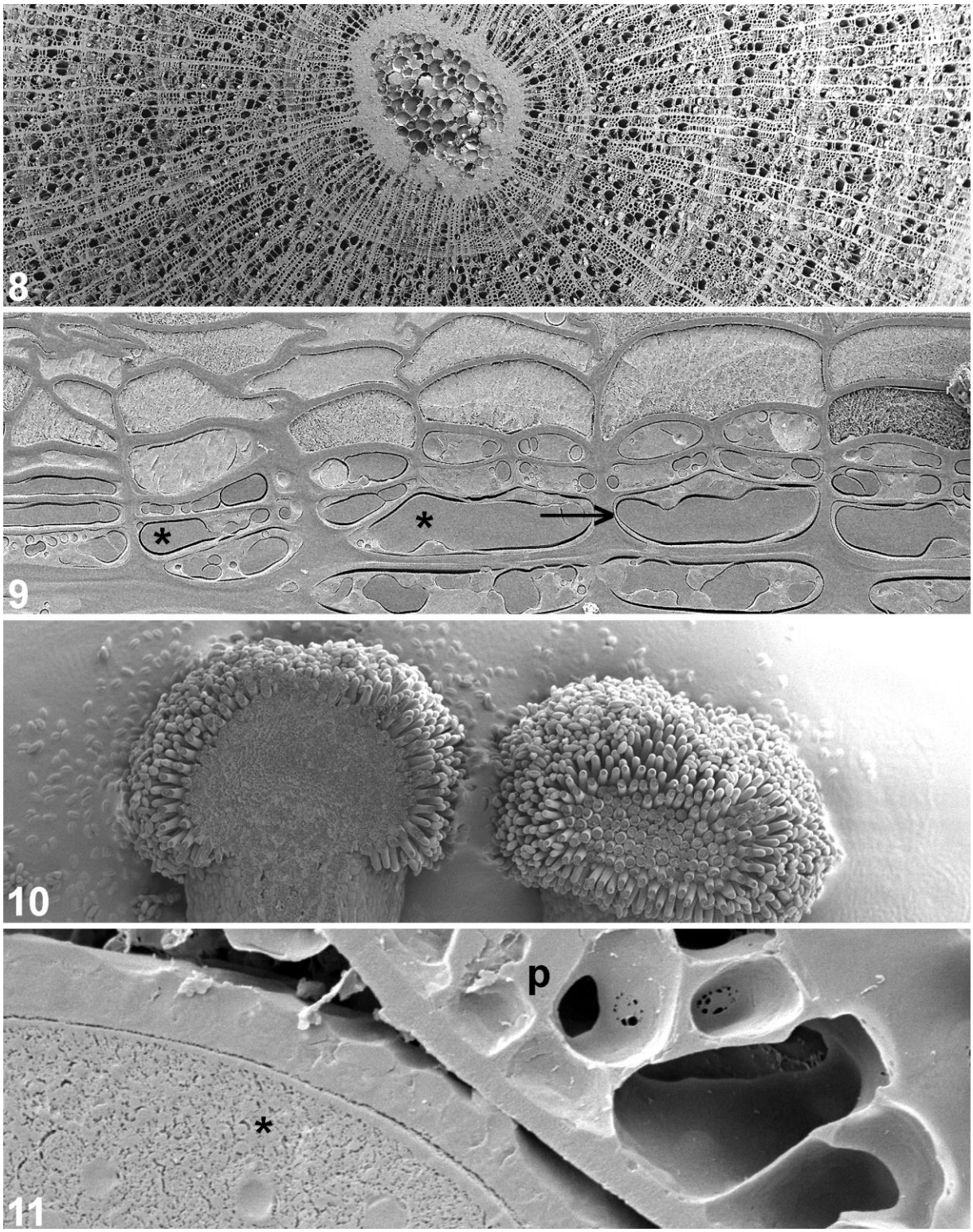


Fig. 8. Transversally planed surface of a 'woody' Hazel stem using indium in the clamp holder and planed in a cryo-ultramicrotome (Reichert Ultracut E/FC4D) with a glass knife and finally with a diamond knife (Histo no trough, 8 mm 45°, Drukker International, The Netherlands). Horizontal field width = 3014 µm. **Fig. 9.** Detail of the cork cambium in the bark of the Hazel stem. The cytoplasmic components in the cells

are visible. The free water content of the cambial zone is relatively low and large non-etchable areas (*), which may consist of oil, can be seen in the cambial cells (arrow). (The planed stem was harvested in winter). Horizontal field width = 174 µm. **Fig. 10.** Longitudinal planed surfaces of two rapeseed stigmas (*Brassica napus* L.) using the 'cup shaped' sample holder and the cryo-ultramicrotomy planing technique. To avoid artificial hydration of the stigma papillae the sample is frozen "halfway sunken" in tissue freezing medium. Well controlled planing is essential for stigma-pollen interaction analysis. Horizontal field width = 2391 µm. **Fig. 11.** Detail of fig. 10. A planed dry pollen grain (*) closely attached to a papilla (p) of a rape seed stigma. Horizontal field width = 15 µm.

In addition, this technique can cause rupturing and “smearing” of tissues across the plane. A transverse plane of a chrysanthemum stem as obtained with the cryo-milling procedure is shown in Figure 5. The general quality of the specimen is acceptable, but structural details, such as bordered pit pairs, are barely visible due to smearing.

Cryo-planing by microtomy is more widely used and probably more useful and cheaper than cryo-ultramilling. The procedure is similar to cryo-microtomy, which is used to produce frozen thin sections. However, for cryo-SEM the thin sections are ignored and the face of the frozen block is observed. Two variations of this technique are currently being employed. With the first method, cryo-microtomy is performed on a normal sledge microtome, which is equipped with a steel knife, in a low-temperature (-20°C) room (Sano *et al.*, 1995, Utsumi *et al.*, 1998). This method allows planing of large areas of high quality, but because of the relatively high temperature, re-crystallisation can take place (Willison and Rowe 1980). With the second method, a cryo-ultramicrotome is used. As a result, a block can be faced at considerably lower temperatures. This method, which has also been used to prepare planed flat surfaces for ambient temperature SEM (Alphenaar *et al.*, 1994), is clearly described by Huang *et al.*, (1994) who used a Tucson cryo-ultramicrotome that was equipped with a glass knife and operated at -80°C. He describes the procedure in the following way: “The sample was planed with slow cutting strokes, first removing thick sections to clear away the tissue damaged in cutting the piece, and finally with 1 µm sections to produce a smooth surface. The tissues removed by the knife were brushed away with a cold, fine brush to keep the knife edge clean”. In more recent investigations, Canny (1997) and Tyree *et al.* (1999) equipped the cryo-ultramicrotome with a diamond knife.

One advantage of cryo-ultramicrotomy is that the temperature of the specimen and knife is adjustable and does not rise above e.g. –80°C during the procedure. Consequently, redistribution of structures is avoided. However, examination of published figures of specimens prepared by this method frequently reveals that planing marks occur on the face of the block (see e.g. McCully *et al.*, 1998, Pate and Canny 1999). Planing marks result from scratches or chatter. Scratches, perpendicular to the knife-edge, are the result of improper knife angles or knife imperfections. Our experience indicates that planing marks most frequently occur due to chatter. This chatter, audible as scratching noise, is visible as line marks parallel to the knife-edge. Besides tight fitting of the knife, the sample has to be mounted firmly into the specimen holder. Additionally, at -80°C the sample is much harder and brittle than at -20°C, resulting in a lower grade of elastic deformation

during planing. Chatter can be avoided by decreasing the cutting speed and by reducing the section thickness to 0.1 µm or less**.** We successfully used indium to increase the fitting between the sample and the holder (Figs. 6 and 7: Fibrous chrysanthemum stem, Fig. 8: Large area of hard woody hazel stem, Fig. 9: Detail of cork cambium in the bark of the hazel stem). With this modification, no planing marks are evident even at high magnifications. Cryo–planing by ultra-microtomy of small delicate structures, embedded in Tissue Tek, prior to freezing, produced good results (Fig.10: Pistils of rapeseed, *Brassica napus* L., Fig 11: Detail of fig 10, pollen grain attached to a papilla). Our best results were obtained with a specimen temperature of – 90°C and a knife temperature of –100°C.

Planing with a cryo-ultramicrotome causes section fragments to adhere to the surface of the block and the knife due to electrostatic charging. This problem has been solved in part by using a de-ioniser or anti-static gun (McCully *et al.*, 1998). However, natural depressions, such as empty xylem vessels (McCully *et al.*, 1998), remain prone to this problem. We have had some success in eliminating the contamination by “washing” the block in LN_2 or by exposing it to sudden pressure fluctuations such as those that occur during cryo-transfer. This problem is apparently less severe or absent when the block is planed with a sledge microtome in a cold room (see figures in Utsumi *et al.*, 1998).

No single *a priori* cryo-planing method can be recommended for all samples. However, planing with cryo-ultramicrotomy appears to produce the most reliable results and avoids several artefacts because of the very low temperature at which it is performed. Firm mounting of a sample in the cryo-holder is essential for producing a flat and mark free planing surface. For planing large areas, the use of a sledge microtome in a cold room is a good alternative. Cryo-milling should be used only when very large areas, which are beyond the limits of the other techniques, need to be planed and examined at low resolution.

Investigation and further opportunities

The ability to produce flat, planed surfaces for observation with cryo-SEM has several advantages over freeze-fracturing. Well-planed surfaces allow reliable morphometry and X-ray analysis (Canny and Huang 1993, Huang *et al.*, 1994). Structural details, such as cell lumina, are sufficiently resolved so that digital image analysis is possible (unpublished results). In a flat plane, identification of tissues and other ultrastructural detail is often much easier than in a fractured plane (Van Cauwenberghe *et al.*, 1993). Cryo-planing has been shown to be suitable for studying delicate structures (Huang *et al.*, 1994), frozen hydrated biological specimens, food, and natural ice (Mulvaney *et al.*, 1988). Planing can be done on various planes through the sample, as well as in successive serial planes (Van Cauwenberghe *et al.*, 1993). This technique is currently receiving considerable attention to study the mechanism of xylem vessel embolism repair (Utsumi *et al.*, 1998, McCully *et al.*, 1999, Tyree *et al.*, 1999). No other high-resolution techniques are currently available to visualise embolisms. Recently, Holbrook *et al.* (1999) postulated that during embolism repair, the geometry of the bordered pit prevents

water from entering the pit channel until the lumen is entirely filled. Cryo-SEM and cryo-planing can be used to prove this hypothesis. Finally, samples that are prepared for cryo-planing can be stored indefinitely in LN_2. This enables several samples to be planed at one time and to be observed or re-observed at a later time.

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Chapter 4

Dynamics of embolism induced at the cut surface

Overview

If a flower is cut off, the open vessels at the cut surface can aspire air. Uptake of air depends on the suction force and the capillary force in the xylem vessel (4.1). The suction force is a result of the xylem pressure and of the cross-sectional surface area of the vessel. The capillary force results from the surface tension of the xylem sap, the perimeter of the xylem vessel and the contact angle between xylem sap and cell wall at the meniscus. The capillary force is directed opposite to the suction force and is stronger in narrow vessels than in wide vessels. Even under moderate desiccation of the cut flower, the suction force is strong enough to overcome the capillary force, thus enabling air entrance, even in the narrower vessels. Early leaf wilting was directly related to embolism, for moderately desiccated flowers which restored their water balance showed a removal of embolisms, while flowers that could not restore the water balance still were embolised (4.2). Under normal conditions, aspired air only enters the cut open vessels and does not move into intact vessels (4.3). The xylem vessel length determines the height of air entrance in cut flowers.

4.1

Induction of air embolism in xylem conduits of pre-defined diameter

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Abstract

A new method is presented that enables the induction of embolisms in a fraction of all xylem vessels, based on diameter, at one cut end of a stem segment. The method is based on the different capillary characteristic of xylem vessels of different cross-sectional size. To verify the method, air embolisms were induced in cut xylem vessels of chrysanthemum (*Dendranthema x grandiflorum* Tzvelev cv Cassa) stem segments at different xylem tensions and compared with the distribution of gas-filled vessels as visualised by cryo-scanning electron microscopy (Cryo-SEM). At -6 kPa xylem pressure, air-entrance was only induced in large diameter vessels (>30 μm), while at -24 kPa embolisms were induced in almost all xylem vessels (>10 μm). Although the principle of the embolisation method worked well, smaller diameter vessels were observed to be embolised than was expected according to the calculations. The role of cross-sectional shape and contact angle between xylem sap and vessel wall at the menisci are discussed. After correction for the observed (diameter independent) deviation from circularity of the cross-sectional vessel shape the contact angle was calculated to be approximately 55°. Hydraulic resistance (R_h) measurements before and after embolisation showed that embolising only large diameter cut xylem vessels had only a small influence on overall R_h of a the stem segment. Embolising all cut xylem vessels at one cut end almost trebled overall R_h. The difference was discussed in the light of the networking capacity of the xylem system.

Introduction

Air embolisms in xylem conduits can partially or completely block the water transport path between roots and sinks for water in plants. The consequent increase in hydraulic resistance (R_h) of the xylem may cause severe water stress and in extreme cases even plant death (Pickard, 1981). In intact plants air embolisms can result of cavitation, due to subsequent freeze-thaw cycles or severe water stress (Sperry and Sullivan, 1992). Air embolisms may also occur as a result of mechanical injury of xylem conduits, for example due to storm. Besides these natural reasons, well-considered human action such as for instance the harvest of cut flowers by

cutting them in air may also lead to air embolisms in xylem vessels that are later supposed to conduct water again. Insufficient removal of these air embolisms may result in serious water stress which may lead to early leaf wilting as previously has been shown in chrysanthemum cut flowers (Van Meeteren, 1992). Until now, little is known about the physiological background of this phenomenon, despite its common occurrence. Possibly vessel characteristics play a role. Large diameter vessels are thought to be responsible for a disproportional fraction of the overall flow through a stem because of the r^4 relation of flow through circular pipes (Hagen-Poiseuille relation; Dimond, 1966; Gibson *et al.*, 1984; Pickard, 1981). Consequently, large diameter vessels are important to prevent water stress, although it has to be mentioned that in chrysanthemum stems bordered pits at vessel-to-vessel connections significantly add to the hydraulic resistance of the xylem system (van Ieperen *et al.*, 2000; Nijsse *et al.*, 2001). However, large diameter vessels are also thought to cavitate more easily than narrow vessels (Netting, 2000). The bordered pits, on the other hand, restrict the spread of air in the cut flower stem and therefore the increase in hydraulic resistance due to air emboli, because they do not allow the passage of air-water interfaces (Zimmermann, 1983).

There is a lot of evidence that embolised vessels may be repaired. Various studies on different plant species using Cryo Scanning Electron Microscopy (cryo-SEM) or measuring hydraulic resistance show that xylem vessels can recover from air embolisms (Canny, 1997; McCully *et al.*, 1998; Nijsse *et al.*, 2000; Sperry *et al.*, 1987; Utsumi *et al.*, 1998; Yang and Tyree, 1992). The mechanism by which the plant manages to remove these air embolisms is still under debate (Holbrook and Zwieniecki, 1999; Tyree *et al.*, 1999). Yang and Tyree (1992) developed an interesting mechanistic model for air removal after cavitation. In this model capillary forces compress the air inside the trapped air bubble and thus generate the driving force for the removal of these air embolisms. A simple form of the capillary equation was used to calculate the driving force for air removal, assuming the vessels circular and the contact angle between xylem cell wall material and water at the meniscus 0º. Model calculations showed that stems with large diameter vessels recover slower from air embolisms than stems with small diameter vessels (Yang and Tyree, 1992). Thus, vessel diameter probably not only influences the hydraulic resistance of the xylem system but also the recovering process from air embolism. Unfortunately, reality is more complicated since plants usually contain vessels in a wide range of diameters (Zimmermann, 1983), which considerably complicates the process. Furthermore, the model calculations depend on the assumed cross-sectional circularity of the embolised vessels and on the assumed 0º contact angle. These assumptions are both disputable (Pickard, 1981).

Until now experimental evidence for the role of vessel diameter in recovering from air embolisms is poor, due to the lack of a good method to embolise vessels of different diameter within samples of comparable xylem structure. The aim of this paper is to present and verify a method that enables controlled induction of air embolisms in cut xylem vessels at one cut end of a stem segment, which are larger than a certain pre-defined cross-sectional size. The embolisation method is basically verified by visualisation of the water and air-filled cut xylem vessels at the cut surface and measuring their cross-sectional size using Cryo Scanning Electron Microscopy (cryo-SEM) (Canny, 1997) in combination with image analysis techniques.

Additionally, hydraulic resistance (R_h) measurements were done before and after embolisation to investigate the effect of partial blockage of the xylem on overall R_h. Image analysis techniques were also used to measure the cross-sectional shape of the actually embolised vessels to determine their deviation from circularity.

Materials and methods

Method basics and theoretical background

The embolisation method basically consists of the controlled suction of air into a pre-defined fraction of all initially water-filled cut xylem vessels at one cut end of an excised stem segment. This pre-defined fraction consists of vessels that are larger than a certain cross-sectional size. The controlling factor for water entry is the actual xylem pressure at the cut end of the stem segment where water usually enters, but which is temporary exposed to air to enable air entrance. The capillary characteristics of the vessels, which strongly depend on their cross-sectional size, are used as the distinguishing factor that determines whether a vessel embolises or not. A common measurement system for R_h measurements is used to control the xylem pressure during embolisation. The use of such a measurement system also gives the possibility to measure R_h before and after embolisation to determine the effect of embolisation on R_h of the stem segment.

After cutting, air-water interfaces (menisci) appear at all ends of cut xylem vessels. Upon exposure to air, such a meniscus only starts to move inwards if the inward force exceed the outward force on the meniscus ($F_{inw} > F_{outw}$). This could be the case if the xylem is under tension (negative pressure). However, xylem pressures are not always low enough to enforce the entrance of air in all diameter vessels at the cut end. Capillary characteristics of the xylem vessels tend to oppose the entrance of air. F_{inw} depends on the pressure in the xylem vessel (ψ_{xp} [Pa]) and on the cross-sectional surface area of the vessel (A_c, [m^2], Eqn 1). F_{outw}, the capillary force that tends to keep the meniscus of the water column at the cut end of the vessel, is generated by the outward component of the surface force along the edge of the meniscus (Nobel, 1983). F_{outw} depends on the surface tension (γ, [N m^{-1}]) of the xylem sap, the perimeter of the xylem conduit (P_c, [m]) and the contact angle (Θ, [°]) between xylem sap and cell wall at the meniscus (Eqn 2).

$$F_{inw} = \psi_{xp}.A_c \quad (1)$$

$$F_{outw} = P_c.\gamma.\cos(\Theta) \quad (2)$$

Combining Eqn 1 and 2 gives Eqn 3 where ψ_{xp} is the xylem pressure at the cut surface at which a xylem vessel with a perimeter-area ratio P_c/A_c starts to embolise.

$$\psi_{xp} = -\frac{P_c}{A_c}.\gamma.\cos(\Theta) \quad (3)$$

Xylem sap is usually very dilute. Therefore γ is assumed to be equivalent to that of water (0.072 N m^{-1} at 20 ^{o}C). Xylem vessels are often assumed to be circular in cross-section and the contact angle is often assumed to be 0° (Pickard, 1981). Following this simplification substitution of the perimeter ($2 \cdot \pi \cdot r_c$) and the cross-sectional area ($\pi \cdot r_c^2$) of circular shapes into Eqn 3 gives Eqn 4, in which r_c [m] is the radius of the xylem vessel at its cut end.

$$\psi_{xp} = \frac{2}{r_c} \cdot \gamma \qquad (4)$$

Thus, according to the last equation, if ψ_{xp} is –6 kPa only cut vessels with a radius larger than 25 μm should embolise upon exposure to air. If ψ_{xp} is –24 kPa, all vessels with a radius larger than 6 μm should embolise. Deviation of the cross-sectional shape of the meniscus from circularity tends to increase the calculated critical radius, while an increased contact angle tends to decrease the calculated critical radius. These pressures are certainly much smaller than what could normally be expected in xylem conduits of transpiring plants (Nobel, 1983). However, xylem pressures between 0 and –50 kPa may easily be established in water filled xylem vessels in excised stem segments using a simple laboratory system for hydraulic resistance (R_h) measurements. In fact, the use of small pressure differences to induce flow (< 50 kPa) is common practise during hydraulic resistance determinations (e.g. Chiu and Ewers, 1993; Sperry *et al.,* 1988; van Ieperen *et al.,* 2000), although not always negative xylem pressures are induced. In contrast to the pressure gradient required to induce flow for R_h measurements, induction of air embolisms depends on the negative xylem pressure located at the cut end of the stem segment where water usually enters. During R_h-measurements with negative xylem pressures actual xylem pressure at this point is usually close to atmospheric pressure. Fortunately, the pressure gradient during R_h measurements is clearly related to flow. Withdrawal of the water supply from the cut end of the stem segment stops the flow of water through the stem segment and forces the xylem pressure in the whole sample to equilibrate to a constant uniform negative xylem pressure (ψ_{xp}), caused by the pulling pressure at the other cut end of the stem segment. If ψ_{xp} is low enough to induce air entrance into a cut xylem vessel (Eqn 4), air enters only the cut open vessel because capillary forces in pit membranes of bordered pits do not allow the passage of air-water interfaces at these relatively small xylem pressures.

Plant material and sample preparation

Samples were collected from mature chrysanthemum (*Dendranthema x grandiflorum* Tzvelev cv. Cassa) plants. Plants were propagated via stem cuttings, transplanted into pots and grown until commercial maturity according to Van Meeteren *et al.* (2000). The length of the growth period from transplanting to sample collection was approximately 12 weeks. One night before cutting the plants were well irrigated and put in dark for 12 hours to ensure maximal turgidity and minimal presence of natural air embolisms. The plants were approximately 90-100 cm long and fully flowered. Each sample (stem segment) originated from an individual plant and was collected from the same position on the plant. All manipulations with plants

and stem segments in the laboratory were done under water to prevent the entrance of air into the vessels at their cut ends. Stem segments of 40 cm length were cut from the plants at 5 cm above the root/shoot junction with sharp shears and re-cut with a razor blade to approximately 30 cm length by cutting off 5 cm from both ends. A new razor blade was used for each cut. The length of the stem segments was exactly measured. Stem segment was always longer than the maximal length of cut open vessels (approximately 25 cm), which was determined using the latex particle method of Zimmermann and Jeje (1981) on three similarly grown chrysanthemum plants (Van Ieperen *et al.,* 2000). Leaves were removed from the stem segment with a razor blade, leaving 1 cm of the petioles on the stem. Each stem segment had 5-6 petiole stubs and was approximately 5-6 mm in diameter. A silicone tube was pushed over the upper cut end of the stem segment (cut end at largest distance from the roots) and attached to the R_h-measurement system (see below and Fig. 1). During preparation a small positive pressure was applied to the upper side of the stem segment to avoid penetration of air into open vessels at the lower cut-end. The time between collection from the plant and the start of the R_h-measurement was approximately 15 minutes.

Rh measurements

To determine R_h of a stem segment, the water flow rate was measured by pulling water through the stem segment at a known pressure gradient. To standardise the R_h measurement a hydraulic pressure difference of -24 kPa was used. During measurements, flow was always in natural direction i.e. from the lower cut end (closest to the roots) to the upper cut end of the stem segment. A hanging water column of degassed water in a flexible tubing system provided the pulling pressure at the upper cut end of the stem segment (Fig. 1A). The pressure gradient was calculated from the length of the stem segment and the difference in height between the water level in the container on the balance and the lower open end of the tubing system. Changes in the pressure gradient during R_h measurements were negligible because the water level in the container changed less than 3 mm during each individual measurement.

The rate of water flow through the stem segment was calculated from weight changes measured with a Sartorius LC3200D balance interfaced to a personal computer. Weight was measured at 1 s^{-1} sample rate and averaged over 30 seconds. Two subsequent averaged weights were used to calculate the rate of water flow. Calculated flow rates were corrected for direct evaporation from the container, which was measured before and after each individual measurement and during the embolisation treatment. No correction was done for the change in submerged volume of the stem segment in the solution because the calculated error on the rate of water flow (of a 5 mm diameter stem segment submerged in a solution in a 9.5 cm diameter container) is lower than 0.5%. This error could even be lower due porosity of the stem segment. Flow measurements were done using an aqueous solution containing 1.49 mol m^{-3} $NaHCO_3$, 0.67 mol m^{-3} $CaCl_2$, 4.8 mmol m^{-3} $CuSO_4$, (pH=6.6; 20 $\pm$2°C). This solution sufficiently delays the long term increase in R_h, which, as in other species (Sperry *et al.*, 1988), also occurs in excised Chrysanthemum stem segments when using deionised water as the flowing solution (Van Meeteren *et al.,*

2000). Due to the almost neutral pH and low concentration of solutes, this solution is likely to have a surface tension and contact angle similar to water or xylem sap.

To justify the use of the standard pressure of –24 kPa during R_h measurements and the use of defoliated stem segments, tests were done to check whether measured R_h was affected by the magnitude of the pulling pressure and whether leaf removal influenced R_h. Two sets of four different pressure gradients were subsequently applied on a single stem segment (and repeated on four different stem segments): one set of pressures was applied before and one after leaf removal. To check if all vessels near the lower cut surface were involved in water transport, tests were done with a 1% acid Fuchsin solution (Sigma Chemical Co., St. Louis, USA). Stem segments were harvested, prepared and attached to the measurement system as described above. Flow was permitted during 2 minutes after dye appeared at the upper cut end of the stem segments. Fresh sections were cut at several distances from the bottom cut surface with a razor blade and examined under a light microscope.

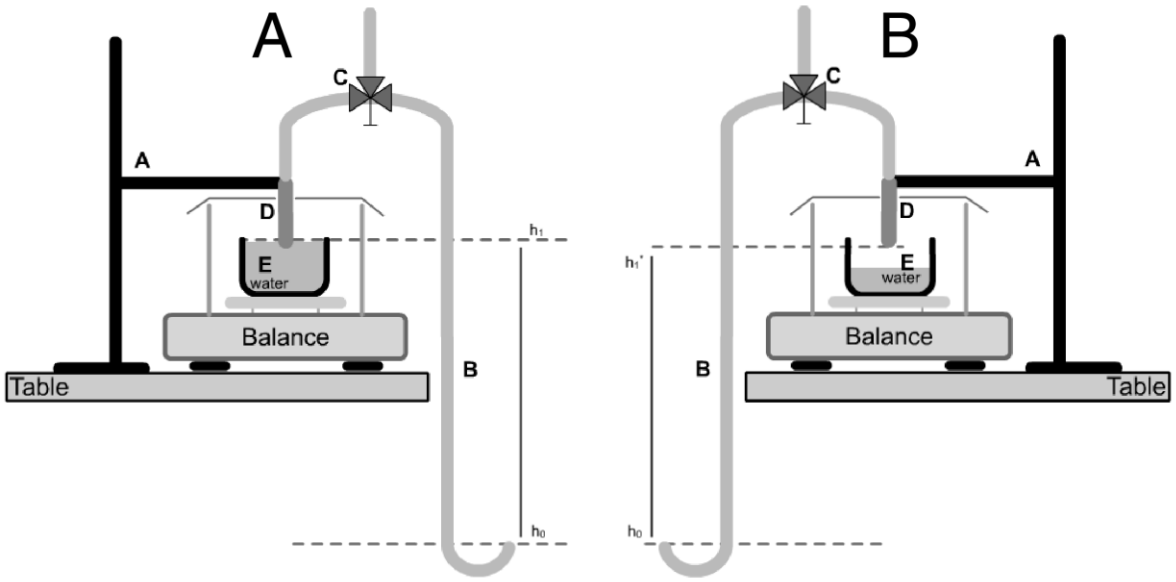

Fig. 1. Apparatus for **(A)** measuring hydraulic resistance (R_h) and **(B)** controlling the xylem pressure at the basal cut end of excised stem segments during exposure to air (**B** is **A** but without water supply to the basal cut end). To induce a pressure gradient (of below atmospheric xylem pressures), the upper part of a stem segment was connected to a flexible silicon tube holding a hanging water column of de-gassed water, which could be varied in length. During R_h measurements the basal cut surface of the stem segment was positioned approximately 0.5 cm below the surface of the water in the container on the balance. The pressure gradient to induce flow was determined by the length of the water column (h_1-h_0) and the length of the stem segment. The length of the water column which induced the pressure at the basal cut end during embolisation was slightly smaller (h_1^*-h_0). (A: lab stand, B: Flexible silicon tube with degassed water (hanging water column), C: 3-way valve to switch between below and above atmospheric pressure, D: stem segment, E: glass container (diameter = 9.5 cm) filled with solution)

Air embolisation treatment

After the first R_h measurement on a stem segment without initial air embolisms (at -24 kPa) one of the embolisation pressures (-6.0, -10.4 or -24.0 kPa) was established. Then the water level in the container was lowered just below the lower cut end of the stem segment in order to: 1) stop the flow of water, 2) induce equilibration of ψ_{xp} along the stem segment and 3) allow air entrance into cut vessels at the lower cut end. After a period of approximately 6 minutes of exposure to air the water level was raised again and a second R_h measurement (at -24 kPa) was made.

At –6 and –24 kPa four replicate cycles (R_h-measurements - embolisation -R_h-measurement) were done, each cycle on a new stem segment. The whole procedure was repeated after approximately 3 weeks with new stem segments collected from a new batch of plants. During the second batch of measurements an extra embolisation pressure (–10.4 kPa) was added.

Cryo Scanning Electron Microscopy

Stem segments were prepared for visual analysis of embolised vessel ends using cryo-SEM. The basal 10-15 cm of these stem segments were carefully frozen into liquid nitrogen at the following stages during a cycle:

1) Immediately after harvest to check for the presence of initial air-embolisms
2) After 6 minutes of exposure to air to verify the effect of ψ_{xp} on the embolisation of xylem vessels.

The frozen stem segments were stored in liquid nitrogen (-196 ^{o}C) prior to final preparation for cryo-SEM. To visualise the contents of the xylem vessels in the stem segments, the lower 1 cm portions of the frozen stem segments were mounted on hollow brass stubs using Tissue Tek adhesive (No. 4583 Miles Inc., Elkhart, Indiana, USA) with the lower cut ends positioned upwards. Cut-surfaces of stem segments that had been frozen immediately after harvest were not suitable for direct examination of vessel content due to adhering, frozen water. Therefore, these samples were sawn at 2 mm and 3 cm from the basal cut surface and planed with steel blades or a glass knife in a cryo-microtome at a maximum temperature of -30°C. The cut surfaces of samples taken immediately after air entrance were examined without any re-cutting. The surface was etched for 5 min at -89°C and 10^{-4} Pa in an Oxford CT 1500 HF cryo-transfer unit and sputter-coated with platinum. Vessel contents were investigated at the cut surface in a JEOL 6300F field emission scanning electron microscope at -170°C and 2.5kV. Digitised images of the xylem were recorded at several magnifications.

Cryo-SEM images from the stem segments that were frozen immediately after embolisation at -6 and -24 kPa, were analysed using image analysis software (Scion Image, a free PC version of NIH-Image, Scion Corporation, Maryland, USA). Each of the selected images contained at least four and maximal six vascular bundles with embolised vessels. The cross-sectional surface area (A_c), the perimeter (P_c) and the smallest and largest diameters of the inscribed ellipses of all embolised vessels were analysed. The critical diameter for air-entrance (which equals $2r_c$) was defined as the average of the largest and smallest diameter of the inscribed ellipses (Sperry and Tyree, 1990; Stamm, 1966). To check the circularity of the embolised vessels,

($2/r_c$)-ratios (see Eqn 4) of embolised vessels were calculated, and compared with the actual measured P_c/A_c-ratios.

Results

Stem segment characteristics

After applying dye at the basal cut end of the stem segments, stain was found in walls of xylem vessels of all sizes. Most coloured vessels (small and large diameter) were located in the approximately 20 to 25 vascular bundles in a cross-section. However, not all vascular bundles were coloured at all heights in the stem segments. At 5 mm above the basal cut surface 4-5 vascular bundles were unstained, probably because they were previously connected to leaves that were removed from the stem segment before the measurement started. These bundles are disconnected from the negative pressure applied at the top of the stem segment. This explanation was confirmed by observations of colouring above the highest, second highest and third highest petiole stubs on the stem segments, where respectively, all, all minus one and all minus two vascular bundles were stained. No dye was found in the central pith area of the stem segment except close (< 2 mm) to the basal cut-surface.

The length distribution of the xylem vessels, determined by the latex particle method (Zimmermann and Jeje, 1981), and measured up from the cut-surface, was found to be most accurately described by the exponential curve $N = 100 \cdot e^{(-\lambda x)}$, where N is the percentage of vessels longer than length x (measured from the cut-surface). (R^2>0.95 in all individual determinations; λ = 0.0275 $\pm$ 0.0021 (mean and standard deviation); n=3). From this relationship it can be calculated that 75% of the vessels were longer than 10 mm and 50% longer than 25 mm. The maximal vessel length was between 24 and 28 cm. Observations during the embolisation treatment confirmed that in none of the stem segments xylem vessel length exceeded stem segment length because no air appeared at the upper cut end during and after exposure of the basal cut end to air.

Verification of the method by cryo-SEM

In the cryo-SEM images water- and air-filled vessels could be easily distinguished by their structure and contents. No embolised vessels were observed in stem segments, which were frozen immediately after harvest (Fig. 2A). This strongly indicates the absence of natural air embolisms before embolisation. Xylem pressure during embolisation clearly influenced the cross-sectional size fraction of embolised vessels in a stem segment. After embolisation at -6 kPa, emboli were almost exclusively observed in large diameter vessels (Fig. 2B and C), while after embolisation at -24 kPa many of the small diameter vessels also became embolised (Fig. 2D and E). Not all 20-25 vascular bundles in a cross-section contained embolised vessels: the vessels in four or five vascular bundles remained completely water-filled. This is in agreement with the results of the staining experiment, which made clear that probably a few bundles at the lower cut surface are disconnected from the pulling pressure at the upper cut end of the stem segment.

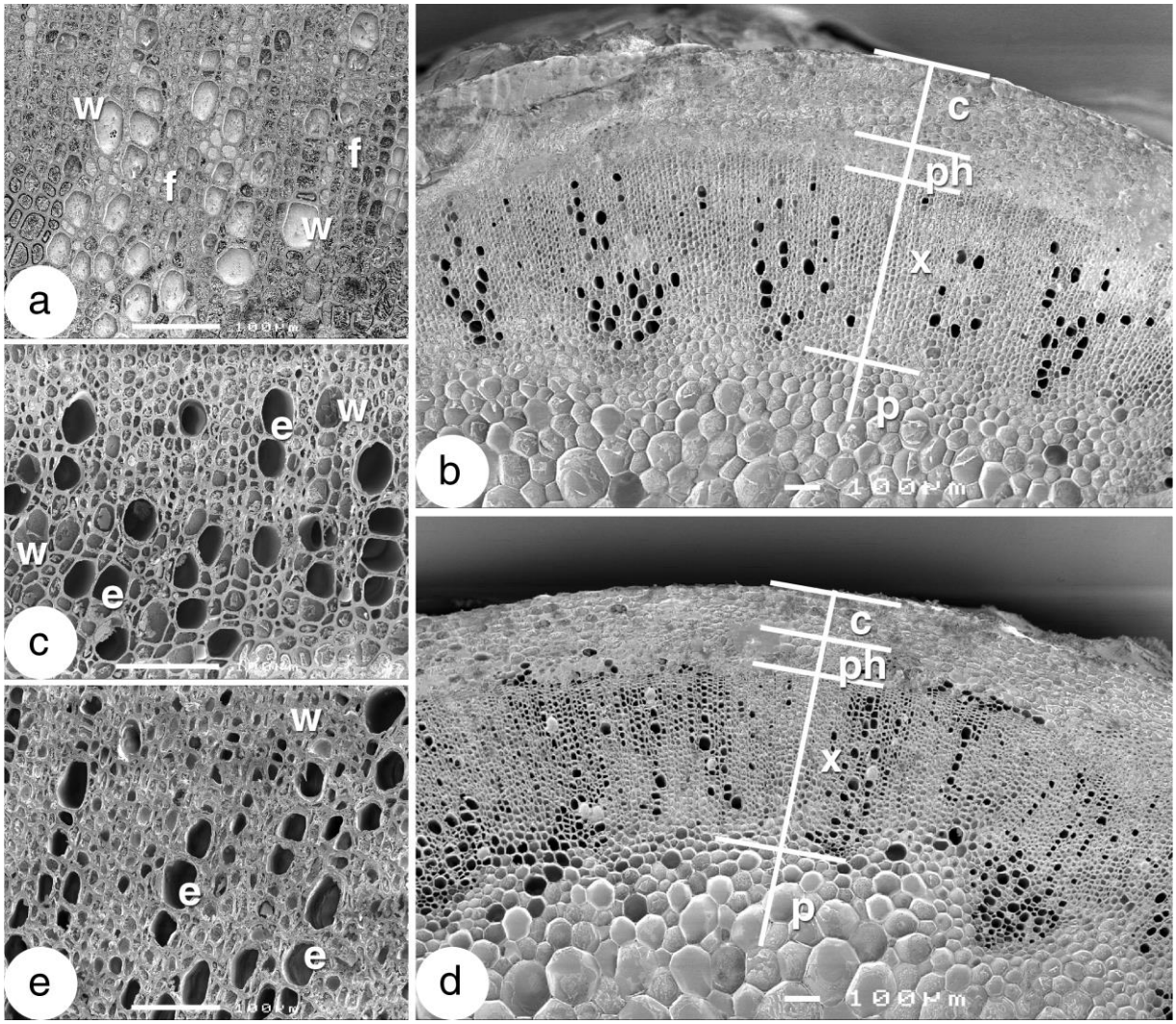

Fig. 2. Cross-sectional faces of *Dendranthema x grandiflorum* Tzvelev c.v. Cassa stem segments examined by cryo-SEM. Figures b and d show a sector of a whole stem cross-section containing several vascular bundles with water- and air-filled xylem vessels. Figures a, c and e show details of the xylem tissue.
a: 5 mm above basal cut surface after harvest under water.
b: basal cut surface after embolisation at -6 kPa. **c**: detail of b.
d: basal cut surface after embolisation at -24 kPa. **e**: detail of d
(c: cortex, e: empty xylem vessel, f: xylem fiber cells, p: pith, ph: phloem, w: water filled xylem vessel, x: xylem. All bars represent 100 µm).

Consequently these disconnected bundles do not add to the water flow through the stem segment in this experimental set up.

The number of embolised vessels at -24 kPa was much larger than at -6 kPa. This was almost completely due to the large number of embolised vessels with diameters lower than 20 µm. Frequency distributions of measured diameters of embolised vessels after air-entrance at -6 and -24 kPa are shown in Fig.3. The distribution at -24 kPa reflects more or less the diameter distribution of all vessels since almost all vessels were embolised. The distribution at -6 kPa shows a break between the 25-30 and 30-35 µm diameter classes: instead of an increasing percentage of embolised

vessels in the lower diameter classes (below 25-30 µm) a decreasing percentage was observed. This shows that most vessels with a diameter smaller than approximately 30 µm did not embolise at –6 kPa while almost all larger diameter vessels did. This critical diameter for embolisation at –6 kPa (30 µm) is smaller than was calculated by Eqn 4 (50 µm).

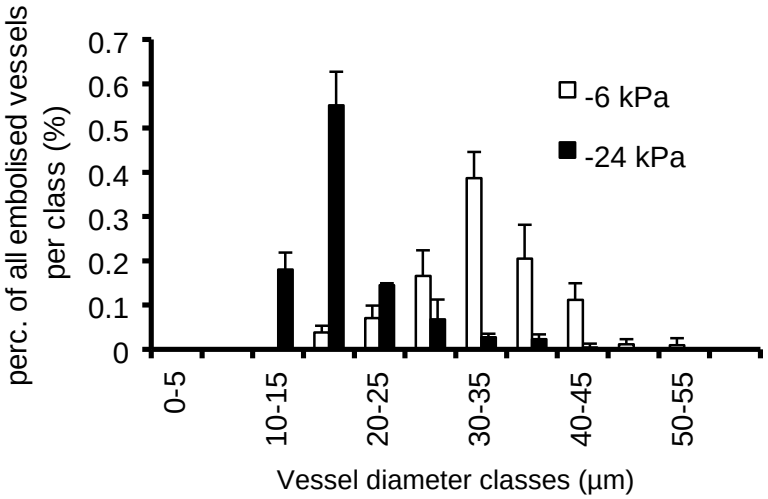

Fig. 3. Percentage of all embolised vessels per diameter* class after air-entrance at -6 kPa and -24 kPa xylem pressure, obtained from 3 images per pressure containing each at least four vascular bundles.
*diameter defined as the average of the largest and smallest diameters of the inscribed ellipse in an embolised vessel.

The cross-sectional shape of embolised conduits was clearly not circular (Fig. 4) as was assumed in Eqn 4. The actual measured P_c/A_c ratio of embolised vessels after embolisation at –6 and –24 kPa was on average 1.13 times the calculated P_c/A_c ratio (= $2/r_c$). The difference between calculated and measured P_c/A_c was independent of vessel diameter and critical cross-sectional vessel size (Fig. 4).

R_h test measurements

The test measurements revealed that measured R_h was independent of the applied negative pressure and of leaf removal (Fig. 5). Linear relationships were found between the applied pressure gradients and observed rates of water flow, while the absence of leaves did not affect the slope of the pressure-flow rate relationships (Fig.5). The positive intercept at the flow-axis of the curve of the stem segment with leaves agreed with the measured transpiration of the stem segment with leaves (which was measured when no pressure was applied).

After starting an R_h measurement with a new stem segment, the measured flow rate of water sometimes started at approximately 85-90% of its final flow rate. The final flow rate was reached within 10-40 minutes and afterwards remained constant for a period of at least 3 to 4 hours. During this period sudden changes in pressure

gradient usually caused transient responses in flow rate, which however never lasted longer than two minutes (results not shown).

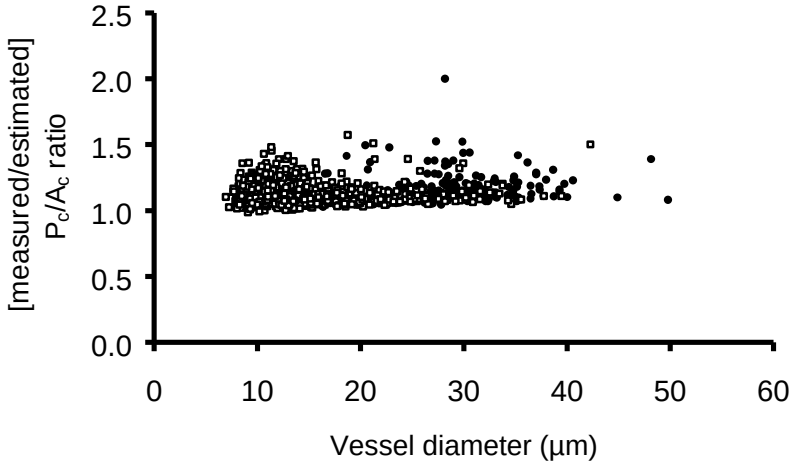

Fig. 4. Relationship between vessel diameter* and the quotient of the actual measured and the calculated perimeter/cross-sectional area ratio's (P_c/A_c) of the same conduits. Calculated P_c/A_c is based on vessel diameter* and assumed circularity. Circles: embolised conduits after air-entrance at -6 kPa (n = 273). Squares: embolised conduits after air-entrance at -24 kPa (n= 610).
*diameter defined as the average of the largest and smallest diameters of the inscribed ellipse in an embolised vessel.

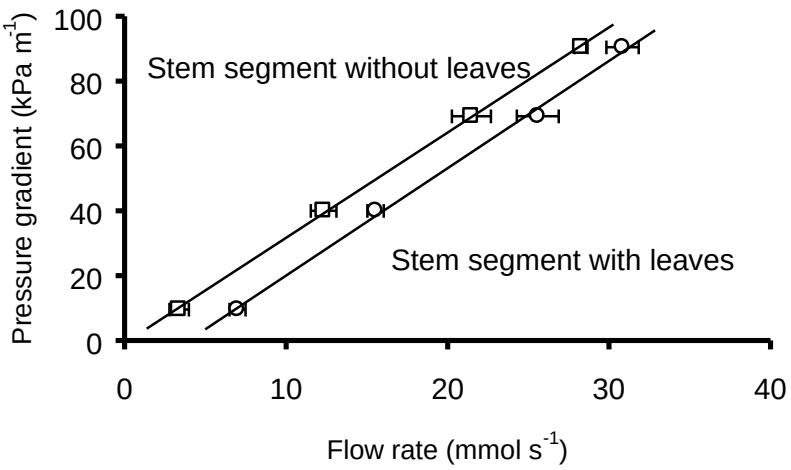

Fig. 5. Pressure4-flow relationships measured on individual *Dendranthema x grandiflorum* Tzvelev cv. Cassa stem segments before (circles) and after (squares) leaf excision (averages of four stem segments).

R_h before and after embolising the xylem at different xylem pressures

Typical courses of measured water flow rates before and after air-entrance at different xylem pressures are shown in Fig. 6. Calculated hydraulic resistances before and after air-entrance are presented in Table 1. R_h before embolisation was

approximately similar in all treatments and replicates, although the variability between individual stem segments was substantial. Surprisingly, measured water flow rate and R_h were not decreased after embolisation at -6 kPa. After embolisation at -24 kPa in contrary, R_h almost trebled. Based on means and standard deviations presented in Table 1, R_h seem to be hardly changed after embolisation at -10.4 kPa. However, comparisons of paired measurements of R_h (before and after air-entrance) on individual stem segments showed consistent increases in R_h (see also Fig. 6). The increase in R_h after embolisation at –10.4 kPa appeared in all stem segments and ranged between 8 and 23%.

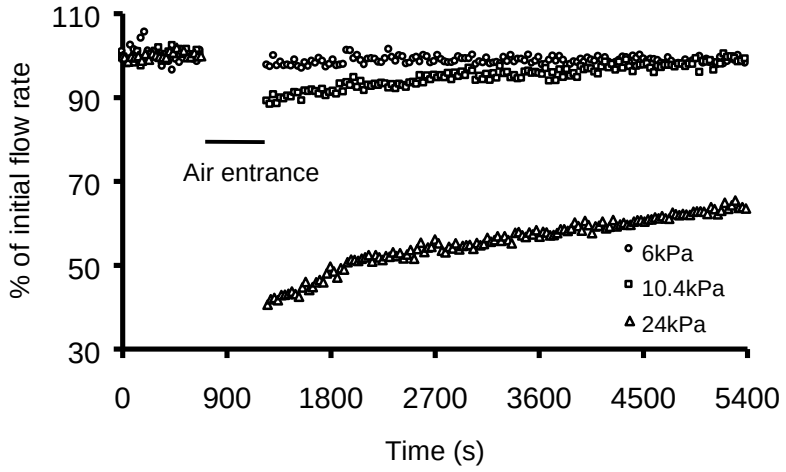

Fig. 6. Comparison of typical water flow rate patterns through stem segments before and after embolising different parts of the xylem. Embolising xylem pressures: -6 kPa (only large vessels), -10.4 kPa (large and intermediate diameter vessels) and -24 kPa (large, intermediate and small diameter vessels). The pressure gradient to induce flow during the flow rate measurement was 80 kPa.m^{-1}.

Table 1. *The effect of xylem pressure during air-entrance on changes in hydraulic resistance (R_h) due to air-blockages*
Stem segments were collected from two groups of plants (A and B) which were grown separate in time. R_h was determined before and after air entrance on each individual stem segment. Mean and standard deviation of R_h (here expressed as hydraulic resistance per meter) before and after air-entrance at each pressure and group combination are indicated

Xylem pressure during air-entrance [kPa]	R_h before [kPa m^{-1}µmol^{-1}s]	R_h after [kPa m^{-1}µmol^{-1}s]	Group	n
-6.0	2.1 ± 0.32	2.0 ± 0.31	A	4
	2.2 ± 0.49	2.2 ± 0.52	B	3
-10.4	-	-	A	-
	2.1 ± 0.49	2.4 ± 0.64	B	8
-24.0	2.1 ± 0.28	5.7 ± 1.41	A	4
	2.3 ± 0.11	7.2 ± 1.10	B	4

Discussion

The technique of embolising cut xylem vessels strongly depends on the actual established hydraulic pressure at the lower cut surface where air could possibly enter the cut xylem vessel ends. It is therefore important to be sure about the magnitude of hydraulic pressure localised at that specific position. The absence of flow and the continuity of the water column between the basal cut end of the stem segment and the lower open end of the flexible tube (Fig. 1) ensure this well-defined xylem pressure at the cut end of stem segment. Immediately after the start of air entrance in a vessel the pressure on the moving meniscus of the water column further decreases which is due to the vertical position of the stem segment in the system (Fig. 1). This facilitates further movement of the meniscus inward the embolising vessel.

Presented results show that the proposed method indeed enables the controlled embolisation of xylem conduits larger than a certain diameter within samples of comparable xylem structure (Fig. 2). The diameters of embolised vessels in Cryo-SEM images only roughly agreed with calculated critical vessel diameters (Fig. 3). This indicates that Eqn 4, which reflects the simple capillary equation, could not be used as such. The simplification of Eqn 3 to Eqn 4 is based on two assumptions: cross-sectional circularity of the vessels and a 0° contact angle between xylem cell wall and meniscus at the air water interface. The analysis of the cross-sectional shape of a large number of embolised vessels (Fig. 4) revealed that the assumption of circularity in Eqn 4 was not justified. However, the diameter independent (Fig. 4) deviation from circularity could not explain the difference between the observed and the calculated critical diameter for embolisation (approximately 30 µm versus 50 µm at -6 kPa). Instead the observed deviation from circularity even increased the difference between observed and calculated critical diameter. A larger then 0° contact angle on the other hand decreases the difference between observed and calculated critical diameter. Taking into account the observed deviation from circularity and the observed critical diameter of approximately 30 µm, the contact angle must have been approximately 55°. Remarks of Pickard (1981) concerning the contact angle at xylem walls strengthen this explanation; he mentioned substantially higher contact angles than 0° between water and wall material of xylem conduits (up to 45°). Recent measurements of the contact angle in six species using a complete different approach, showed that it varied between 42° and 55° (Zwieniecki and Holbrook, 2000). The deviation of the contact angle from 0° could be of importance for the removal of air embolisms. According to the model of Yang and Tyree (1992) this larger contact angle could significantly decrease the rate of recovering from air embolisms.

The general response of flow during the R_h determination, including the decline three-to-four hours after applying the pressure gradient on the stem segment, was about the same as reported in literature when using de-ionised water or a low concentration sodium chloride solution (Sperry *et al.*, 1988). They explained the long-term decline by microbial clogging. A simple explanation for the rise of the flow rate during the first 30-40 minutes of the measurement period could not be given. A non-degassed solution was used to prevent undesired rapid removal of the air embolisms in the xylem sap after reapplying the solution to the basal cut end of the stem

segment. The possibility of artefacts due to the use of non-degassed water needs to be considered. It seems, however, that the use of non-degassed solutions did not result in extra embolisms, as the flow rate initially increased instead of decreased and remained constant during the following three to four hours. Solutions with low pH (Sperry *et al.*, 1988) were not used in order to prevent unexpected effects on forces between the cell wall and the water: low pH could possibly change the contact angle between wall and water, which affects the embolisation treatment. It is possible that some kind of adaptation appeared at the start of the R_h determinations in response to the change in content of the xylem vessels. It is known that the R_h of chrysanthemum stems could be influenced by the ionic composition of the xylem fluid (Van Ieperen *et al.*, 2000). This explanation is strengthened by the fact that no transient responses were observed after changes in pressure gradients applied after the initial period of increase of flow rate during the test measurements on stem segments with and without leaves.

The R_h of a non-embolised stem segment is the collective resistance of many xylem conduits in parallel, which are interconnected and which have different lengths. In chrysanthemum stem segments 50% of the cut vessels at the basal cut surface were shorter than 25 mm. Vessel-to-vessel connections significantly add to R_h. Prediction of the overall R_h of whole stem segments is difficult even without including air-embolisms, due to the non-normal distribution of vessel lengths and vessel diameters, and the unknown correlation between vessel length and diameter (Chiu and Ewers, 1993; Van Ieperen *et al.,* 2000). The effect of air embolisms in the cut vessels at the basal end of the stem segment on overall R_h is therefore difficult to assess. Usually, only a small portion of the path for water flow is blocked. Even if all the severed vessels are blocked by air, water can move via cell walls along the shortest distance to the intact vessels whose ends are near the cut surface. Once water gets into these vessels it may flow via the normal network of functioning vessels. The following simple calculation, ignoring the actual vessel lengths, diameters and resistances of pit membranes, shows that the impact of embolisms on overall hydraulic conductance (the reciprocal of R_h) is limited. Suppose that air embolisms drop the hydraulic conductance of the lowest 2.5 cm of a 30 cm long stem segment to 5% of its value before air entrance, and that the hydraulic conductance in the remaining part of the stem segment is unchanged. In that case, overall hydraulic conductance of the 30 cm long stem segment decreases with 61%. If hydraulic conductance in the lower 2.5 cm of the stem segment drops to 90%, overall hydraulic conductance decreases with 1%. These calculations partially explain why the effect of embolisation at –6 kPa (Table 1) on R_h was very small, although the Cryo-SEM images obtained from samples frozen immediately after the embolisation treatment (Fig 2B and C) clearly show that most large diameter vessels were embolised at the cut surface. On the other hand, embolisation of small and large diameter vessels at -24 kPa xylem pressure (Fig 2C and D) was accompanied with a considerable increase in R_h (Table 1).

Theoretically there are several explanations for the observed discrepancy between the visually observed embolisation and measured changes in R_h in the -6 kPa treatment: An unlikely explanation is that large diameter vessels are not important for water flow. This is in contradiction with the generally assumed analogue with Poiseuilles law, and with the observed colouring of the large diameter vessels in

present experiments. Moreover, the fact that the vessels were embolised implies that they were connected to the main longitudinal water transport path and therefore did add to longitudinal water transport. A second explanation could be that all large diameter vessels were short or only embolised for a small percentage of their length. In both these cases, water could have followed relative short side-paths via adjacent non-embolised conduits before entering large diameter vessels, thus not significantly changing the overall hydraulic resistance. It is, indeed, possible that in the -6 kPa treatment the large diameter vessels were not completely embolised due to longitudinal tapering of the vessels. A third explanation could be that small diameter vessels, which were still water filled after embolisation at -6 kPa, provided the short side-paths to large diameter vessels that start just above the cut surface after air-penetration at –6 kPa. These short-side paths were not available after embolisation at -24 kPa, which might have been the reason for the much larger increase in R_h after embolisation at -24 kPa.

Verification of the presented embolising technique by cryo-SEM showed that embolising a part of the cut xylem vessels, discriminating on diameter, is well possible. Based on present experiments it can be concluded that the effect of embolising only all large diameter vessels at one cut end of a chrysanthemum stem segment hardly changes overall R_h.

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4.2

Air in xylem vessels of cut flowers

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Abstract

Until now all studies on the role of air emboli in the water uptake of cut flowers describe indirect methods to demonstrate the presence of air in the plant tissues. Using cut chrysanthemum flowers, this report is the first one that directly visualises both air and water in xylem ducts of cut flower stems, by observing transversal sections of frozen stalks in a cryo scanning electron microscope (cryo-SEM). After cryo-fixation frozen stems were sawed transversally at a desired height, polished with a cryo ultra mill, lightly etched, sputtered with platinum and investigated with the cryo-SEM. This method allows for detailed studies on the origin and fate of air emboli on water balance in cut flower stems

Introduction

Water stress during vase life is often the cause of shortened vase life of cut flowers (Halevy and Mayak, 1981). Loss of turgidity results in wilting of flowers, leaves or stems. A high level of turgidity is necessary for the development of flower buds into full-bloom maturity, and for maintaining normal metabolic activity in the cut flower. The main reason for the occurrence of a water deficit, apparently is some kind of blockage to water flow that develops in the stem (Halevy and Mayak, 1981). The nature of this blockage can vary widely (Van Doorn, 1997). Factors such as micro-organisms (Marousky, 1969; Van Meeteren, 1978; Zagory and Reid, 1986; Put and Jansen, 1989), physiological responses to cutting of the stem (Marousky, 1971; Fujino and Reid, 1983; Van Doorn *et al*., 1991), and the presence of gas bubbles in the xylem conduits (Durkin, 1979; Dixon *et al*., 1988; Van Meeteren, 1992; Van Doorn and Otma, 1995) have been suggested as causes for the decline in hydraulic conductance. For cut roses, Dixon and Peterson (1989) proposed a combination of effects of wounding at the cut end along with increasing numbers of micro-organisms that induce an initial decline in stem water potential resulting in cavitation in the xylem vessels. Thereafter, further decline in stem conductance by xylem cavitation occurs. De Stigter and Broekhuysen (1989) also found cavitation in rose stems gradually developing during vase life when the cut surface of the stem became

clogged by microbial growth. However, there are conflicting reports about the relationship between bacterial numbers in the vase solution and the occurrence of cavitation. For example, Williamson and Milburn (1995) using Acacia found no clear relationship between the number of cavitation events as measured by audible acoustic emissions and the number of bacteria in different solutions.

Air entering the xylem vessels via the surface of the cut stem at the moment of harvesting and/or during dry storage of cut flowers can seriously block water uptake, as shown for chrysanthemums (Durkin, 1980; Van Meeteren, 1989, 1992) and roses (Durkin, 1979, 1980). Scholander *et al*. (1957), however, held liana stems in air for 15 minutes, during which 80 ml of air was taken up, and found that the subsequent rate of water uptake was as high as before. Considerable differences exist between rose cultivars in the degree of inhibition of water uptake upon exposure of the cut stems to air (Van Doorn and Reid, 1987). Van Doorn (1990) concluded that the presence of air in the lumen of the xylem elements in itself is not an obstacle to water uptake.

Only indirect methods have been used to establish the role of emboli on the water uptake of cut flowers: recutting under water (Durkin, 1979), removal of gas from water used for rehydration (Durkin, 1979; Slootweg, 1995), uptake of fluorescent dye or suspensions of macromolecular matter (Dixon and Peterson, 1989; Van Doorn and Otma, 1995), vacuum rehydration (Aarts, 1957; Van Meeteren, 1989), ice-cold vase water for a short period (Van Meeteren, 1989, 1992; Slootweg, 1995), detection of cavitation by acoustic emissions (Dixon *et al*., 1988), the volume of air taken up at the cut stem end (Van Doorn, 1990), and the conductivity to air in short stem segments (De Stigter and Broekhuysen, 1989). Methods to directly observe emboli in ducts from the outside of the stem are scarce due to the woody status of most cut flower stems. The only really non-destructive method is nuclear magnetic resonance (NMR) imaging (Van As, 1992). However, the resolution of NMR imaging is limited. For optical or electron microscopy, sectioning is indispensable, which would replace water columns and thus emboli. To avoid this problem, the water has to be immobilised and this can be done by freezing. In this communication we report on the application of the cryo scanning electron microscope (cryo-SEM) to visualise the presence of air in the water conducting elements of cut flower stems. Initial attempts to cryofracture the stems were unsuccessful because the tissues tended to fracture longitudinally instead of transversely due to the arrangement of the wood fibres. Therefore we developed a technique to obtain transverse segments of the xylem tissue. We demonstrate the usefulness of the method with an experiment on rehydration after desiccation of cut chrysanthemums.

Materials and methods

Plant material

Chrysanthemum (*Dendranthema x grandiflorum* Tzvelev cv. Cassa) cuttings were grown in pots with a commercial potting soil in a greenhouse at a day/night temperature of 16°C/18°C and a photoperiod of 16 hours during weeks 0-4 and 8 hours during weeks 5-11. The flowers were harvested at commercial maturity by cutting the stalks under water at soil level. To start the experiment with flowers of the same water status, the stalks were placed for 2 hours in a mixture of ice and water in

dark in a cold room at 5°C. During this treatment the flowers got full turgidity. Thereafter, 13 cm of the stems was cut off under water, and leaves were removed till 12 cm above the cut plane. On these cut flowers the influence of dry storage after a desiccation treatment on vase life and vessel contents was studied.

Desiccation and storage

Dehydration of the flowers was accomplished by placing the individual flowers for 1 h horizontally on two wires in a conditioned room at 20°C, 60% relative humidity (RH) and a light intensity of 14 µmol $m^{-2}s^{-1}$. During this desiccation treatment, flowers lost 4-6% of their initial fresh weight. Thereafter half of the flowers were stored dry for 24 hours under non-transpiring conditions in plastic bags at 20°C, cut off 2 cm, and placed for 2 hours in a mixture of ice and water at an air temperature of 20°C (rehydration treatment). The other half of the flowers were cut off 2 cm directly after desiccation, and placed for 2 hours in the ice water. After these pre-treatments the flowers were placed in vases to observe their fresh weight changes.

Vase period

During the vase period, each flower was individually placed in a vase with water of defined quality (demineralised water + 124.8 mg/l $NaHCO_3$ + 99.2 mg/l $CaCl_2$ + 1.2 mg/l $CuSO_4$) in a conditioned room at 20±1°C, 60%RH and a light intensity of 14 µmol $m^{-2}s^{-1}$ (Philips, TLD 50W/84HF) with a photoperiod of 12h.

Cryo Scanning Electron Microscopy

To investigate the contents of the xylem, two flower stalks were sampled before and after desiccation and after the rehydration treatment. They were cryo-fixed by plunging them into liquid nitrogen. Still under liquid nitrogen the lower 7 cm of the stems were sawed off with a diamond circle sawing device (Minicraft high precision drill, Black and Decker) and stored in a cryo vessel. Before investigation a selected stem part of 1 cm height was sawed out and mounted on a hollow messing stub with Tissue Tek (NR. 4583 Miles Inc., Elkhart, IN-USA.) with one of the transversal sawing surfaces up. This sawing surface was polished with a diamond cryo ultra mill (Reichert-Jung Polycut E, equipped with a cryostage). The surface was etched for 5 min at -89°C at 10^{-4} Pa in an Oxford CT 1500 HF cryo transfer unit and sputtercoated with 3 nm platinum. We studied the vessel contents at 2 cm above the stem base (after the recutting) in a JEOL 6300F field emission scanning electron microscope at -170°C at 2.5kV. After investigation samples could be stored and investigated for another time.

Fresh weight measurements

To observe changes in the water status of the cut flowers, stalks not used for cryo-SEM investigation (10 for both treatments) were weighed after each treatment (desiccation, storage, rehydration) and during several days of vase life at noon.

Results

Fresh weight measurements

Figure 1 shows the changes of fresh weights after harvest for the two treatments. The flowers that were stored for 24 hours after the desiccation period showed a decrease of fresh weight, even when the flowers were placed in vase water. The leaves of these cut flowers were visibly wilted. During the 24 hours of storage the flowers did not lose weight as much, but apparently they lost the ability to restore the water uptake. The non-stored flowers totally regained their starting weight during the rehydration treatment. During vase life these flowers had turgid leaves, indicated by a positive water balance during the first days.

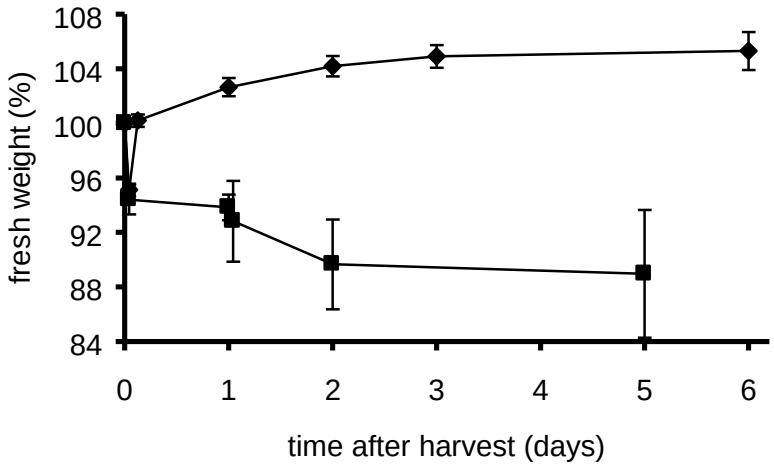

Fig. 1. Time course during the vase period of the fresh weight of flowers given a rehydration treatment (2 hours in water of 0°C; air temperature: 20°C) after either 1 hour desiccation and 24 hours dry storage under non-transpiring conditions (■), or immediately after the 1 hour desiccation period (◆). Vertical bars represent the standard error of the mean.

Cryo Scanning Electron Microscopy

The results of the cryo-SEM are illustrated in Fig. 2. An overview of a transverse section as seen in cryo-SEM is shown in Fig 2a. The pith of the chrysanthemum stem consists of large dead cells of which only dry walls remain. Only the outer pith cells are still filled. The xylem consists of primary vessels, secondary vessels and libriform. The primary vessels can be discerned from the secondary vessels by their helical cell walls and their more centred position. The vessels lie embedded between libriform fibres. Most secondary vessels are situated within the former vessel bundles. Around the xylem the cambium, phloem and cortex are visible. The primary phloem has developed to strong sclerenchymatous fibres (these are the fibres that can be peeled off from a fresh stalk). As a result of freezing and etching, the lumina of the xylem fibres show a lattice of solutes as a result of the crystallisation of the cytoplasm during freezing. Vessels contain less solutes and show therefore a flat ice-surface. Fig. 2b demonstrates at high magnification the distinction between fibres, empty

vessels (air filled) and water filled vessels. Figs. 2c-f show the results of the different treatments at 2 cm above the cut plane. The situation before desiccation is shown in Fig 2c. All xylem vessels are filled. After the desiccation period numerous primary and secondary vessels are found empty (Fig. 2d). After 24 hours storage followed by the rehydration treatment still a lot of vessels remain empty (Fig 2e), while the xylem vessels of the non-stored stems have been refilled (Fig 2f). However, approx. one fifth of the vessel bundles still contained empty vessels (not shown).

Discussion

The cryo-SEM technique presented here allows observation of undisturbed hydrated plant parts, and hence a precise detection of liquids and gases in plant tissues as earlier demonstrated by Canny (1997) in non-fibrous tissues (excised sunflower petioles). In a strict sense, only water is visible (as ice), whereas air that is present during the liquid nitrogen fixation is removed at the (usual) vacuum condition in the SEM. There may be argued that the freezing method will cause cavitation or embolism. Indeed this might be the case when the samples are thawed back. Figs. 2c and 2f show only filled vessels, thus proving that freezing itself does not induce empty vessels. The presented method allows polishing and investigating areas of even more than 1 cm^2. In this technique, the use of chemical fixatives is circumvented, thereby avoiding several artefacts. In cut flower research the appearance and disappearance of embolisms can be studied by comparing either cross fractures at different heights in the stem or by comparing different stages after harvesting and various treatments. The cryo-SEM method is destructive to the chrysanthemum flowers, so the vase life of the same flower cannot be tested. To compare xylem contents of flowers with the vase life of other, similar treated flowers, homogeneous plant material is required. Of course flowers can also be cryo-fixed after vase life.

Apart from water, the technique also allows for detection of other factors, such as micro-organisms that have been mentioned as a cause for changing water potentials (Marousky, 1969; Van Meeteren, 1978; Zagory and Reid, 1986; Put and Jansen, 1989). In this case water (ice) has to be removed by deep etching (Van Doorn *et al*., 1991).

This is the first experiment where the presence of air embolism in xylem of cut chrysanthemum has been directly observed. Approximately 50% of the xylem vessels at the original cut plane will reach more than 2 cm higher up in the stem (data not shown). Therefore, at the height of sampling, 2 cm above the cut plane, ±50% of the vessels is in open contact with the cut plane and may contain entered air. The 1 hour desiccated flowers (Fig. 2d) and the non-restored flowers (Fig. 2e) exhibited a percentage of empty vessels close to 50%, pointing that most of the cut open vessels contained air at 2 cm above the cut surface. More sampling is needed to get reliable percentages of open vessels. The flowers that were stored were no longer able to restore their water balance. It was argued before that this absence of restoration was due to a lost ability to refill the embolised xylem vessels (Van Meeteren, 1992).

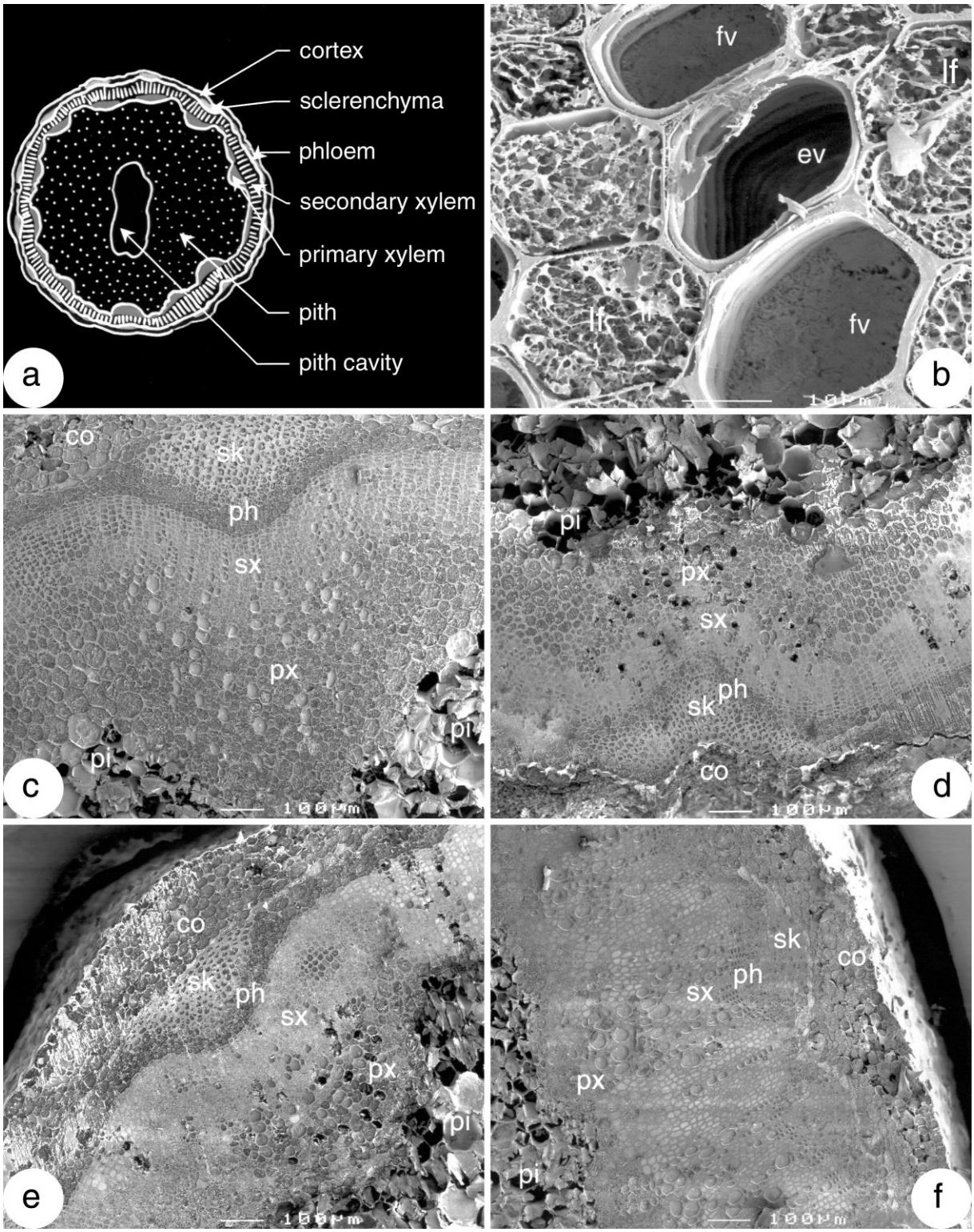

Fig. 2. a: schematic overview of a transversal section through a chrysanthemum stem.
b: High magnification cryo-SEM image of the xylem showing two filled vessels (fv), one empty vessel (ev) and libriform fibres (lf). Note the different crystallisation patterns in the lumina of the fibres and the filled vessels

c-f: Cryo-SEM transversal images of vessel bundles 2cm above the original cut plane of a cryo-fixed stalk after the following treatments:
c: harvested and 2 hours in ice water.
d: harvested, 2 hours in ice water and 1 hour desiccation.
e: harvested, 2 hours in ice water, 1 hour desiccation, 24h storage and 2 hours in ice water
f: harvested, 2 hours in ice water, 1 hour desiccation, and 2 hours in ice water
(co: cortex, sc: sclerenchyma, ph: phloem, sx: secondary xylem, px: primary xylem, pi: pith).

Our cryo-SEM results agree very well with this hypothesis. In addition the non-stored flowers had a positive water balance during the first days of vase life and showed refilled xylem vessels. That some vessel bundles still contained some open vessels may be caused by the fact that de lowest leaves were taken off.

Our future work using this cryo-SEM technique on air embolism in cut chrysanthemum stems will focus on the following questions: 1) Under what conditions will air enter the stem? 2) To what height and how fast can air enter the stem? 3) What is the mechanism resulting in refilling xylem vessels? 4) Genetic and environmental conditions can influence early leaf wilting (Van Meeteren, 1989). Which of these influences are directly related to the behaviour of air in the vessels?

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4.3

Quantification of emboli by visualisation of air filled xylem vessels

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Abstract

Between harvest and vase life the cut surface of most cut flowers is exposed to air for a longer or shorter period. It was hypothesised that under normal harvest and transport conditions air only enters the cut open vessels and does not move to non-cut vessels. The vessel length distribution of chrysanthemum stems was analysed with the latex particle method and compared to the distribution of air embolisms in 5% (w/w) desiccated stems, visualised using cryo-scanning electron microscopy. It was concluded that by moderate desiccation all cut open vessels are completely air-filled and that intact vessels are not embolised.

Introduction

In higher plants water transport from roots to leaves occurs mainly through the xylem vessel system. Xylem vessels consist of one to numerous tracheary elements, which are interconnected by inter-vessel pits. Xylem vessel lengths can vary from shorter than one millimeter to several meters. The diameter of xylem vessels varies from a few micrometers to a few hundreds of micrometers.

Between harvest and vase life the cut surface of most cut flowers is exposed to air over a shorter or longer period. In theory even a moderate water tension in the xylem vessel system is sufficient to allow air entrance into cut open vessels. The resulting embolisms can block the water transport system, which results in early leaf wilting or a shortened vase life (Van Meeteren, 1992). In theory, based on estimated pore-sizes in the inter-vessel pits, for induction of air embolism through an inter-vessel pit, a much higher water tension is required. Zimmermann (1983) estimated the tension needed for air entrance in non-cut vessels at -15 Atm for a maximal pit membrane pore size of 0.2 µm. Therefore it is generally hypothesised that under normal harvest and transport conditions only the cut open vessels should be air filled. If so, the vessel lengths will determine how far above the cut surface air-blockage can occur.

By visualising the embolised vessels, it was tested in this study whether air enters the xylem vessels at moderate desiccation (5% w/w), and whether the entrance of air is restricted to the cut open vessels. In chrysanthemum cut flowers the vessel length distribution was characterised using the latex particle method and compared to the

distribution of air emboli in a moderately desiccated stem using cryo-scanning electron microscopy (cryo-SEM).

Materials and methods

Plant material

Chrysanthemums (*Dendranthema x grandiflorum* Tzvelev cv. Cassa) were grown in pots in a greenhouse of Wageningen University and investigated at the stage of commercial maturity. Early in the morning, the chrysanthemums were transported to the lab and cut under water at 25 cm above soil surface.

Vessel length determination

Stem segments of 30 cm length were cut out the chrysanthemum stem under water. The upper part of the segments was attached to a vacuum system, while the lower part was placed in an aqueous red latex solution (1% w/v). Latex particles can easily move through vessels, but are far too big to pass inter-vessel pits. After overnight uptake of the latex solution, the particles completely clogged (and thereby coloured) the cut open vessels. Out of every internode two transverse sections (2 mm thick) were cut out and photographed with a digital camera attached to a microscope. Using a digital image analysis computer program (SCIL_Image 1.3, University of Amsterdam, Faculty of Mathematics and Computer Science, Amsterdam, The Netherlands) the amount of red-coloured vessels was quantified as function of the distance to the cut surface (modification of the method of Zimmermann and Jeje; 1981).

Detection of embolised vessels

Stems were air-dried under ‘vase life conditions’ (20°C, 60% RH) until a weight decrease of 5%, which took around one hour. After this desiccation treatment, the stems were plunge-frozen in liquid nitrogen (-196°C) to immobilise the water in the vessels. As a control stems were frozen directly after cutting under water, without the desiccation treatment.

At different distances from the cut surface stem transversal sections were prepared for investigation in a cryo-scanning electron microscope (cryo-SEM) as described in Nijsse and Van Aelst (2000). In short, stem pieces of about 1 cm were sawed out under liquid nitrogen using a circular diamond saw. These stem pieces were cryo-planed at -90°C in an ultra-microtome (Reichert-Jung Ultracut E/FC4D) equipped with an 8 mm wide diamond knife (Histo no trough 45°, Drukker International, The Netherlands). The planed surfaces were freeze dried for 3 minutes at –89°C (to gain contrast) and photographed in a cryo-SEM (Jeol 6300F) at –190°C. The digital photographs were analysed on the number of embolised vessels per transversal section.

Results

Figure 1a shows an overview of a cryo-planed section through a chrysanthemum stem. The vascular bundles are neatly arranged in a circle close to the cortex. In the centre of the pith (consisting of dead, empty cells) a hole is made during preparation to avoid a rapid pressure increase of the boiling liquid nitrogen during cryo-planing. Figure 1b shows a detail of the xylem tissue of a desiccated stem. Xylem vessels can be distinguished from other cells by the solid grey appearance of their lumina, which is a result of the low solute contents of xylem sap. Embolised vessels are mostly filled with sawing debris and can easily be recognised. Figures 1d, 1f, and 1h show the distribution of embolised vessels in a desiccated stem at distances of respectively 1, 8, and 16 cm above the cut surface. The higher above the cut surface, the less embolised vessels are found. The control treatment (no desiccation) showed no embolised vessels. Figures 1c, 1e, and 1g show the results of red latex uptake at similar heights above the cut surface and show the same decrease of marked vessels as the cryo-planed sections do. The relative amount of vessels filled with red latex as function of the distance to the cut surface is shown in figure 2. In the same figure the decrease of embolised vessels is plotted. As found in other experiments (chapter 2.2) the amount of red vessels (open at cut surface) can be characterised by an exponential relation with an ever repeating distance called ‘half-length value’ over which the amount of red vessels halves. The half-length value of the latex treatment was on average 28.5 mm and corresponded very well with the half-length value of 27.8 mm of the embolised vessels in the desiccated stem.

In another group of stems the non-desiccated (control) stems contained air filled vessels. The desiccated stems of this group showed the exponential decrease of embolised vessels close to the cut surface, but higher up in the stem still a number of embolised vessels was visible. Both in the control and higher up in the desiccated stems the embolised vessels were clustered within one ore more vessel-bundles. This was in contrast to a random distribution in the desiccated stem of which the control did not contain embolised vessels.

Discussion

The close similarity between vessel length distribution and distribution of embolisms points out that air completely fills all cut open vessels. If any cavitation occurs, this must be rare, otherwise more embolised vessels should have been found higher in the stem. These results agree with the common hypothesis, based on estimated pit membrane pore sizes and physical laws, that the air-water meniscus will not pass inter-vessel pits at moderate water tension. In case the control (non-desiccated) already contained embolised vessels, the desiccated stems showed close to the cut surface a clear exponential decrease, but higher up still open vessels occurred in clusters in one or more vessel-bundles. These pre-harvest embolisms could be due to drought-stress, mechanical damage or due to insects. It can be questioned whether pre-harvest embolisms can cause vase life problems.

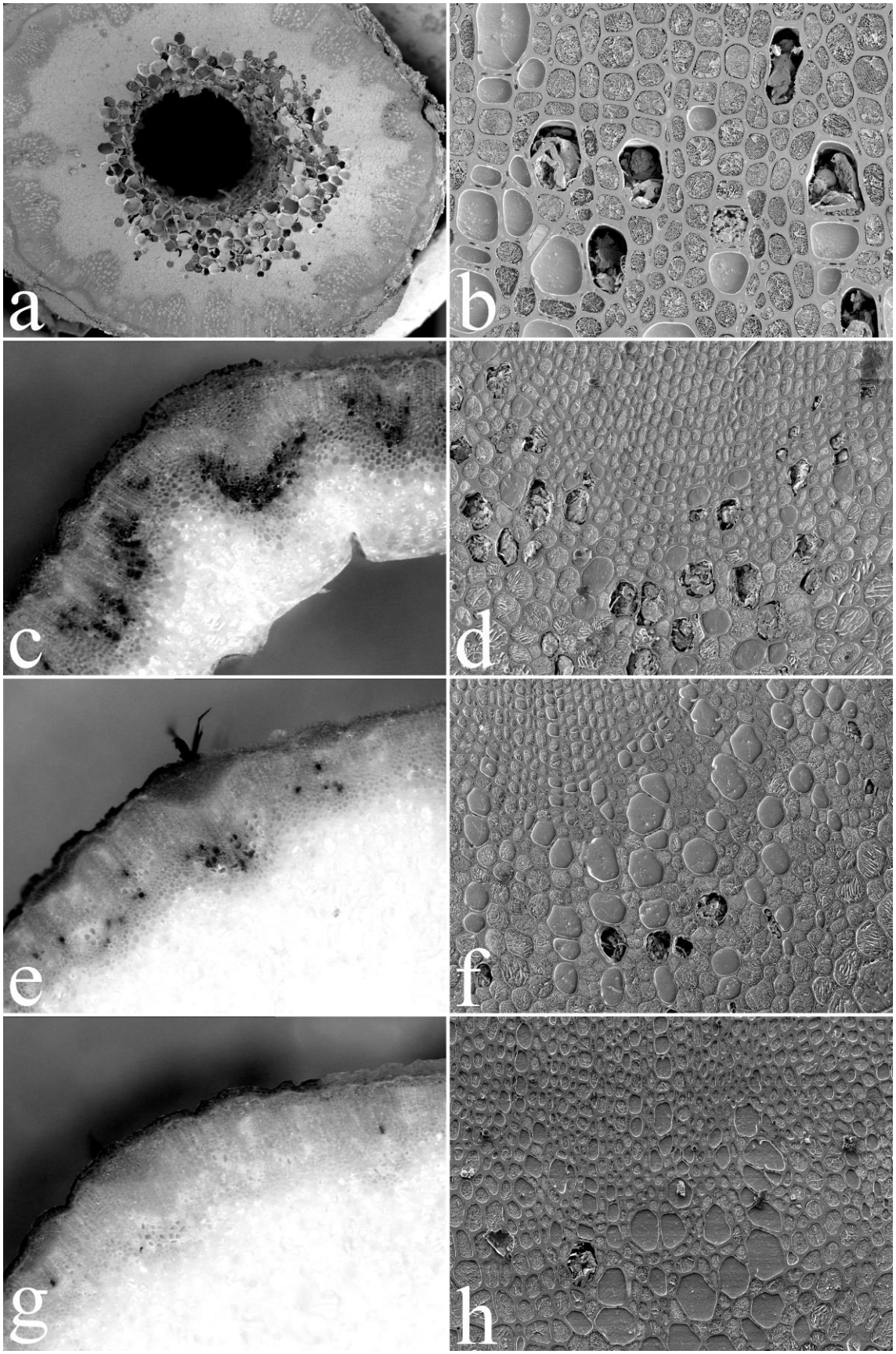
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Fig. 1. (left) Transverse sections through chrysanthemum stems.
a: Overview of a cryo planed section. The vascular bundles are neatly arranged in a circle close to the cortex. The centre consists of empty pith cells in which a hole is made during preparation.
b: Detail of cryo-planed xylem tissue of a desiccated stem. Xylem vessel lumina can be distinguished from other cell lumina by their solid grey appearance, which is a result of the low solute contents of xylem sap. Embolised vessels are mostly filled with sawing debris and can easily be recognised.
c-h: comparison of stems after red-latex uptake (left) and stems after desiccation and subsequent cryo-planing (right); at 1 cm (**c,d**), 8 cm (**e,f**) and 16 cm (**g,h**) above the cut surface.

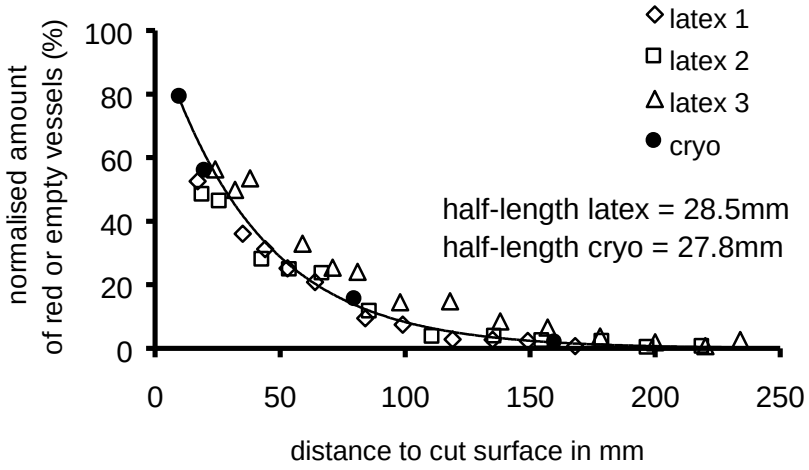

Fig. 2. Comparison of red latex uptake (vessel length distribution) and embolism distribution after desiccation. Open symbols: percentage of vessels filled with red latex in three chrysanthemum stems versus distance to the cut surface. Closed symbols: percentage of embolised vessels in a 5% (w/w) desiccated stem versus distance to the cut surface.

The used cryo-SEM technique is applicable for a wide range of embolism studies. For example the severity of desiccation at which cavitation starts to occur can be determined. Also the process of restoration of desiccated stems during subsequent vase life can be studied with this method. A disadvantage of the cryo-SEM method is the problem that not a great series of stems can be investigated due to the extensive preparation and investigation procedure.

In general, vessels are shorter higher up in the plant (Zimmermann and Potter, 1982; own results, chapter 2.2). This can be one of the reasons that higher-cut chrysanthemum flowers have a better restoration of their water uptake after dry

storage than lower-cut flowers (Van Meeteren and Van Gelder, 1999). At this time no data are available about variation of vessel length between cultivars in the same cut flower species. Neither the influence of growing conditions on vessel length is known.

If just before vase life the stem is re-cut under water (Van Meeteren and Van Gelder, 1999), the half-length value of the vessel length distribution can be used to predict how much of the stem has to be cut off to restore a certain percentage of the vessels at the new cut surface. For example, to gain 75% of directly water containing vessels at the cut surface, a piece with a length of two times the half-length value has to be cut off under water, i.e. about 6 cm in case of the plant stems studied here.

Conclusion

Xylem vessel length determines the height to which air, aspired via the cut surface, can block the xylem system in cut flowers. After moderate desiccation (5% w/w) of cut flowers, air embolises all cut open vessels. Intact vessels do not get filled with air by moderate desiccation.

Acknowledgements

We thank Annie van Gelder for practical assistance.

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Chapter 5

Embolism repair related to stem hydraulic architecture

Overview

A positive water balance of a cut flower only can be obtained if water uptake is sufficient to exceed the water loss by transpiration. Sufficient water uptake is only possible if at least a part of the cut open vessels is refilled with water. The refilling of embolised xylem vessels is discussed from a physical point of view (5.1). The processes of refilling are dependent on the anatomical properties of the xylem vessel system. A compact vessel system is concluded to refill better than a vascular system consisting of relatively long, wide and loosely connected vessels. The anatomy of the stem xylem vessels clearly influences the process of embolism repair. The possibility of an easy and accurate measurement of the anatomical characteristics was studied in order to come to a prediction of the vulnerability to embolism related problems on vase (5.2). Clearly no absolute prediction of vase life is possible from one anatomical characteristic, however, breeders and growers now can optimise the stem xylem anatomy to acquire flowers that are less vulnerable to embolism.

5.1

Embolism repair in cut flower stems: a physical approach.

W. van Ieperen, U. van Meeteren, J. Nijsse

(Submitted)

Abstract

The role of xylem anatomical properties on air emboli removal and xylem hydraulic conductance recovery from cut flower stems during the first hours of vase life was studied from a physical point of view. A new theoretical model based on physical processes was developed and tested using chrysanthemum (*Dendranthema x grandiflorum* Tzvelev) cut flowers. The model predicts that the repair process takes place in two phases. During the first phase of a few seconds, air from all cut open xylem vessels redistributes to a smaller number of mainly large diameter cut open vessels. Consequently, the connection between vase water and the non-cut water filled xylem vessels in the upper part of the cut flower stem is partly restored and hydraulic conductance partly recovered. The second phase may last several hours. During this phase, remaining air partly or completely dissolves into its surrounding water and further hydraulic conductance recovery gradually takes place. The results of the model agreed very well with dynamic measurements of hydraulic conductance recovery on chrysanthemum stem segments after aspiration of air. Visual detection of air embolisms after cryo-fixation by cryo scanning electron microscopy (cryo-SEM) showed that during the repair process, air indeed gathered in larger diameter vessels at a relatively high position from the cut surface of the stem. According to model calculations, air emboli removal and hydraulic conductance repair is improved in stems with smaller diameter vessels. The model also predicted that the height of the water level in the vase would influence air emboli removal much more in stems with larger diameter vessels than in stems with smaller diameter vessels. This prediction was experimentally confirmed. It is concluded that anatomical differences in xylem structure play an important role in rehydration capability of cut flowers. Moreover, xylem anatomical properties interact with rehydration treatments.

Introduction

Insufficient water uptake due to xylem occlusion is one of the main reasons for inferior cut flower performance during vase life. Causes of xylem occlusion are various: microbial growth, deposition of materials such as gums and mucilage in the lumen of xylem vessels, formation of tyloses and the presence of air emboli in the vascular system (Van Doorn, 1997). Air emboli are a special case of xylem occlusion

for two reasons: firstly, because air emboli are present in almost all cut open vessels at the base of flower stems after cutting, and secondly because air emboli presence is (partly) reversible. Sufficient removal of air emboli has been proposed to be a prerequisite for restoration of water uptake and a positive water balance during vase life (Durkin, 1979, 1980; Van Doorn, 1990; Van Meeteren, 1992; Van Meeteren and Van Gelder, 1999). Besides its direct effect on xylem hydraulic conductivity, the presence of air emboli might also be a prerequisite for the formation of other, permanent types of xylem occlusion (Van Doorn and Cruz, 2000). Variations in rate and level of embolism removal are thought to be (partly) responsible for differences in water uptake between different cultivars of cut roses (Evans *et al.*, 1996). Previous calculations with a physical model (Yang and Tyree, 1992) showed that the process of air removal from embolised xylem vessels in trees depends on a combination of physical and plant anatomical factors. In cut flowers, similar factors in both plant material and vase water may play a role. However, instead of the usually profound negative xylem pressure in trees, xylem pressures around air emboli near the cut surface of a flower stem are relatively high (presumably even positive instead of negative). In addition, the distribution of air emboli due to cavitation in trees and the clearly located emboli in cut flower stems after severance are rather different.

It was shown by cryo scanning electron microscopy (cryo-SEM) in moderately desiccated chrysanthemum cut flowers (Nijsse *et al.*, 2001a) and in detached stem segments (Van Ieperen *et al.*, 2001) that almost all cut open vessels fill with air after cutting, provided that the cut open vessels were connected to non-cut water filled xylem and under a minimal tension. Cut open vessels completely embolised while no air passed into non-cut xylem vessels as could be expected because air-water interfaces cannot pass vessel-to-vessel connections (Zimmerman, 1983).

At the start of vase life, air cannot be simply pressed out of the cut open vessels. Instead, it will be trapped between the entering water column and the pit membranes of the vessel-to-vessel connections. Dissolution of air into surrounding water is necessary for removal of air from these cut open vessels. In chrysanthemum a positive correlation between emboli presence and a subsequent negative water balance during vase life was shown (Nijsse *et al*., 2000). The water balance and water uptake after entrance of air in chrysanthemum cut flowers is positively influenced by cutting height (Marousky, 1973; van Meeteren and van Gelder, 1999). This might be related to the gradual basipetal increase in xylem diameter in chrysanthemum cut flower stems (Nijsse *et al*., 2001b).

The aim of the present paper is to describe and explain the processes involved in air embolism removal in cut flowers at the start of vase life from a physical point of view. A model is used to simulate the complex interactions between air removal and physical and anatomical factors of the xylem and vase water properties. Simulation results are tested by measuring the dynamics of hydraulic conductance of chrysanthemum stem segments, by visualisation of air emboli presence in recovering chrysanthemum stem segments using cryo-SEM, and by a vase life experiment in which the water height in the vase was varied.

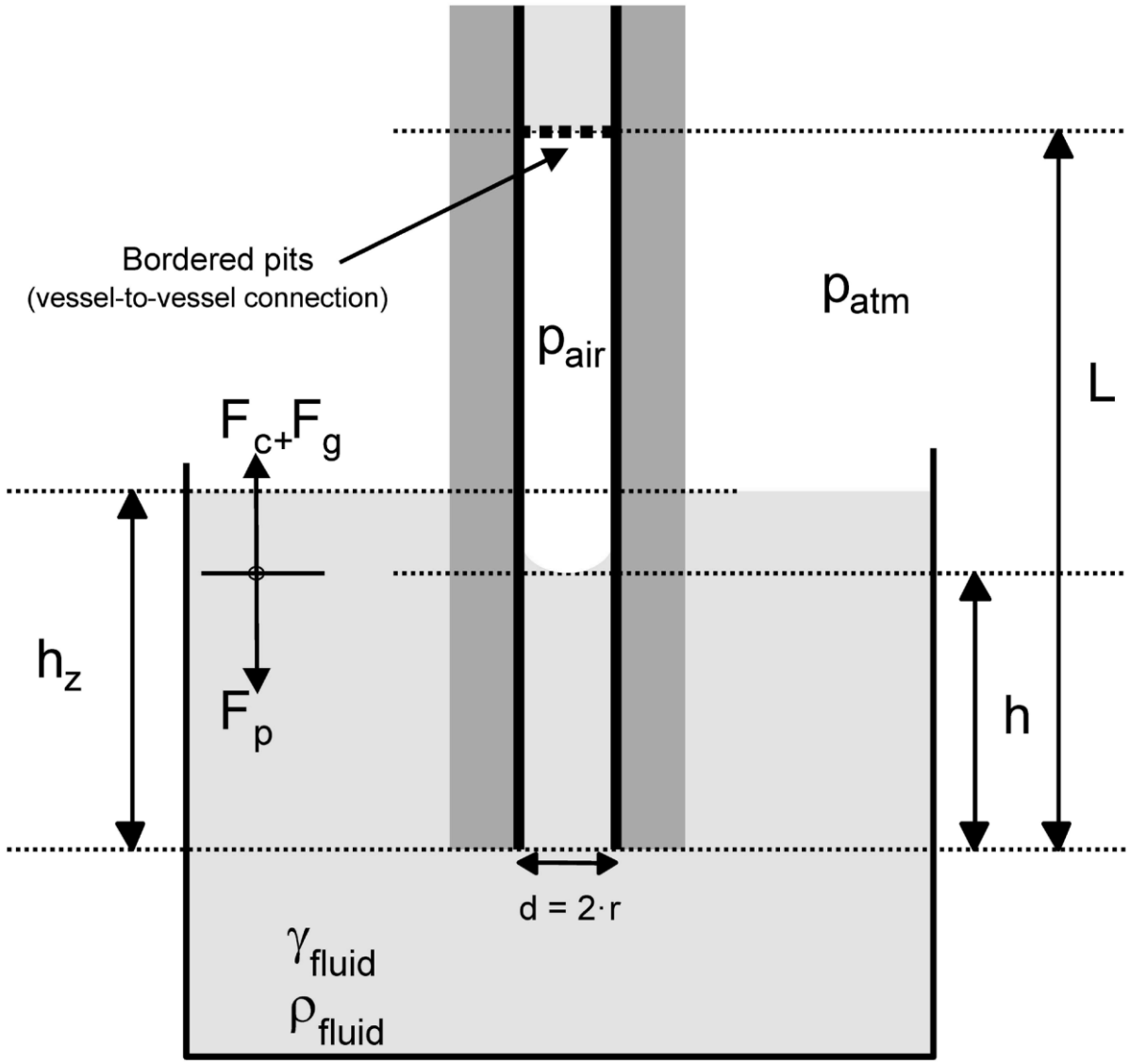

Fig. 1. Schematic representation of physical forces and parameters involved in rise of water (*h*) in an individual air-filled cut open vessel (Parameters see text and Table I).

The model

Model description and simulation results

The process of hydraulic conductance recovery in the basal cut-end of a flower stem at the start of vase life can be separated into two sub-processes:

1. Reconnection of the vase water with the xylem water columns in intact xylem vessels through redistribution of air and water in the network of previously air-filled xylem vessels ends, and
2. Dissolution of trapped air from (partly) embolised xylem vessels into their surrounding and subsequent further re-filling with water.

The processes under 2 are derived from a previously described model for embolism recovering in intact trees after cavitation (Yang and Tyree, 1992). The processes under 1 are specific for cut flowers at the start of vase life and are newly described here. Immediately upon reapplying water at the start of their vase life, water tends to rise in the cut open xylem vessels of flower stems. This tendency is caused by capillary forces in the cut open vessels and the positive pressure due to the level of the vase water above the cut surface of the flower stem. The rise of water compresses the air in the cut open vessels and generates a super atmospheric air pressure, which is the driving force for the later dissolution of air. Because xylem vessels act in a network in which they are interconnected, both sub-processes influence the hydraulic conductance of the xylem in the flower stem. The model below describes these processes, and enables some quantification of air removal as dependent on xylem anatomical properties. To enhance clarity about factors and processes involved, a simplified system description concerning one individual vessel is presented first and later this model is extended to a multi-vessel system.

Table I: Description of input variables and parameters including standard values[1] for the parameters used in model calculations

γ	Surface Tension of the solution in the vessels (water at 20°C).	0.0728	$N.m^{-1}$
η	Viscosity of the solution in the vessels	0.001	Pa.s
ρ	Density of the solution in the vessels	998.2	$kg.m^{-3}$
Θ	Contact angle between meniscus and xylem cell wall	50	^{o}
g	Gravity constant	9.8	$m.s^{-2}$
P_{atm}	Atmospheric air pressure	101325	Pa
h_z	Height of the water level in the vase above the cut end of the flower stem	0.1	m
r	Radius of the vessel	20	µm
L	Length of the vessel	0.03	m
k_a	Solubility of air in water	$7.75x10^{-6}$	$mol.m^{-3}.Pa^{-a}$
D_a	Diffusion constant for air in water	$1.95x10^{-9}$	$m^2.s^{-1}$

[1]Standard values are used in the model calculations unless explicitly mentioned else.

-Simplified system description: initial rise in an individual air-filled cut open vessel

At the start of vase life of a flower stem, an individual air-filled cut open vessel can be regarded as a closed chamber for air (Fig. 1). Upon reapplying water to the cut end of such a vessel, air becomes trapped because air-water interfaces cannot pass the bordered pits at vessel-to-vessel connections. However, partial refilling of such a cut open vessel still occurs due to a combination of forces, which force the air-water meniscus to rise in the vessel. These forces include the capillary force (F_c [N]; Eqn 1), promoting partial refilling, and the gravitational force (F_g [N]; Eqn 2), de- or promoting partial refilling depending on the height of the water level in the vase (h_z [m]). Refilling also causes compression of air inside the captured air bubble, which results in an additional force that counteracts the rise (F_p [N]; Eqn 3).

$$F_c = 2 \cdot \pi \cdot r \cdot \gamma \cdot \cos\theta \qquad (1)$$

$$F_g = \pi \cdot r^2 \cdot (h - h_z) \cdot \rho \cdot g \qquad (2)$$

$$F_p = \left(\frac{L}{L-h} - 1 \right) \cdot p_{atm} \cdot \pi \cdot r^2 \qquad (3)$$

A description of the variables and parameters including their standard values used in model calculations, is presented in Table I. The contact angle of the water-wall interface, (Θ [º]) has often been assumed 0º. However, recently it was shown in various plant species that Θ might be much higher (42º-55º; Zwieniecki and Holbrook, 2000). In this model Θ is assumed 50º as has previously been estimated for xylem vessels of chrysanthemum cut flowers (Van Ieperen *et al*., 2001). Vessels are assumed circular in cross-section (with radius *r* [m]).

Providing no air disappears from the trapped air volume, a static equilibrium is reached when the net force on the meniscus is zero (F_c+F_g+F_p=0). This situation is accomplished when the meniscus has reached height *h* [m] in a cut open vessel of length *L* [m] (Eqn 4).

$$h = \frac{-\left(p_{atm} \cdot r + (L + h_z) \cdot g \cdot \rho \cdot r + 2\gamma \cdot \cos\theta\right)}{-2 \cdot g \cdot \rho \cdot r} + \cdots$$

$$\cdots + \frac{\sqrt{\left(p_{atm} r + (L + h_z) \cdot g \cdot \rho \cdot r + 2\gamma \cdot \cos\theta\right)^2 - 4\left(g \cdot \rho \cdot r\right) \cdot \left(L \cdot 2\gamma \cos\theta + h_z \cdot L \cdot \rho \cdot g \cdot r\right)}}{-2 \cdot g \cdot \rho \cdot r} \qquad (4)$$

Calculations with Eqn 4 show that the rise *h* increases with vessel length *L* and decreases with vessel radius *r* (Fig. 2A). However, if expressed as percentile of vessel length, the rise is mainly influenced by vessel radius. Consequently, air pressure at static equilibrium mainly depends on vessel radius (Fig. 2B). The influence of the contact angle on rise is substantially, especially in small diameter vessels (Fig. 2C). The influence of the water level in the vase (h_z) on rise (*h*) is small in small diameter vessels (results not shown). However, increasing h_z from 1 to 10 cm increases the air pressure inside the air bubble more than 30% in large diameter vessels (r>30 µm). This substantially increases the driving force for air solution from trapped air in large diameter vessels into the surrounding.

Not only air pressure and rise at the (semi) static equilibrium are important but also the time to reach it. Besides the forces F_c, F_g and F_p, an important other force influences the dynamics of rise: the friction force (F_w [N]; Eqn 5). F_w describes the friction that a flowing water column in a circular tube experiences.

$$F_w = \frac{8 \cdot \pi \cdot d \cdot \rho \cdot h \cdot v^2}{Re} \qquad (5)$$

$$Re = \frac{d \cdot \rho \cdot v}{\eta} \qquad (6)$$

Re is the Reynolds number (Eqn 6), a dimensionless number that describes flow characteristics; e.g. it indicates whether flow will be laminar or turbulent. The Reynolds number depends on the diameter of the vessel (d=2·r [m]), the velocity of the moving water column (v [m.s^{-1}]), and the density ρ and viscosity (η [Pa.s]) of the

fluid (vase water). The position of the meniscus in the vessel determines the length *h* of the water column, which in turn influences the F_w. The model calculates the acceleration (*a* [m.s^{-2}]) of the rising water column based on the sum of all forces (F_{net}) and the mass of the moving water column (Eqn 7).

$$F_{net} = \frac{a}{h \cdot \pi r^2} \qquad (7)$$

The acceleration a is used to calculate the velocity *v* of the meniscus, which in turn is used to calculate the rise of water and the position of the meniscus in the vessel.

Computer simulations with a model based on Eqn 1-3 and 5-7 show that initial rise completes within a relatively short time period of usually a few seconds (maximal minutes) and is mainly influenced by the length of the air-filled vessel (Fig. 3).

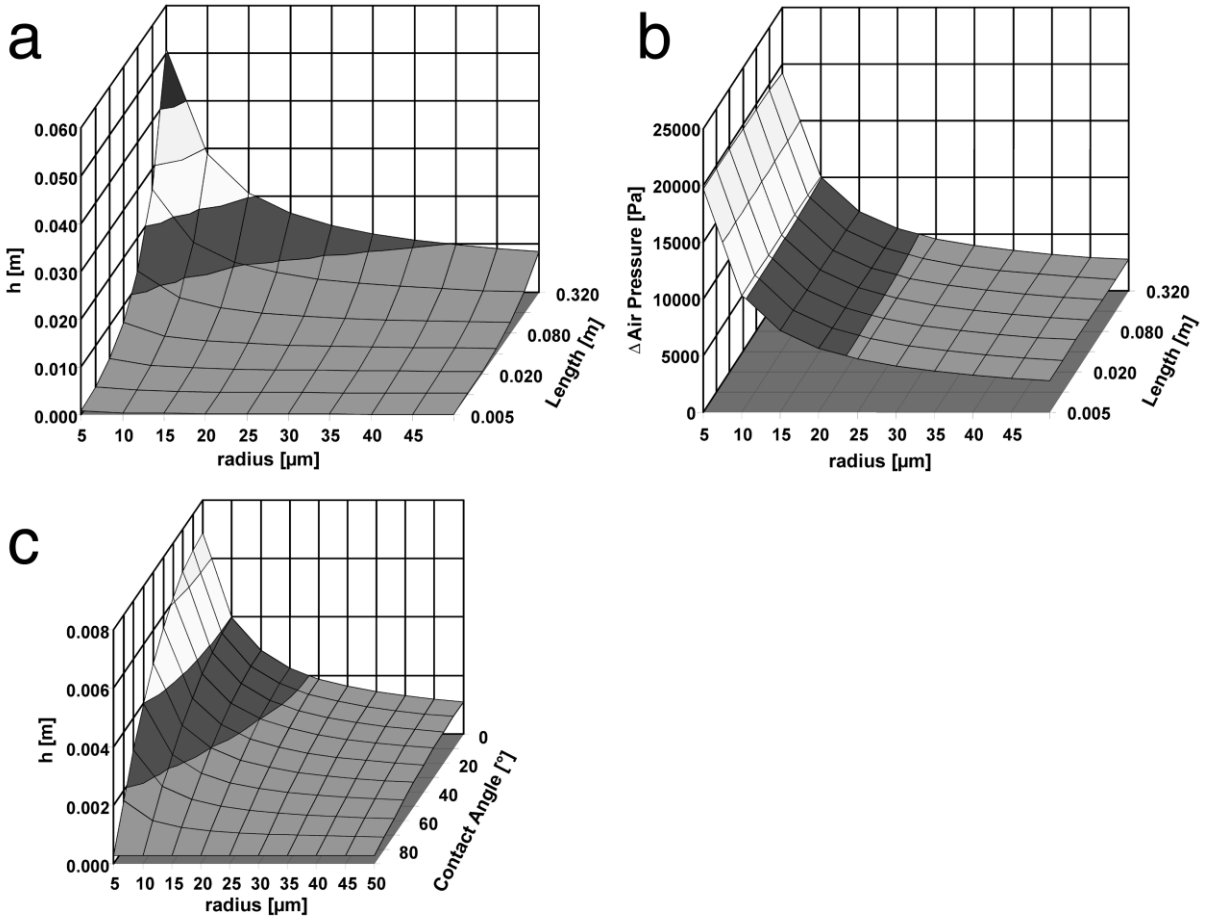

Fig. 2. Simulated effects of length and radius of individual embolised cut open xylem vessels on the rise of water (*h*) in the vessel (A) and on the realised super-atmospheric air pressure inside the trapped air bubble (B) at static equilibrium after applying water at the cut surface. Effect of the contact angle on the relationship between radius and rise *h* (C).

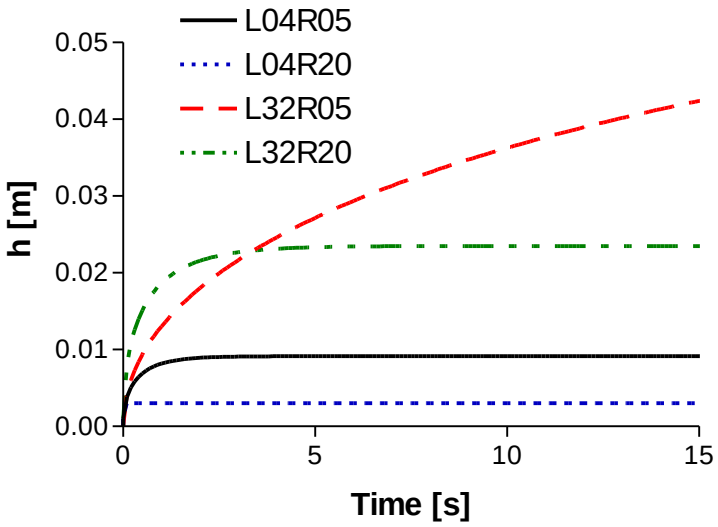

Fig. 3. Simulated time course of rise of water (*h*) in individual, initially air filled cut open xylem vessels of different anatomical properties (L32R20 = Length 32 cm, Radius 20 μm and so on; other parameters see Table I).

Table II: *Simulated water height (h), percentile refilling (R_{fp}) and above atmospheric air pressure (ΔP) in two adjacent, initially air-filled cut open vessels after a 60 s simulation.*

(A) Effect of vessel radius in a system containing a short and long vessel.

Radius [μm]	Length [cm]	*h* [cm]	R_{fp} [%]	ΔP [Pa]
5	4	4.0	100	-
30	20	0.7	3.3	4035
5	4	4.0	100	-
20	20	0.8	4.1	5581
5	4	4.0	100	-
10	20	0.9	4.7	10251

(B) Effect of vessel length in a system containing a narrow and wide vessel.

Radius [μm]	Length [cm]	*h* [cm]	R_{fp} [%]	ΔP [Pa]
5	4	4.0	100	-
30	4	0.1	1.3	4095
5	20	5.8	28.9	17166
30	4	0.1	1.8	4093

-Extended system description: initial rise in a set of adjacent air-filled cut open vessels with different diameter and length

When two adjacent vessels refill with water, free air exchange via bordered pits can occur if the bordered pits allow air transport, and if an appropriate driving force exists.

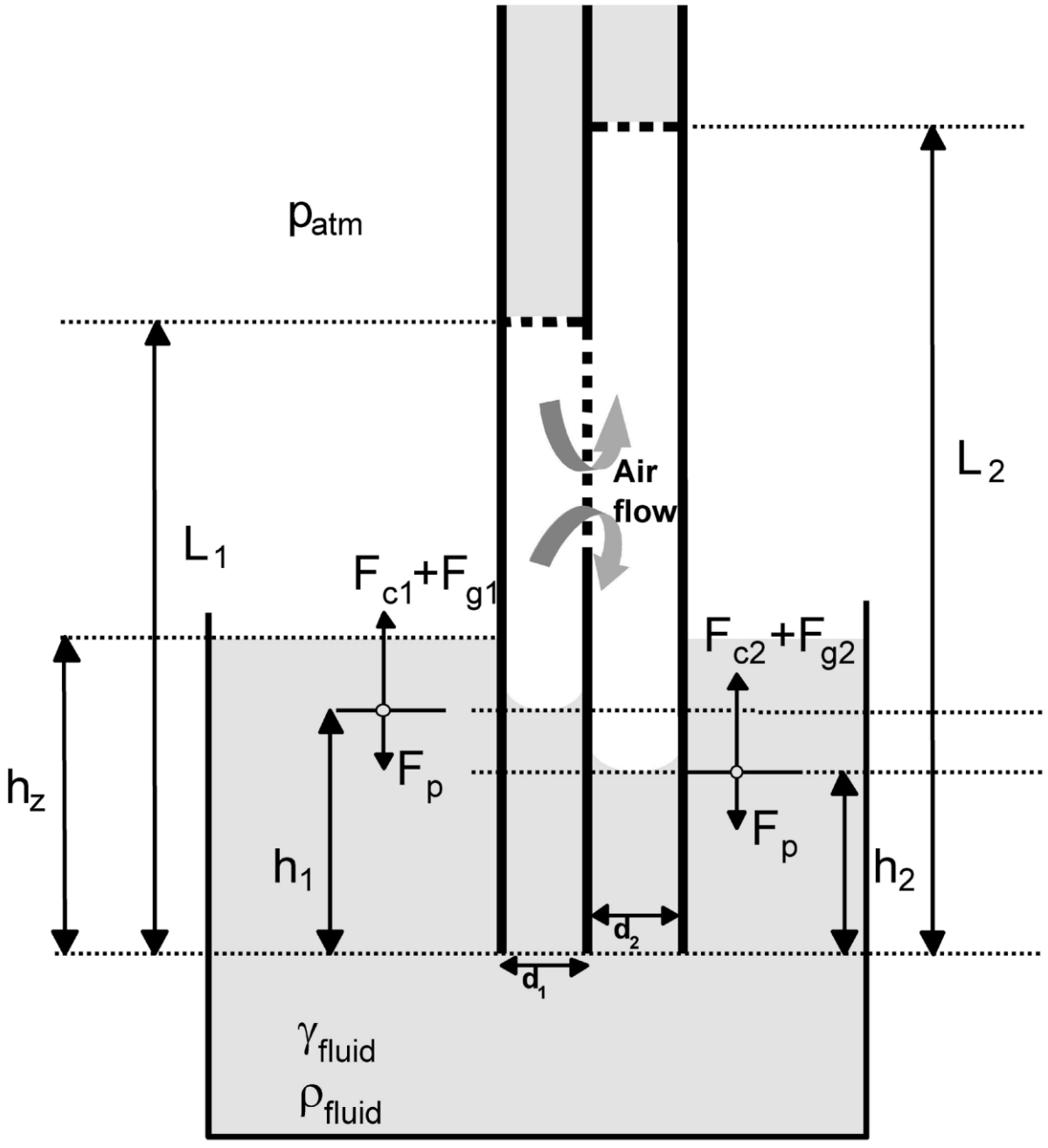

Fig. 4. Schematic representation of rise of water in a system of two adjacent communicating cut open xylem vessels (parameters see text and Table I).

First prerequisite is fulfilled if no air-water meniscus is present in the pits which is assumed to be the case at sites were two air-filled vessels connect. Consequently, the total volume that is compressed in the two vessels might be considered as one, and the air pressure counteracting the rise of the menisci in the two vessels is similar in both vessels (Fig. 4). Hence, refilling patterns and capillary rise in adjacent communicating vessels may largely differ from non-communicating vessels of similar

dimensions. To test this, a model was developed to simulate the refilling process due to capillary rise in clustered vessels. Compared to the single vessel version of the model the calculation of F_p is changed. As long as the rise in one vessel not exceeds the vessel top of the other vessel, F_p is determined by the increase in air pressure of the total volume in both vessels. Several scenarios of different vessel diameter and length combinations of two adjacent air-filled vessels were simulated to investigate the time course and final height of the water columns due to initial rise in both previously air-filled vessels (Table II). These simulations show that if a small diameter vessel is shorter than the adjacent larger diameter vessel, the small diameter vessel always completely refills by expulsing its air into the adjacent vessel. If the smaller diameter vessel is longer than the adjacent vessel, it stops refilling shortly after the meniscus passed the top of the adjacent vessel. This may result in important short distance side-paths for water transport to other, water-filled vessels, which start close to the basal cut surface of the flower stem (Fig. 5).

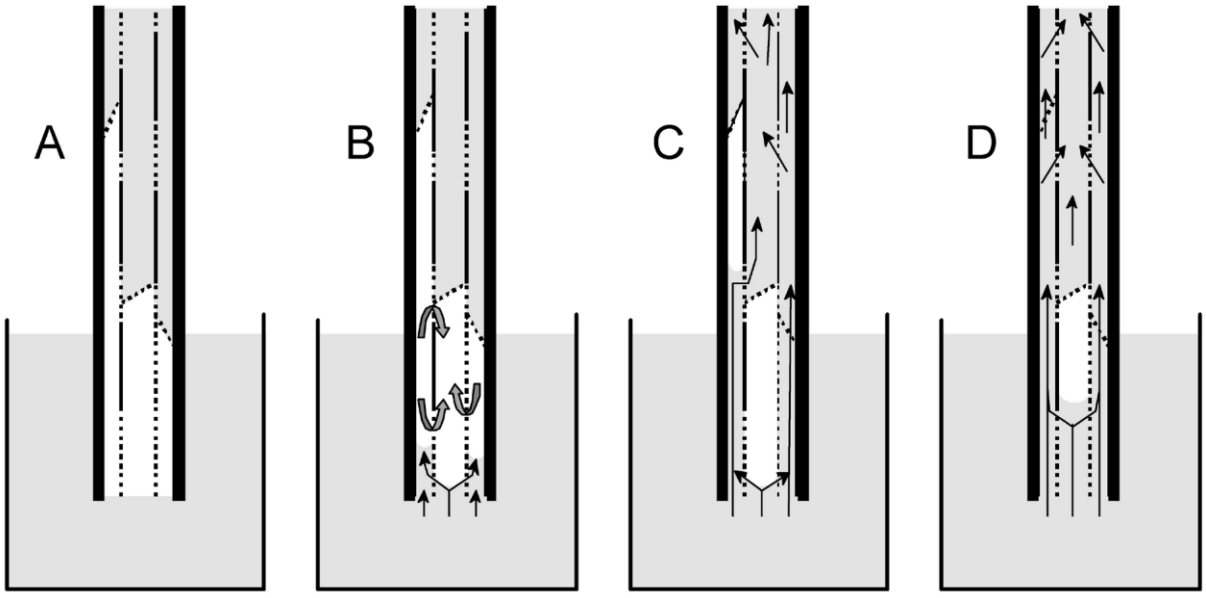

Fig. 5. Schematic representation of different subsequent phases of hydraulic conductance recovering after reapplying water to the basal cut surface of a system of three communicating cut open xylem vessels of different diameter. A, B and C occur during the first few seconds to minutes after reapplying water and show the processes of redistribution of air (grey arrows in B) and water in the communicating cut open vessels, which results in partial restoration of the water connection between vase water and xylem water column above the embolisms (visualised by the arrows in C). The time between C and D may be several hours. D shows the (partial) dissolution of trapped air.

It is possible that the larger diameter vessel completely empties again due to the entrance of air originating from an adjacent refilling smaller diameter vessel. The model does not account for the eventual release of air into the vase solution due to this phenomenon. Air pressure inside the remaining air bubbles in cut open vessels depends mainly on the diameter of that vessel. The time to reach maximal h due to

initial rise varies between seconds to maximal a minute and mainly depends on the length and diameter combinations of the vessels involved.

-The last step: Air solution and diffusion from trapped air-bubbles in cut open xylem vessels.

The mechanism of air removal from trapped air inside an embolised xylem vessel revolves two steps. 1: the solution of trapped gasses into water at the wet surface of the air bubble, followed by 2: the diffusion of solved gas to the surrounding environment around the trapped air bubble. Due to air removal, the water column in the vessel rises.

According to Henry's law, the equilibrium solubility of gas at a gas-water interface is proportional to the gas pressure. The equilibrium air concentration (C_a [mol.m^{-3}]) at the air-water interface depends on the air pressure (p_{air}) and the solubility of air in water (k_a [mol.m^{-3}.Pa^{-1}]) as shown in Eqn. 8 and 9.

$$C_a = \mathrm{k}_a \cdot p_{air} \qquad (8)$$

$$\mathrm{k}_a = 0.8 \cdot \mathrm{k}_{N_2} + 0.2 \cdot \mathrm{k}_{O_2} \qquad (9)$$

The solubility of air in water (k_a) is defined as the average solubility of the two major gasses in air (N_2 and O_2). The time to reach equilibrium between gas and dissolved state in the liquid at the air-water interface is assumed negligible. The above atmospheric pressure (p_{air}) in the air bubble generates a concentration gradient between the water interfaces at the air bubble and the surrounding tissue, which is the driving force for dissolved air movement by diffusion. The rate of diffusion ($\Delta N_a/\Delta t$ [mol.s^{-1}]) is determined by the concentration gradient ($\Delta C/\Delta x$ [mol.m^{-4}]), the diffusion area (A [m^2]) and diffusion constant (D_a [m^2.s^{-1}]) for dissolved air in surrounding tissues, according to Fick's Law (Eqn 10).

$$\frac{\Delta N_a}{\Delta t} = D_a \cdot A \cdot \frac{\Delta C}{\Delta x} \qquad (10)$$

The diffusion area (A) is an uncertain variable since the transport path between air-filled vessels and surrounding tissue involves transport through cell walls. These walls are highly lignified and have a low water content and therefore a very low diffusion constant for air. Likely only direct water connections via bordered pits and at the cross sectional area of the rising water column are available for diffusion. Diffusion area (A) will also decrease in time, when the water column rises due to dissolution and diffusion of air from the air bubble into the environment.

Model Summary and Discussion

Repair of cut open xylem vessels in the basal part of a cut flower stem may be split into two processes. A fast repair of mostly small diameter vessels by expulsion of their air into adjacent larger diameter vessels that were also cut. This process takes place during the first seconds to minutes after re-applying water. It results in a xylem network in the basal part of the cut flower stem in which most small diameter vessels conduct water while larger diameter vessels remain at least for a part of their length air-filled (Fig. 5). The effect on hydraulic conductivity is complex. In xylem systems without air emboli far most of the water is transported through only a few large diameter vessels as follows from the Hagen-Poiseuille equation (Zimmermann,

1983; Gibson *et al*., 1984). Upon this, it may be expected that especially removal of air emboli from large diameter vessels contribute to the recovering of water uptake. However, the xylem system usually exists of many parallel interconnected xylem vessels of different lengths (Zimmermann, 1983). Therefore, complete recovering of a small diameter vessel, or a small rise of water in some large diameter cut-xylem vessels may already lead to substantial partial recovery of hydraulic conductance, due to the connection of the water in the mentioned vessels with neighbouring not-cut-vessels (water filled).

The process of hydraulic conductance recovery continues with the much slower processes of solution and diffusion of trapped air from the captured air bubbles into their surroundings. It was calculated before that in trees the process of air removal from embolised vessels has a time constant of several hours (Yang and Tyree, 1992). Anatomical properties of xylem vessels clearly influence the long-term dissolution processes: Vessel diameter strongly influences p_{air} in the trapped air bubble. Small diameter vessels also have a longitudinal surface area-volume ratio that enhances the rate of air removal compared to large diameter vessels. Vessel length is less important: increasing vessel length almost proportionally increases the diffusion area and therefore rate of air removal. This compensates for the larger volume of air in longer air-filled vessels. The contact angle between water and wall material in embolised vessels also strongly influences p_{air} in the trapped air bubble and thus the processes of solution and diffusion. Chemical contents of the cell wall as well as chemo-physical properties of the entering fluid (vase water) might influence this contact angle, and thus the rate of air removal.

When postharvest treatments will affect the physical processes involved in embolism repair (like vase water height, water temperature, surfactants) it may be expected that the outcome of these treatments interact with the anatomical properties of the flower stems.

Materials and methods

Plant material

For all types of measurements chrysanthemum (*Dendranthema x grandiflorum* Tzvelev cv. Cassa and Vyking), plants were grown in a greenhouse at Wageningen University in 14-cm diameter plastic pots containing a commercial potting soil. The average temperature was 18^{o}C and an 18 h photoperiod was maintained until the plants had formed 15-17 leaves longer than 0.5 cm (3-4 weeks). Thereafter, an 9 h photoperiod was maintained until harvest. When necessary, lengthening of the natural photoperiod was achieved by high-pressure sodium lamps (Philips SON/T). Conversely, shortening of the natural photoperiod was by use of black screens.

Measurement techniques

-Hydraulic conductance dynamics

Stem segments for hydraulic conductance measurements were collected from plant material ('Cassa'), which was grown as described above and prepared (under water) according to van Ieperen *et al*. (2001) to prevent initial air embolisms. The hydraulic conductance of 20 cm long stem segments, cut at 15 cm height above the

roots, was measured by pulling water through a stem segment at known pressure difference (40.0 ± 0.01 kPa) using the apparatus as described by van Ieperen *et al*. (2000). Initial hydraulic conductance was measured during 15 minutes. Then air entrance at the basal part of the stem segment was allowed during 3 minutes by removing the water supply from the basal cut surface while maintaining the pressure. Afterwards water was reapplied to the stem segment and the restoration of the hydraulic conductance (K_h) was measured during approximately 1.5 hours at 30 s time intervals according to van Ieperen *et al*. (2000).

-Visualisation of air embolisms by cryo scanning electron microscopy (Cryo-SEM)

After 1.5 hours of measuring hydraulic conductance the negative pressure was released from the top of the stem segments that were later used for visual detection of air embolisms by Cryo-SEM. After the water uptake of the stem segment had completely ceased, water supply was carefully removed from the basal cut end of the stem segment, while leaving a drip of water at the cut surface to prevent occasional air entrance. Then the stem segment was plunge-frozen in liquid nitrogen (-196°C) to immobilise the water in the vessels. At different distances from the cut surface stem transversal sections were prepared for investigation in a cryo scanning electron microscope as described in Nijsse and Van Aelst (1999). In short, stem pieces of about 1 cm were sawed out under liquid nitrogen using a circular diamond saw. These stem pieces were cryo-planed at -90°C in a cryo-ultramicrotome (Reichert-Jung Ultracut E/FC4D) with a diamond knife (8 mm wide; Histo no trough 45°, Drukker International, The Netherlands). The planed surfaces were freeze-dried for 3 minutes at –89°C and 10^{-4} Pa and sputter-coated with platinum in an Oxford CT 1500 HF cryo transfer unit. The surfaces were photographed in a cryo-SEM (Jeol 6300F) at –190°C using a digital imaging system.

-Water balance during vase life

For the vase life experiment to investigate the effect of vase water height on restoration, chrysanthemum cut flowers ('Vyking') were treated as described before (Van Meeteren and Van Gelder, 1999). In short, flowering stems were harvested at commercial maturity by cutting the stalks at their root-shoot junction (soil level) and brought to the laboratory as soon as possible. Thereafter, lower leaves were removed, cut stem ends were trimmed by 1 cm in air, and the stalks were placed for 3 h in a bucket with a mixture of ice and water (3:1 by volume) in darkness at 4°C to regain full turgidity. The lower 12 cm of half of the flower stems was cut off in air after the hydration treatment. Thereafter, the fresh weight of the flowers was determined as the initial weight, flowers were moderately desiccated by horizontally placing the individual stems for 1 h on two wires (weight loss was approximately 5%). Afterwards the cut ends were re-cut in air by 2 cm, and the flowers were placed in glass tubes (the vases) containing a solution of $CaCl_2$, $NaHCO_3$ and $CuSO_4$ (Van Meeteren *et al*., 2000) at 20 ± 1°C, 60 ± 5% RH and a light intensity of 14 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Philips, TLD 50W/84HF) with a light period of 12 h. The height of the solution in the vases was set to 5 or 18 cm above the cut surface of the flowers. When the water level had lowered approximately 1 cm extra solution was added to the tubes to re-establish the correct water height. Water uptake after 24 hours was calculated from the volume of added

solution per individual flower. The percentage of initial weight of the flowers was determined after 24 hours. Initial weights were corrected for the weight of the cut 2-cm stem pieces. The design was randomised complete block with three replicates per treatment in each of four blocks. Analysis of variance was applied to the percentage of initial flower weight and water uptake per flower after 24 hours of vase life.

Experimental results

Dynamics of hydraulic conductance restoration curves of stem segments.

A typical time curve of hydraulic conductance (K_h; percentage of the initial hydraulic conductance) of a 20-cm long stem segment during a rehydration experiment is shown in Fig. 6A. The hydraulic conductance without air in the xylem vessels was measured at the start of the measurement and set to 100%. Thereafter, air was aspired at the basal cut-surface for 3 minutes followed by reapplying of water. The hydraulic conductance showed an initial fast recovery after the replacement in water (Fig. 6A). Like a saturation process, after a few hundreds of seconds, the increase levelled off and the hydraulic conductance tended to a plateau lower than 100% of the initial conductance before air entrance. As can be seen from the residuals (Fig. 6B), the data could not be fitted with a simple rectangular hyperbola or binding isotherm ($K_h = \frac{K_{h,max} \bullet time}{T_{half} + time}$; $K_{h,\ max}$ = plateau of hydraulic conductance, T_{half} =time required to reach half of $K_{h,\ max}$). However, extending the curve with a second hyperbola resulted in a close fit ($K_h = \frac{K_{h,max1} \bullet time}{T_{half\,1} + time} + \frac{K_{h,max\,2} \bullet time}{T_{half\,2} + time}$; R^2=0.9915; Fig. 6A) and a Gaussian distribution of the residuals (P>0.10; Fig. 6C). The 95% confidence intervals of the parameters (insert Fig. 6A) show a short half time of a few seconds for the first hyperbola and a long half time of approximately 2600 s for the second hyperbola.

Effect of de-gassed water on hydraulic conductance restoration curves.

Changing the concentration gradient of air (between trapped air and its surrounding) will effect the dynamics of hydraulic conductance restoration, if dissolving of air plays a role in the restoration of hydraulic conductance. Therefore, an experiment was carried out in which the amount of air dissolved in the vase water was affected by de-gassing the water. Comparing the reapplication of de-gassed water after air aspiration with tap water (Fig. 7) demonstrated a large increase in the plateau of the restoration curve. When a double hyperbolic function is used and $T_{half,\ 1}$ is set to 7.4 s (because the first measured data after reapplying water were disturbed), the best fits of the curves for de-gassed and tap water are respectively $K_h = \frac{26.47 \bullet time}{7.4 + time} + \frac{88.23 \bullet time}{2215 + time}$ and $K_h = \frac{25.67 \bullet time}{7.4 + time} + \frac{20.95 \bullet time}{684.7 + time}$, both with a Gaussian distribution of the residuals. A fast increase in hydraulic conductance occurred when tap water was replaced by de-gassed water at 1930 s after reapplying water (Fig. 7).

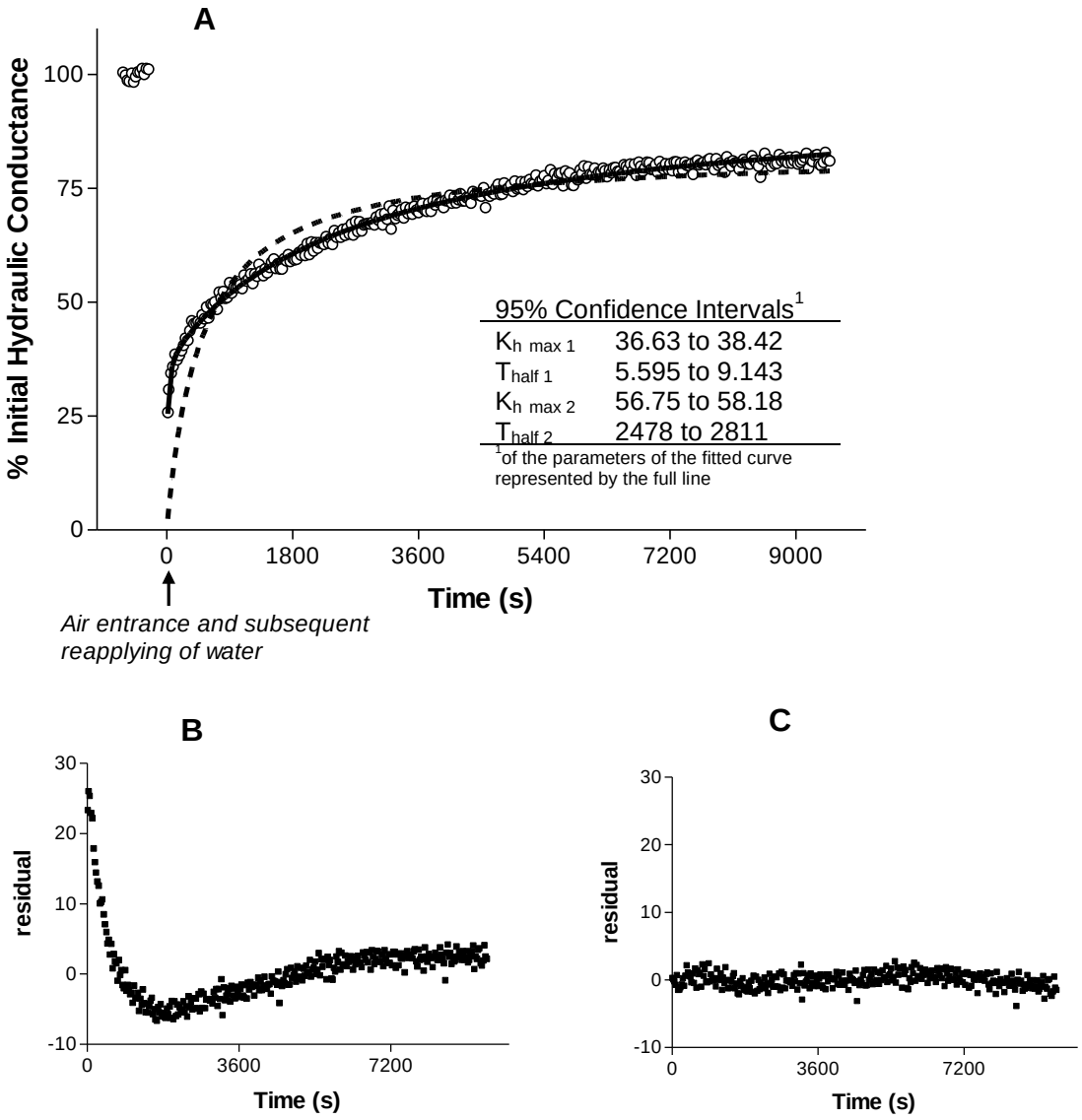

Fig. 6. A: Time curve of hydraulic conductance of a 20-cm chrysanthemum stem segment cut 15 cm above the roots. After measuring the initial hydraulic conductance without air in the xylem vessels, air was aspired at the basal cut-surface for 3 minutes. Thereafter water was reapplied. Time 0 is the time of reapplying water. o = measured data, dashed line = fitted rectangular hyperbola ($K_h = \frac{82.83 \bullet Time}{487.1 + Time}$), full line = fitted curve using a combination of two hyperbola ($K_h = \frac{37.53 \bullet Time}{7.37 + Time} + \frac{57.47 \bullet Time}{2644 + Time}$).
B. Residuals of the fitting with one hyperbola. **C.** Residuals of the fitting with two hyperbola.

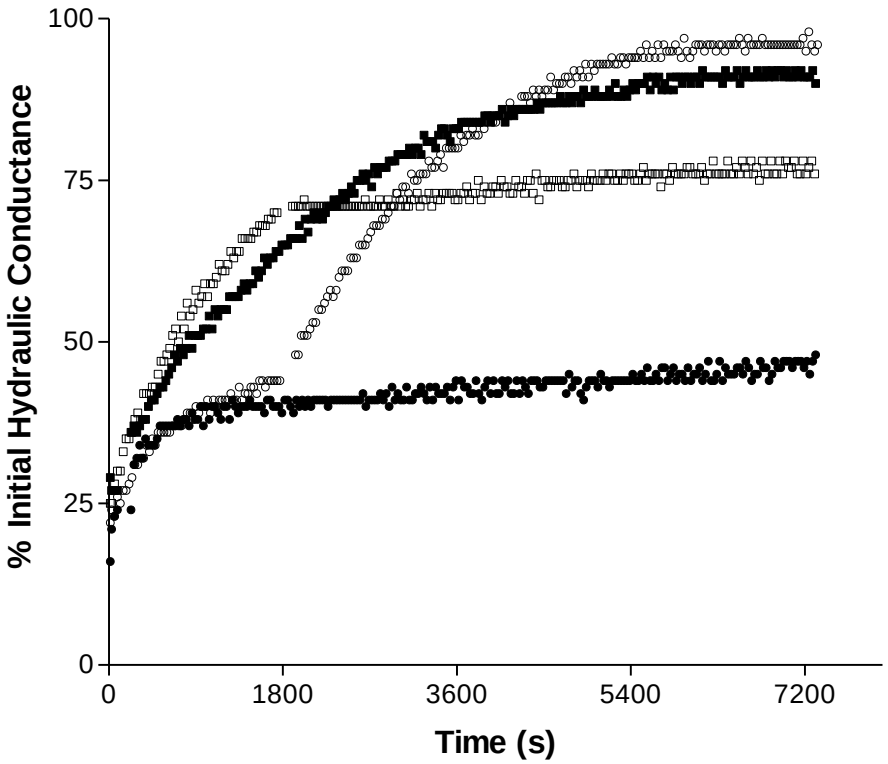

Fig. 7. Effect of the use of de-gassed water on time curves of hydraulic conductance of a 20-cm chrysanthemum stem after air aspiration via the cut-surface. After measuring the initial hydraulic conductance without air in the xylem vessels, air was aspired at the basal cut-surface for 3 minutes. Thereafter water was reapplied. Time 0 is the time of reapplying water. The water used was either tap water (●, ○) or de-gassed water (■, □). In two treatments, 1930 s after reapplying water it was changed: tap water to de-gassed water (○) or de-gassed water to tap water (□).

About half an hour after this change, it resulted in a conductance similar to that of the stem segment immediately placed in de-gassed water after the air aspiration. The increase in hydraulic conductance halted instantaneously when de-gassed water was replaced by tap water (Fig. 7).

Presence of air at different heights above the cut surface.

Transversal sections of the frozen stem segments were cut at 0.1, 1, 2 and 7 cm above the original base-cut-plane and studied by cryo-SEM. At 0.1 and 1 cm nearly all vessels were ice filled. However, 2 and 7 cm above the cut-plane a number of xylem vessels was clearly without ice. Fig. 8 shows details at 1 (A) and 2 cm (B) above the cut-plane. In total, there were respectively 8, 29, 92 and 54 vessels without ice at 0.1, 1, 2 and 7 cm respectively. They all belonged to the widest vessels of the particular stem segment. There was no ice absent in small diameter xylem vessels.

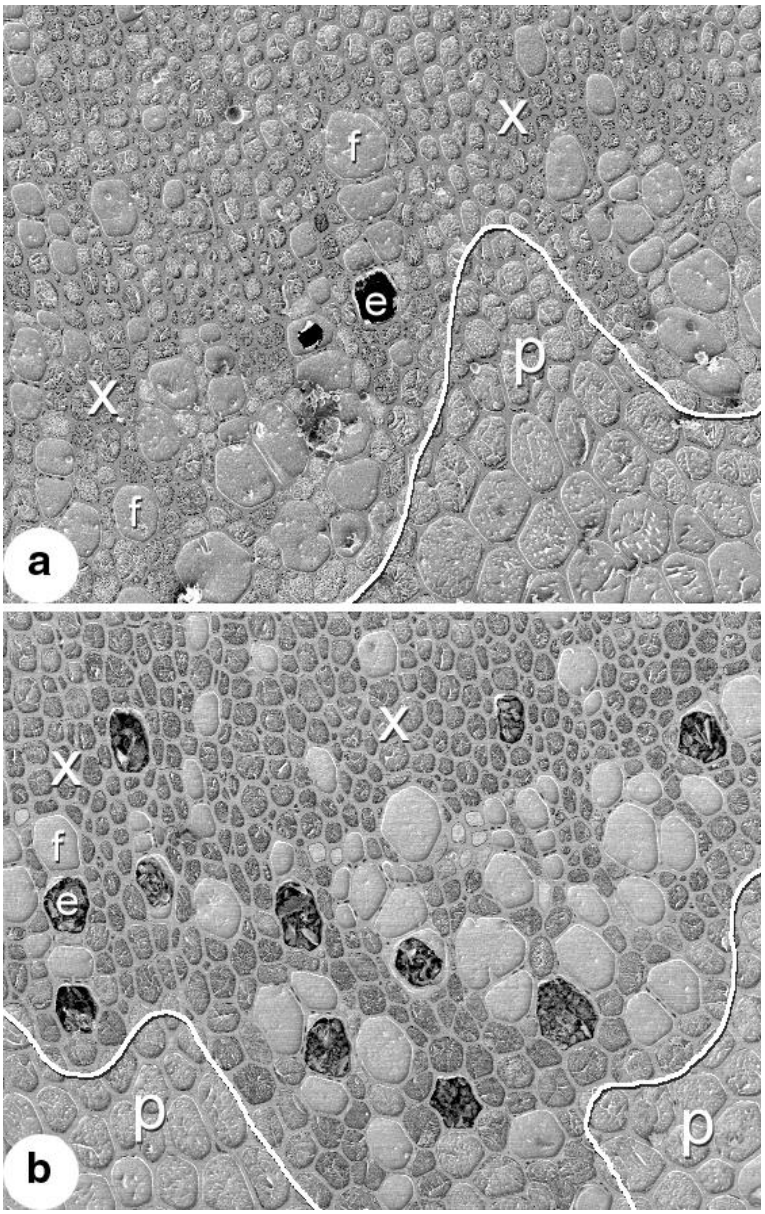

Fig. 8. Transversal sections of chrysanthemum stem segments examined by cryo-SEM. Segments were cryo-fixed by plunging into liquid nitrogen 1.5 h after placing the segment in water subsequent to air uptake. Frozen segments were planed at 1 cm (A) and 2 cm (B) above the original cut-plane for cryo-SEM study. Xylem fibres contain more solutes than vessels and show a dense crystallisation network. This network is absent in vessels. Vessels without ice can be recognised by a dark black colour in contrary to the smooth grey colour of the ice filled vessels. e: empty vessel, f: filled vessel, p: pith, x: xylem fibres. Horizontal image widths are 500 µm.

Water height.

Water height (h_z) in a vase will affect air pressure inside xylem vessels and therefor it maybe expected that water height would affect the dissolution of air. The simulation results showed that the effect would be small unless large diameter vessels are present. Fig. 9 shows the effect of vase water height on fresh weight and

water uptake during the first 24 h of rehydrating cut chrysanthemum flowers that were kept dry for one hour before rehydration. There was a positive effect of water height, especially with flowers cut near their root-shoot junction. The interaction between vase water height and cutting height of the flowers at harvest was significant ($p<0.001$).

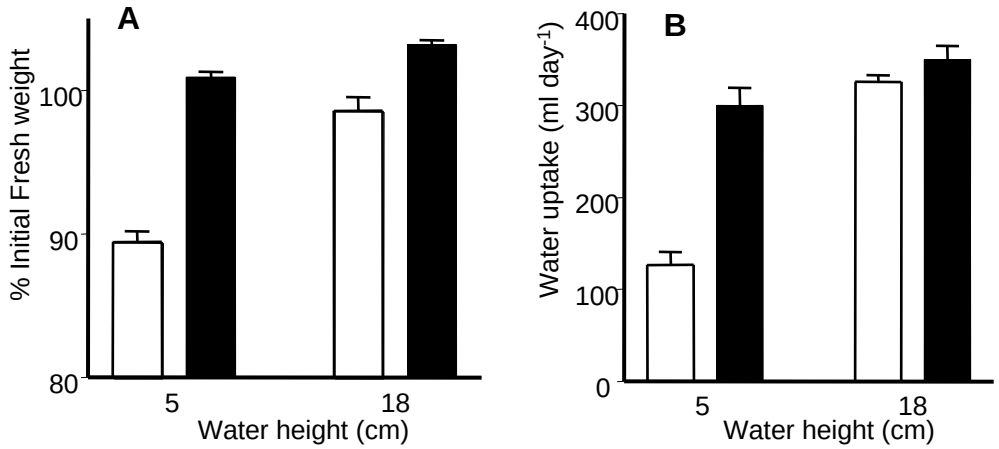

Fig. 9. Effect of height of vase water on fresh weight, expressed as percentage of initial weight (A) and on water uptake (B) during the first 24 h after dry storage of chrysanthemum flowers. Water height was 5 or 18 cm. Flowers were cut at 3 (open bars) or 15 cm (filled bars) above the root-shoot junction. Vertical bars represent standard error of the mean.

Discussion

The physical description of the embolism repair processes in xylem vessels near the cut base of a cut flower, predicted that within hours only a part of the vessels would be completely refilled with water and other vessels partly. Consequently, hydraulic conductance will only be partly repaired as compared to the conductance without any air present. This agrees with the measurements of hydraulic conductance of stem segments (Fig. 6). The large improvement of conductance repair by the use of de-gassed water (Fig. 7) strongly indicates that the loose of conductance was due to the presence of air in the vessels. Computer simulations based on the physical description showed that refilling of xylem vessels will be due to two processes (redistribution of air and water followed by dissolution of trapped air) with large differences in their time constants (respectively seconds to minutes and several hours). This agrees very well with the measured dynamics of hydraulic conductance of stem segments after applying water after air aspiration (Fig. 6). The results of cryo fixation showed that near the stem base nearly all vessels were water filled, while some centimetres above the cut surface large diameter vessels were air filled (Fig. 8). The number of air filled vessels after a 1.5 hours recovering period increased between 0.1 and 2 cm, while at 7 cm above the cut surface the number was decreased. Taking into account the exponential decay in the number of air filled vessels with height from the cut surface before re-applying water (Nijsse *et al*., 2001a

and b), the amounts of air-filled vessels at different heights support the model calculations. Almost no air-filled vessels were observed close to the cut surface while higher in the stem a large percentage of the vessels that could contain air (since they were open from the cut surface) still contained air after 1.5 hours of recovering. Furthermore, only large diameter vessels showed to be air filled after the 1.5 hours of recovering.

Vase water height had a positive effect on rehydration of flowers after air aspiration due to a short dry period (Fig. 9). Water height could have increased the air pressure inside xylem vessels and therefore the dissolution of air. Simulation results showed that the effect would be small unless large diameter vessels are present. In general, there is a gradual basipetal increase in xylem vessel diameter in plants (Aloni, 1987). This is also demonstrated in cut chrysanthemum flowers (Nijsse *et al*., 2001b). It explains the interaction between vase water height and cutting height (Fig. 9). Another reason of the water height effect on recovering could have been water uptake via the stem epidermis in contact with water. Van Doorn (1994) showed with roses that stems could take up water by exposure of more than about 60 mm^2 of xylem wall surface to water by girdling. The absence of air emboli is suggested to be a prerequisite for a positive water balance during vase life of cut flowers (Durkin, 1979; Van Meeteren, 1992). Van Doorn (1990) concluded that the presence of air in the lumen of the xylem elements in itself is not an obstacle to subsequent water uptake. In his experiment, however, the aspiration of air into the stem was measured and not the presence of air. The air could be (partly) removed from the vessels after replacing the flower stems in water.

We conclude that anatomical differences in xylem structure, especially vessel diameter distribution, play an important role in rehydration capability of cut flowers after air aspiration. During plant growth, new vascular tissues develop continuously in dynamic relationships to one another. This continuous development of new vascular tissues enables regeneration of the plant and its adaptation to interruptions and changes in the environment (Aloni, 1987). Therefore, it may be expected that differences in rehydration abilities will exist due to variations in growth conditions in the preharvest phase of cut flowers. Moreover, it can be expected that rehydration treatments will have different outcomes using different genotypes of flowers or even different lots of the same flower cultivar, as treatments will largely interact with xylem anatomical properties.

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5.2

Characterisation of stem hydraulic architecture in relation to embolism repair

J. Nijsse, H. van Gelder, U. van Meeteren

Abstract

Early leaf wilting in chrysanthemum (*Dendranthema* x *grandiflorum* Tzvelev) cut flowers has previously been related to the occurrence of emboli in cut open xylem vessels. Stems with a more compact vessel system (short, narrow, and densely connected vessels) are in theory less vulnerable to early leaf wilting. The possibility of an easy characterisation of the vascular anatomy was investigated, in order to come to a reasonable prediction of the vulnerability to embolism. Fresh weight restoration on vase after a dehydration treatment was measured in different experiments. Within the same batches, stem segment conductivity before and after induction of emboli was measured and several dimensions of the wide xylem vessels (with a cross-sectional area larger than 700 μm^2) and stem tissues were analysed. Xylem vessel length distributions of four cultivars were compared. A clear relationship between restoration of conductivity and several anatomical parameters was found. However, no single relation exists for the relation of one anatomical characteristic and restoration of conductivity over all experiments. Even without information of absolute influences of xylem anatomical properties, the results indicate that breeders and growers can optimise these properties to obtain chrysanthemums that are less vulnerable to embolism. Xylem thickness and some other tissue characteristics are easy to determine and may reflect some characteristics of the stem anatomy and the vulnerability to air embolism.

Introduction

Different species of cut flowers can show a fairly different keepability on the vase. Within species, different cultivars also can show considerable differences of vase behaviour (Van Meeteren, 1989). Natural flower senescence is often not the reason for termination of vase life, because of premature water shortage due to problems as disruption of water uptake by bacterial plugging, wound reactions or air emboli (Van Doorn, 1997). Other problems that shorten vase life are *Botrytis* infections, disruption of stomatal closure, decolouring of flowers or leaves, etc. In chrysanthemum air embolism of xylem vessels often causes a problem called 'early leaf wilting' (Van Meeteren, 1989, 1992; Van Meeteren and Van Gelder, 1999; Nijsse *et al*., 2000). Air aspired at the cut surface blocks the water uptake into the xylem vessels, resulting in

wilting of the leaves within the first hours or days of vase life. In intact plants air normally cannot enter xylem vessels, only in case of cavitation at a high water tension (Zimmermann, 1983). In contrast, a relatively weak sub-atmospheric pressure in the xylem is needed to enable air entrance into vessels that have been cut open (Van Ieperen *et al*., 2001a). Air aspired at the cut surface only fills the cut open vessels. Air cannot move into intact vessels, for the air-water meniscus cannot pass the membranes of bordered pits under normal postharvest conditions (Nijsse *et al*., 2001a). Embolism of (a part of) the cut open vessels often occurs. Vase behaviour is strongly dependent on the rate and level of recovery from this blockage of the water transport (Van Meeteren and Van Gelder, 1999). Van Ieperen and co-workers (2001b) developed an explanatory model of the recovery from air emboli at the cut surface of cut flowers. Two inter-related processes play a role during the restoration of hydraulic conductivity at the onset of vase life: 1) Initial uptake of vase water and redistribution of air in the xylem vessels. 2) Dissolution of the thus trapped air to the surrounding. In both processes, the driving forces are capillary forces and the pressure of the vase water. Both processes are strongly dependent on the anatomy of the water transport system. The influence of several anatomical parameters on the dynamics of emboli is summed below. We used the term ‘vessels’ for both xylem vessels and tracheids.

-xylem vessel diameter

According to the Hagen-Poiseuille equation, the conductivity of a vessel is related to the fourth power of its diameter. As a consequence, wide xylem vessels can transport much more water than narrow vessels. If a wide vessel is inactivated by embolism, much more conductivity is lost than in case of inactivation of a narrow vessel. Hence, a stem having narrow vessels is less vulnerable to embolism than a stem having wide vessels with the same total conductivity (Zimmermann, 1983).

The capillary force, which prevents air to enter a cut open vessel, depends strongly on the diameter of the vessel. The wider the vessel, the weaker the capillary force, and thus, the lower the xylem tension needed to overcome the capillary force. This makes the wider vessels at the cut surface more susceptible to air entrance (Van Ieperen *et al*., 2001a). When a cut flower is placed on the vase after uptake of air, the vase water will rise higher in the narrower vessels due to the stronger capillary force. More capillary rise results in more hydraulic connections to the intact xylem and a higher pressure of the entrapped air, which in turn results in a better dissolution of the entrapped air (Van Ieperen *et al*., 2001b). Wide vessels have a larger internal volume per length unit. Wide vessels therefore can take up relatively more air than narrow vessels. Dissolution of air out of wide vessels is more difficult, for the diffusion area (the vessel wall) is small per volume of air. Shortly, stems with relatively wide vessels are in all aspects more vulnerable to air embolism than stems with less wide vessels.

-xylem vessel length

The vessel length determines the height of air entrance, for, as stated before, air normally cannot move from a cut open vessel to an intact vessel. Most consumers recut the stem before placing the flower on vase. If a xylem tension is not present, or if the recutting occurs under water, the new cut surface will contain a certain amount

of vessels that are not embolised. This amount is dependent on the length of the vessels. The shorter the vessels, the shorter the stem segment that has to be cut off to obtain a certain percentage of water containing vessels at the cut surface. Further, the longer the vessels, the more air can be taken up (at same diameters). As is true for wide vessels compared to narrow vessels, embolisation of long vessels inactivates a larger volume of the vessel system than embolisation of short vessels does. Additionally, inactivation of a longer vessel causes a greater risk for isolation of a part of the vessel network. Shortly, longer vessels increase the vulnerability to air embolism in cut flowers.

-connectivity of xylem vessels

Van Ieperen *et al*. (2001b) showed that the restoration from emboli is influenced by the way of clustering of vessels. The dissolution of entrapped air will occur faster if a water transporting vessel is located besides the embolised vessel, for the surroundings will saturate slower because of the air dissolution into the water transporting vessel. If two embolised vessels are inter-connected with bordered pits, air can flow to the vessel with the lowest air pressure. This occurs in neighbouring embolised vessels with different diameters. The narrower vessel will force its air into the wider vessel due to the stronger capillary force in the narrower vessel. If the narrower vessel is shorter than the wider one, the narrower vessel will completely refill with water. Further, the connectivity of the xylem vessel network defines in how far inactivated vessels can be by-passed. So, the more connected, the easier the removal of emboli.

-diffusion pathway

If trapped air is dissolved into the surrounding tissues, this air ultimately has to be transported to the atmosphere. The thicker the xylem, the longer the diffusion pathway to the atmosphere, the lower the driving pressure gradient. Thus, a thinner xylem provides an easier dissolution of entrapped air (Yang and Tyree, 1992). In mature chrysanthemum stems the phloem is relatively thin, and the cortex too. Of course, the thicker these tissues, the longer the diffusion pathway. However, the cortex has air spaces, which are in contact with the atmosphere by stem stomata. These air spaces shorten the diffusion pathway. Whether the aerial parts of the pith can take up entrapped air from the vessels is not clear. Theoretically, the air in the pith may be slightly pressurised by the vase water pressure.

Thus, a compact vessel system (short, narrow, densely connected vessels) is less vulnerable to embolism related problems than a vessel system with long, wide and loosely connected vessels. Preliminary experiments have pointed out that the anatomy of the vessel system may vary between cultivars and batches of cut chrysanthemums. Even within a chrysanthemum stem there exists a gradient with wider and longer vessels at the base to narrower and shorter vessels higher up the stem (Nijsse *et al*., 2001b). It is known that higher cut chrysanthemum flower stalks have a higher restoration of fresh weight after desiccation than similar grown and treated lower cut stalks (Van Meeteren, 1989; Van Meeteren and Van Gelder, 1999). It would be useful to be able to compare the hydraulic architecture of different cut flowers in a fast and easy way. Then the xylem vessel anatomy could be monitored

and optimised during breeding and cultivation and the anatomy could be checked to predict the susceptibility to embolism. Until now no such analyses exist. In literature few quantitative comparative studies on xylem anatomy are found (e.g. Darlington and Dixon, 1991; Lovisolo and Schubert, 1998; López-Portillo *et al*., 2000; Vander Willigen *et al*., 2000; Nijsse *et al*., 2001b). But none is both easy to perform and accurate enough to distinguish (slightly) different anatomies in an objective manner. This paper is meant to investigate the possibility of a relatively easy determination of differences in xylem vessel anatomy related to the vulnerability to embolism. The cut surfaces of stems with a known vulnerability to early leaf wilting (i.e. embolism) were analysed on the amount, conductivity, and size distribution of the widest xylem vessels. Xylem thickness and some other tissue dimensions were measured on the same samples, in order to study differences in dissolution pathways. Diversity of vessel length distributions was studied in other batches.

Materials and methods

Plant material and 'treatments'

Chrysanthemum (*Dendranthema* x *grandiflorum* Tzvelev) plants were grown in a greenhouse of Wageningen University. Unless other stated the cultivation was as described by Van Meeteren and Van Gelder (1999). Rooted, 0.05 m long cuttings were transplanted into pots of 14 cm diameter. Total growth period from transplanting the cutting until harvest was about 12 weeks. Several experiments were conducted, within which always two treatments were compared.

-**different cutting heights**. Three identical experiments with cutting heights at 10 and 25 cm above soil surface, using cultivar Cassa:

H1, harvested at 11th May 1999
H2, harvested at 28th May 1999
H3, harvested at 10th June 1999

-**different cultivation conditions.** Two experiments on light conditions:

Cond1, in growth chambers with high light (HL; 68 W/m^2) and low light (LL; 34 W/m^2) conditions, using cultivar Cassa with the cutting height at 15 cm above soil level.

Cond2, in the greenhouse without shading (100%) and with shading to 40% of the natural light, using cultivar Reagan with the cutting height at 15 cm above soil level, harvested at 21 july 1999.

-**different cultivars** (VAR). Two simultaneously grown cultivars: Super Yellow (SY) and Vyking (Vy), with the cutting height at 15 cm above soil level, harvested at 10th november 1999.

Hydraulic conductivity measurements on stem segments were carried out to determine the initial conductivity (without embolism) and subsequently the restoration of conductivity after air entrance via the cut surface. After the conductivity measurements the lower parts of the stem segments were stored in 80% ethanol/water to enable microscopic analysis later on. Within the same batch of flowers the rehydration capability during early vase life was determined. In this study, material was only used from experiments in which the early vase life showed

significant differences between treatments. Vessel length analyses were carried out on batches without conductivity measurements or vase experiments. Vessel length distributions of four cultivars (Super Yellow, Reagan, Vyking, Cassa; eight stems of each cultivar) were compared at 15 cm above soil level.

Determination of rehydration capability during early vase life

For each experiment the vase behaviour was tested on 12 flower stalks per treatment, as described by Van Meeteren and Van Gelder (1999). The fresh weight of the harvested and pre-treated cut stalks was measured. Then the stalks were kept in air to lose 5% of their initial weight (in about 1 hour). After this desiccation treatment the stalks were placed in standard vase water, which contained an aqueous solution of sodium bicarbonate (1.5 mM), calcium chloride (0.7 mM) and copper sulphate (5 µM) at room temperature (20±2 °C; for details see Van Meeteren *et al*., 2000). After 24 hours the restoration of the fresh weight was measured and expressed in percents of the initial fresh weight per hour. Note that the determination of vase behaviour was necessarily on other stems (though of the same batch) than the stems used in the analysis of hydraulic conductivity and anatomy.

Hydraulic conductivity measurements

The hydraulic conductivity of the stem segments was measured as described in Van Ieperen *et al*. (2000). In short, 20 cm stem segments were cut out under water at the desired height in the stem (except for Vyking, of which 30 cm long segments were used, because of its long vessels). The initial hydraulic conductivity (*CS*) was determined by measuring the flow rate (*q*) of vase water through the stem segment with length *x* at a known pulling pressure (ΔP, 40 kPa):

$$CS = \frac{q * x}{\Delta P}, \text{ (in µmol s}^{-1}\text{ kPa}^{-1}\text{ m)}$$

Then the segments were lifted out of the vase water for three minutes, still subjected to the pulling pressure, to induce air aspiration at the cut surface. The segments were placed back in the vase water and, after one hour, the restoration of the conductivity was recorded in percents of the initial conductivity.

Xylem vessel diameter analysis

Transversal sections (40 µm thick) were made with a sledge microtome from the lower cut planes of the stem segments. The sections were embedded in Kayser's glycerol gelatine (Merck 9242, Darmstadt, Germany) on microscope slides. Digital images were made with a Sony 3CCD camera mounted on a light microscope (Leitz Dialux), giving a resolution of 2.9 µm per pixel. About 10 images were needed to photograph the entire xylem area of one transverse section. Using digital image analysis (SCIL_Image 1.3, University of Amsterdam, Faculty of Mathematics and Computer Science, Amsterdam, The Netherlands), the wide xylem vessels were selected and measured on area and longest and shortest axis of the superimposed ellipses. Vessels and other xylem constituents with a cross-sectional area smaller than 700 µm^2 were automatically discarded. The remaining cell-lumina all were xylem vessels; other constituents such as fibres and parenchyma cells were always smaller. Tissues other than xylem (e.g. pith) were manually discarded from the

photographs. Thus from each image, and consequently from each section a list with morphological data was made of all vessels with a cross-sectional area larger than 700 µm^2. The hydraulic conductivity (*CS*) per vessel was calculated according to the formula given by Pickard (1981):

$$CS = \frac{\pi}{4\eta}\frac{a_>^3 a_<^3}{\left(a_>^2 + a_<^2\right)}$$

where $a_>$ and $a_<$ are the semimajor and the semiminor axes of the ellipse and η is the viscosity of the liquid (1.00*10^{-3} Pa s for water at 20°C). For further information on calculations of conductivity on cross-sectional areas, see Nijsse *et al*. (2001). The hydraulic conductivity of all wide vessels in a section was calculated as the sum of the conductivities of the individual vessels, and expressed as a fraction of the measured initial conductivity. The vessels wider than 700 µm^2 were counted and the number of vessels wider than 1000 µm^2 was expressed as fraction of all vessel wider than 700 µm^2, in order to get a quantitative indication of the relative amount of the widest vessels.

Tissue area analysis

The transverse sections made for the vessel diameter analysis were photographed as a whole at low magnification. With the use of SCIL-Image the contours of three areas were marked: area of the whole section (WHOLE), the area surrounded by the cambium (XYLPITH), and the pith area (PITH). From these selections the following areas were calculated:

Area of the whole stem = WHOLE
Area of cortex + phloem = WHOLE-XYLPITH
Area of xylem = XYLPITH-PITH
Area of pith = PITH

The thus determined areas were expressed as fractions of the area of the whole section.

The average thickness of the xylem was determined automatically: First a binary image of the xylem was acquired (the binary image of XYLPITH minus PITH). A skeleton was calculated (the points in the midst of the xylem ring for which the distance to the cambium was the same as the distance to the pith edge). The binary image was processed to a distance-transform image (each pixel in the binary image got the value of the shortest distance to a non-object pixel). For all skeleton-points the shortest distance to the edge of the xylem was thus determined and averaged. The average xylem thickness was defined as the double of the found average distance. The thickness of the combined cortex + phloem was determined with the same method.

Vessel length analysis

Stem segments with a length of 30 cm were cut out under water. The upper part of the segments was attached to a vacuum system (ΔP = 50 kPa), while the lower part was placed in an aqueous red latex solution (1% w/v). Latex particles can easily move through vessels, but are far too big to pass inter-vessel pits. After overnight uptake of the latex solution, the particles completely clogged (and thereby coloured) the cut open vessels. Out of every internode two transverse sections (2 mm thick)

were cut out and photographed with a digital camera mounted on a microscope. Using the SCIL_Image image analysis program, the amount of red-coloured vessels was quantified as function of the distance to the cut surface (modification of the method of Zimmermann and Jeje, 1981). The vessel length distribution was described with its half-length value. This half-length value is the length of a stem segment within which half of the vessels end (Nijsse *et al*., 2001b).

Statistical analysis

Statistical analysis was done with a Student *t*-test at P = 0.05.

Results

In Table 1, the averages are presented on the fresh weight changes during the first day of vase life, with the related experiments on hydraulic conductivity, vessel characteristics, and tissue dimensions. Note that all vase life experiments were carried out with 12 stems per treatment and that the other analyses were repeated as indicated in the last column. The results of vase behaviour differed significantly (+) within each experiment, for this significant difference was the selection criterion to start the other analyses. For all comparisons between treatments, it has been indicated whether the difference was as expected (exp) or not expected (nexp) and whether the difference was significant (+) or not (-) at P = 0.05. The expectations were based on the theory presented in the introduction, and, concerning the initial conductivity and number of vessels, on practical evidence. For each type of measurement was counted how often the expected difference was found and how often this was significant. Clearly, some types of measurements scored better than others did. Only one not-expected significant difference occurred: at comparison of the transverse stem area for the two heights in experiment H2 (in bold). The easy comparisons on xylem thickness and on the fractions of the pith area and xylem area were as accurate as the much more laborious comparisons on initial conductivity, restoration of conductivity, and the number of vessels >700 μm^2.

In Table 1, the comparisons were done only within experiments. Figure 1 shows the relation between xylem thickness and the restoration percentage of the conductivity of the segments over all experiments. This figure makes clear that no single relation between xylem thickness and restoration of conductivity (or fresh weight restoration on vase) can be drawn. The other analyses have a similar cloudy relation with restoration of conductivity and with the fresh weight restoration on vase (not shown). The curve represents a fitted ‘top-bottom’ exponential function on the results of experiments in which the cultivar Cassa was used (H1-H3 and Cond1). This function was chosen because of its assumed functional meaning (see discussion). The residuals of the fit have a normal distribution and R^2 is 30%.

Figure 2 shows the xylem half-length values for four cultivars, all at 15 cm above the soil surface. Super Yellow has shorter vessels than the other cultivars. From table 1 appears that this cultivar has less wide vessels than Vyking, both absolute (number of vessels >700μm^2) and relative (fraction of vessels >1000/>700 μm^2).

experiment	'treatment'	fresh weight restoration (%/hour;1st day)		initial conductivity ($\mu mol.s^{-1}.kPa^{-1}$ m)		restoration of conductivity (% of init. cond		number of vessels >700 μm^2		fraction of vessels >1000/>700μm^2 (%)		fraction of conductivity calc/meas (%)		xylem thickness (mm)		phloem+cortex thickness (mm)		transverse stem area (mm^2)		area fraction phloem+cortex/stem (%)		area fraction xylem/stem (%)		area fraction pith/stem (%)		number of analysed stems	
H1	**10 cm**	**0,165**	0,568	0,568	53,8	284	0,306	0,98	0,49	0,27	33,3	16,2	30,3	53,5	4	4	
	25 cm	**0,241**	0,270	0,270	69,8	152	0,209	1,03	0,35	0,26	30,9	16,3	25,0	58,7	4	4	
		+	exp/+	exp/+	exp/+	exp/+	exp/+	nexp/-	exp/+	exp/-	exp/+	nexp/-	exp/+	exp/+			
H2	**10 cm**	**-0,193**	0,551	0,551	50,3	220	0,307	0,77	0,64	0,29	23,4	20,5	39,3	40,1	4	4	
	25 cm	**0,167**	0,316	0,316	56,5	116	0,204	0,63	0,43	0,23	27,1	15,6	30,0	54,4	4	4	
		+	exp/+	exp/+	exp/+	exp/+	exp/+	exp/-	exp/+	exp/+	**nexp/+**	exp/+	exp/+	exp/+			
H3	**10 cm**	**-0,090**	0,549	0,549	45,0	253	0,282	0,92	0,49	0,28	30,7	17,5	31,6	51,0	4	4	
	25 cm	**0,138**	0,320	0,320	59,5	158	0,214	0,90	0,39	0,28	33,4	17,2	26,2	56,6	4	4	
		+	exp/+	exp/+	exp/+	exp/-	exp/-	exp/-	exp/+	nexp/-	nexp/-	exp/-	exp/+	exp/+			
Cond1	**HL**	**-0,178**	0,317	0,317	54,3	185	0,248	1,13	0,42	0,28	33,5	16,7	26,5	56,8	4	4	
	LL	**-0,100**	0,233	0,233	59,8	105	0,238	0,86	0,31	0,25	25,3	17,6	23,2	59,2	4	3	
		+	exp/-	exp/-	exp/+	exp/-	exp/-	exp/-	exp/+	exp/-	exp/-	nexp/-	exp/-	exp/-			
Cond2	**40%**	**0,176**	0,504	0,504	70,0	158	0,244	0,57	0,62	0,30	30,2	18,5	36,0	45,5	3		
	100%	**-0,096**	0,950	0,950	55,7	405	0,324	0,75	0,79	0,40	50,5	19,2	36,6	44,2	2		
		+	exp/+	exp/+	exp/+	exp/+	exp/-	exp/-	exp/-	exp/+	exp/+	exp/-	exp/-	exp/-			
Var	**SY**	**0,306**	0,300	0,300	70,4	130	0,189	0,75	0,42	0,38	27,2	24,7	27,8	47,5	5		
	Vy	**0,209**	0,617	0,617	65,2	424	0,381	1,49	0,60	0,43	31,0	26,0	33,3	40,8	6		
		+	exp/+	exp/+	exp/-	exp/+	exp/+	exp/+	exp/+	exp/+	exp/+	exp/-	exp/+	exp/+			
			laborious	laborious	laborious	laborious	laborious	laborious	easy	easy	easy	easy	easy	easy			
exp/+				5		4		3		1		3		1		4	
exp/-				1		2		3		4		2		3		2	
nexp/-				0		0		0		1		1		2		0	
nexp/+				0		0		0		0		1		0		0	

Table 1. (left) Comparisons of 'treatments' of cut flowers on fresh weight restoration on vase, stem segment conductivity, several stem xylem vessel anatomical properties, and several stem tissue properties. For all comparisons between treatments, it has been indicated whether the difference was as expected (exp) or not expected (nexp) and whether the difference was significant (+) or not (-) at P = 0.05. Below is indicated how often the different outcomes occur and whether the measurement was laborious or easy to perform. Vase life determinations were always carried out on 12 flowers per treatment. The repetitions of all other measurements are indicated in the last column.

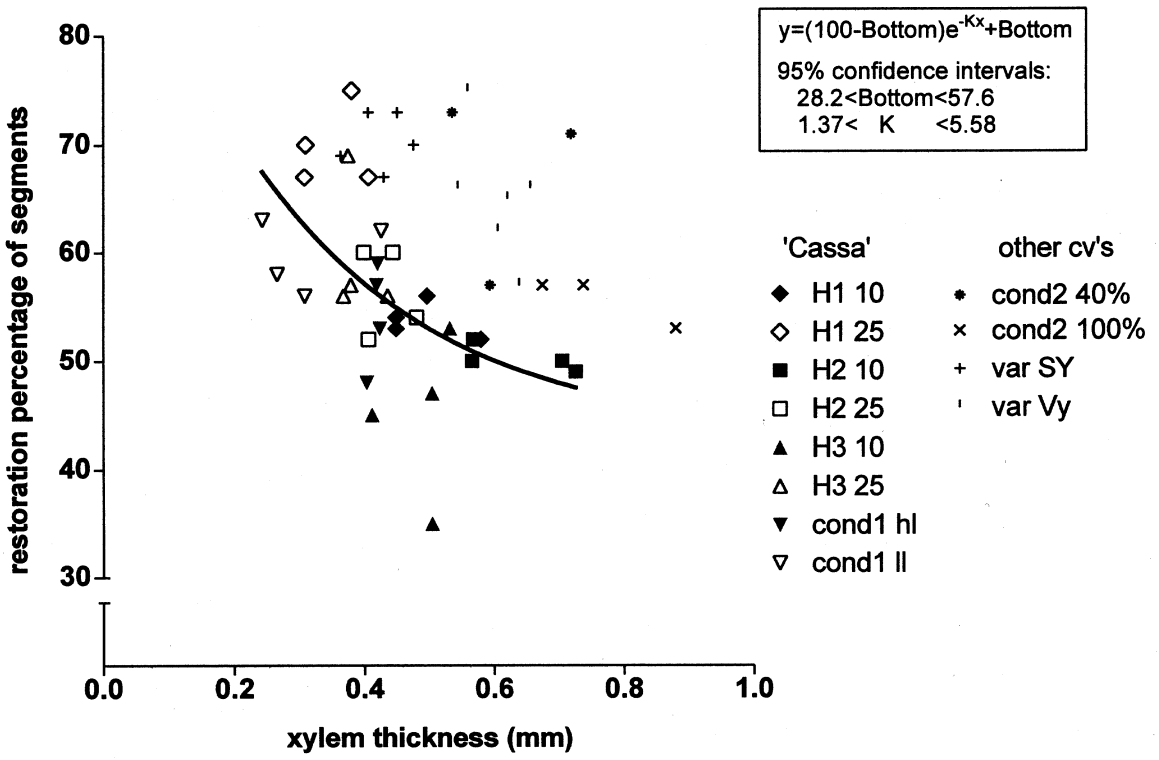

Fig. 1. Relation of xylem thickness and the restoration conductivity in the same stem segments. Different symbols represent different treatments, as indicated in the legend; the open en closed forms of the same symbol represent the two treatments within one experiment. The curve is a 'top-bottom' exponential fit to the data of the cultivar Cassa (H1-H3, and Cond 1).

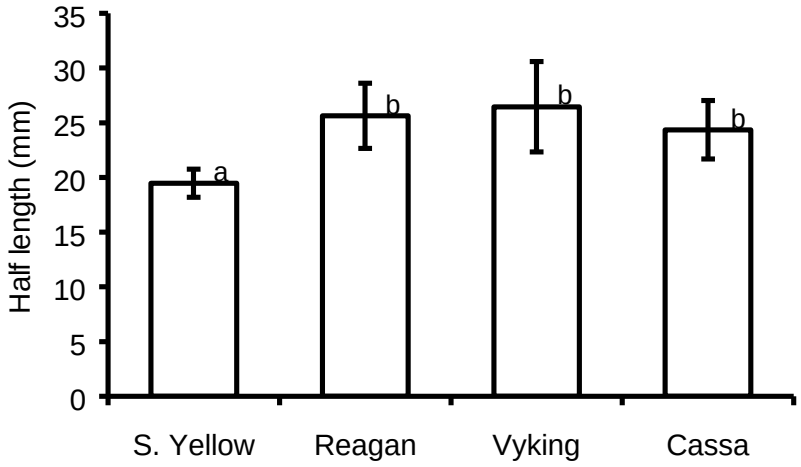

Fig. 2. Xylem vessel length distribution expressed as half-length value, for four cultivars at 15 cm above the soil surface. Bars indicate the standard deviations of the means (N = 8). Half-length values marked by the same letter are not significantly different at P = 0.05.

Discussion

Within experiments, stems with a higher restoration of fresh weight also had a higher restoration of conductivity. This implies that the early vase behaviour indeed is related to the stem properties, for the conductivity measurements were carried out on stem segments without leaves and flowers. Within experiments, the restoration of fresh weight was always higher in the stems with the lower initial conductivity. This was also found in previous experiments (unpublished). A high initial conductivity can be caused by wider vessels, but also by a higher number of vessels. And a higher number of vessels can not directly be associated with a higher susceptibility to early leaf wilting. Thus, in theory the initial conductivity is not a clear predictor of early vase behaviour, but we did find a compelling relationship on several occasions. In theory the fraction “vessels >1000 μm^2/>700 μm^2“ is a good internal standard to estimate the vessel diameter distribution. A high value of this fraction suggests that the vessel diameter distribution tends to wider vessels. In our results this fraction was often not significantly different in the comparisons within the experiments. This may be caused by the fact that the fraction is calculated from two experimental results: the number of vessels larger than 700 μm^2 and the number of vessels larger than 1000 μm^2. Both determinations have a certain variance, caused by measurement 'errors' and by natural heterogeneity, and the combination of these determinations implies a larger variance. The same problem of high variance is true for the fraction of the calculated conductivity on the measured conductivity. Sometimes this latter fraction is higher than 1, which seems strange. But the measured conductivity is determined on stem segments of 20 cm, whereas the calculated conductivity is determined on the lower

cut surface of these segments. It is known that lower in the stem the conductivity is always higher. Further, the calculated conductivity is based on the conductivity of the vessel lumina, but the intervessel connections are not taken into account. Over 20 cm the water has to pass several intervessel pits, which decrease the overall conductivity. The third reason for the relatively high value of the calculated conductivity may be inaccurate measurements of the vessel diameters, even in case of a normal distribution of this inaccuracy. Overestimations of diameters have a larger impact on the calculated conductivity than underestimations, due to the fourth power relation of diameter and conductivity. So neither the fraction "vessels >1000 μm^2 / >700 μm^2" nor the fraction "calculated conductivity / measured conductivity" appears to be a useful predictor of the vulnerability to embolism, although both are theoretically sound.

Though only carried out on the wide vessels, the analysis on individual vessels is still laborious and is not suited for daily use by breeders, growers, and traders. In contrast, the analyses on tissues are easy and rapidly performed. Within experiments, a larger xylem thickness was always related to a lower restoration of conductivity. Using xylem thickness measurements as test for the vulnerability to embolism seems therefore to be both simple and accurate. The measurements of thickness of xylem and other tissue characteristics could possibly be carried out in an even easier and non-destructive way, e.g. by analysing an X-ray projection of the stem. The comparisons on "xylem thickness", "area fraction xylem/stem", and "area fraction pith/stem" followed nicely the expectations. It is often suggested in practice that stem thickness and the vase behaviour of chrysanthemum cut flowers are negatively correlated. This is at least not true in all cases. The stem consists mainly of the xylem and the pith. From our results appears that a larger xylem area is an indicator of a larger susceptibility to embolism, while a larger pith is an indicator of lower susceptibility to embolism. This may be the reason that stem diameter or area is not a good indicator of early vase behaviour. The comparisons on thickness and relative area of "phloem+cortex" were simply carried out because they did not demand additional efforts. However, as discussed in the introduction, the role of the phloem and the cortex on the restoration of emboli could be minor. The results of the measurements on phloem and cortex provide no additional information.

The vessel length measurements indicate that vessel length distributions can vary significantly between cultivars. It is also known that vessel length decreases at increasing heights in the stem of chrysanthemum (Nijsse *et al*., 2001b) as well as other species (Zimmermann and Potter, 1982). No experiments were carried out to directly relate vessel length to early vase behaviour. However, from literature (Van Meeteren, 1989; Van Meeteren and Van Gelder, 1999) and from our results is clear that higher cut flowers have a higher restoration of fresh weight at vase life. Furthermore, in our experiments, the cultivar Super Yellow, which had relatively short vessels, had a better restoration of conductivity and fresh weight on the vase than the cultivar Vyking. A positive relationship between diameter and length of vessels is often found (Nijsse *et al*., 2001b; Zimmermann, 1983). A vessel length determination is less laborious than analyses on vessel diameters. Analysis of the vessel length distribution may therefore be useful to check the susceptibility to early leaf wilting, not only because of the theoretical influence of vessel length, but also because of the correlation to vessel diameter. On the other hand, vessel length can only be

determined on fresh, non-embolised stems, because inactivated vessels do not take up the latex paint. A vessel length determination is therefore only possible at harvest and certainly not after dry storage.

As depicted in the introduction, neighbouring vessels influence the recovery from emboli. It is not known whether the network of vessels can vary as result of variation in genotype, position in the plant, or growing conditions. We did not study the 'connectivity' of vessels. As far as we know, no method exists to distinguish different 'connectivities' of vessels between different batches of stems.

We found a clear relationship between restoration of conductivity and several anatomical parameters. However, the relationship of anatomy to restoration of conductivity (and fresh weight on vase) is not absolute, but relative. Lots of other plant properties and internal and external processes influence the water balance. Some influences might be: the quality of the vase water, the vapour pressure deficit, the temperature and the radiation during the vase life experiments (which are tried to be kept stable), physiological dynamics in the stem as solute balances and surface properties of vessels, dynamics of the stomata, etc. Because of other influencing factors, and probably also because of interaction of different anatomical parameters, an accurate prediction of restoration of embolism only based on one anatomical aspect of the stem is not possible. Possibly a multivariate analysis might help to find the most important (inter-related) factors that define the recovery from embolism. Within one cultivar the relation of xylem thickness and restoration of hydraulic conductivity is much clearer than over several cultivars. We fitted a ‘top-bottom’ exponential equation over the results of the experiments with the cultivar Cassa (Fig. 2). Although having a rather low R^2, this function could have a functional meaning. Xylem thickness is associated with the length of the dissolution pathway from the entrapped air to the atmosphere. Without dissolution (e.g. when the xylem is very thick), still some vessels will be activated on rehydration, caused by the first capillary water uptake and the resulting redistribution of emboli. This restoration of conductivity occurs independently on xylem thickness and is represented by the bottom-value in the fitted equation. The top-value will be 100% at an imaginary xylem thickness of zero. The driving force for dissolution of the entrapped air (i.e. the air-pressure gradient) shows a decay over the xylem thickness. That different genotypes can have a different bottom value (the restoration independent on dissolution), due to vessel anatomical characteristics and physiological properties, may explain that each cultivar has its own relation of xylem thickness on restoration of conductivity.

Breeders now at least can select varieties with a more compact xylem vessel system. Growers should balance the growing conditions to compromise production rate and xylem anatomy. Xylem thickness and some other stem tissue dimensions seem to be good indicators of the quality of the xylem vessel system and its vulnerability to embolism.

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Chapter 6

General discussion

J. Nijsse

Introduction

This chapter is meant to provide a view on the several fields of research covered in this thesis. It must be noted that the contents of this chapter are clearly no resume of the preceding chapters. The next three paragraphs each discuss a field of research and suggest for further research. The chapter finishes with conclusions and some other interesting matter.

Functional anatomy and explanatory modelling of anatomy

To understand the flow of xylem sap in plants a detailed knowledge of the xylem anatomy is indispensable. This knowledge is not only needed to understand flow in intact plants, but also to study the impact of dysfunctioning of parts of the xylem. As clearly shown in this thesis, the impact of air entrance at the cut surface into the xylem of cut flowers depends heavily on the stem xylem anatomy. The length of vessels determines the extent (i.e. the height into the stem) of air entrance. The volume of the vessels determines the amount of air that can be taken up. The width of the vessels is very important during the refilling process. Narrow vessels refill easier than wide vessels. The needed detailed knowledge of the xylem hydraulic architecture should ideally consist of an (explanatory) model that describes all hydraulic properties of the vessel network. These are at least: dimensions and localisation of individual vessels, vessel wall properties, and the connectivity (in terms of water transport) of the vessels. Moreover, the role of the surrounding tissues should be known. An explanatory model of the xylem hydraulic architecture is just a part of an imaginary whole plant model, in which all functions of the plant are described.

The research, as described in this thesis, provides promising new tools both for investigating embolism problems and for further anatomical research. The presented vessel length theory, although not yet validated as the ultimate explanation, gives new fundamental insight into vascular morphogenesis. Another more practical result was the detailed analysis of cross-sectional vessel characteristics. The resulting calculation of conductivity was not entirely new, but unique in its deep detail and new in the integration of conductivity over the length in a non-uniform stem. The results from the anatomical work were implemented into the embolisms studies in this project.

As in most research, a great deal still has to be explored. Many questions that were formulated at the beginning or that arose during the project are still unanswered. A detailed scheme of the vascular bundle network was constructed, but the connectivity of the individual vessels is still unknown. In literature, only rough outlines are given. How many neighbouring vessels has a single vessel? Are there isolated strings of vessels within a vessel bundle? Zimmermann (1983) published some orienting work on wood samples, from which is clear that vessels are not ideal straight pipes. The vessels 'wander' somewhat if visualised in serial sections. But a quantitative description, which is needed to understand the water flow through the vessel network, is missing. The three-dimensional shape of a vessel also remains still uncertain. Do vessels taper at their lower and upper end as has been suggested by Zimmermann (1983)? How does the cross-sectional shape of a vessel vary along its length?

Another important field of research to be done is the comparative anatomy. How does the xylem anatomy of one cultivar differ from the other? Or, what is the influence of a certain change in growing conditions on the width of xylem vessels? To answer these questions a clear description of the anatomy is needed in order to distinguish changes or variations in anatomical properties. In chapter 5.2 we tried to compare the anatomy of the xylem vessels of different genotypes or batches. As became clear in this work, it is possible to trace anatomical differences between different chrysanthemums or at different heights in the plant. However, it is clear that comparative anatomy still needs development.

Digital image analysis becomes increasingly available for non-specialists. In addition desktop computers become more and more powerful. As a consequence, analysis of anatomical data nowadays is less laborious. Even a detailed three-dimensional analysis of the xylem system is practically possible, though still laborious. At this moment no three-dimensional reconstructions of entire vessels have been found in literature. A detailed 3D-reconstruction of a chrysanthemum stem segment would yield interesting results as: the shape of vessels and the connectivity of vessels. In my opinion the whole plant anatomy will be served by such a 3D-reconstruction.

Another approach to reconstruct the three-dimensional architecture of the xylem vessels and their network needs attention. Besides of mapping the hydraulic architecture of plants, a virtual xylem vessel network should be built and optimised. From the results in this thesis and other anatomical investigations an idea has to be formulated on the dimensions of vessels and how they are interconnected. As discussed in chapter 2.3, vessel length distributions might be described by simple exponential functions, which in turn point to a stochastic vessel length determination. As explained in that chapter, the genetic regulation of such a distribution is relatively simple. It would be fruitful to assume similar regulatory mechanisms for other anatomical properties as vessel diameter and connectivity between vessels. Stochastic regulation does not result into strict shapes and dimensions. The plant does not determine the properties of the individual vessels, but rather provides steerable chances for certain morphogenetic processes. Regulation of these chances enables the plant to produce the vascular system that is required at the given conditions. In the field of plant anatomy, which always has been more descriptive than explanative, this vision seems to be speculative. It is true that from the results

presented here no stochastic regulated vessel architecture can be concluded. There are, however, several facts that point to stochastic regulation in nature (and more particular in vessel architecture):

- It is hard to believe that the plant determines the exact shape and position of each cell or vessel.
- Highly ordered structures and completely identical structures are rare in nature. An example of a highly ordered structure is the helical leaf arrangement and the resulting vessel bundle network as revealed in chapter 2.1. At first sight this must be regulated by a very complex mechanism. But in fact, this ordered structure is a result of itself, as is the case with crystals. Once the meristem has started to develop the first leaves there is a limited space for next leaves on the meristem cone. Already in the early 1900s the several types of leaf arrangements were explained by the spatial restrictions on the meristem cones for newly formed leaf primordia (Reinders *et al*., 1943). This explains why chrysanthemums can have both right- and left-handed leaf orientations on different branches within one plant. The right- and left-handed orientations are equivalent and it 'just happens' in what direction the leaf helix is initiated. Thus, even in the highly ordered vessel bundle network, the regulation is not as strictly determined as it seems.
- Most structures have no strict dimensions, location, and shape. Cells within a tissue often have a foamy appearance, which is e.g. clear in cross-sections of the pith in chrysanthemum stems. In general, cells that are located more close to the vascular tissue are smaller than the cells in the stem centre. However, neighbouring cells can exhibit large differences in shape and dimensions. Physical foam can show the same properties. Physical foam can have fine cells in one case, or larger cells in another case, dependent on the environmental conditions or the inherent properties of the constituents. In other words, a biological tissue with certain structural properties can be formed by regulating the stochastic mechanism that rules the not strictly determined formation of individual cells.
- As described in chapter 2.3, the vessel length distribution in chrysanthemum stems can be explained by the mechanism of a steerable chance to fuse for individual vessel elements.
- Organisms grown under the same conditions and having the same genotype are completely different on the cellular level. On the other hand, these organisms can show great similarities at the macroscopic level, which in turn proves that these organisms have a strong ability to determine and regulate their final properties.
- No alternative seems to exist that explains the regulation of the morphogenesis in plants.
- The recently revealed Arabidopsis genome consists of only 119 million base pairs (Walbot, 2000), which is equivalent to about 30 Megabytes of information. The electronic version of this thesis is several times larger! This indicates that a relatively low amount of hereditary information is needed to encode for all essential characteristics and processes within a plant species. Therefore, the regulation of processes must be very densely programmed. The mechanism of stochastic regulation is probably the most simple type of regulation in nature.

Possibly other fields of research may help the plant anatomists (and biologists in general) to prove or falsify this theory of stochastic regulation and to model the

hydraulic architecture based on it. The assumption of stochastic regulation implies restrictions on vessel architecture: generally no strict structures can be expected. For example no population of vessels having all the same length can be expected. An individual vessel cannot have a fixed diameter or cross-sectional shape. Thus, breeders and growers of cut flowers should be aware that not every virtual hydraulic architecture is possible. It is the task of plant biologists to investigate the possibilities and plasticity of the stem hydraulic architecture. Recently Becskei and Serrano (2000) conducted an interesting experiment on a gene construct that coded for a fluorescent protein. The amount of proteins that were produced was different from cell to cell. However, if a negative feedback loop was constructed, the cells produced a much more homogeneous amount of proteins, though still with some spread. This little experiment is a nice example of the possibilities of stochastic regulation. No strict amounts of the fluorescent protein were determined by the gene construct, rather the distribution of produced amounts was regulated.

Starting with the need to come to a good (explanatory) description of the stem xylem anatomy in cut chrysanthemum, the discussion has drifted somewhat away. Stochastic regulation (or 'non-determined determination') may play an important role in regulatory mechanisms in living organisms. As far as I can see, stochastic morphogenesis is also common outside the living world, but stochastic regulation may only be restricted to the living organisms and the technical world (e.g. fuzzy regulation). This discussion about stochastic regulation seems to be novel, although several authors indirectly touch the subject (e.g. Becskei and Serrano with their experiment on the above discussed gene-construct). Now the complete genome of Arabidopsis is revealed, research will focus more and more on the functioning of genes and regulatory pathways. The near future may show the value of stochastic regulation.

As shown with the anatomy of the chrysanthemum stem, the hydraulic architecture is not homogeneous at the stem scale. Clearly a gradient was found for the dimensions of vessels: the higher in the stem the smaller. As postulated by Aloni and Zimmermann (1983) in the six-point hypothesis, an auxin gradient might be the cause for the gradient of the vessel dimensions along the stem. We clearly observed that main leaf traces close to the attachment of the corresponding leaf have narrower vessels than the other vessel bundles in the stem. A model of the stem hydraulic architecture should imply these (local) differences and gradients of vessel dimensions. Both the experiments and discussions presented in this thesis may help in developing a detailed explanatory model of the stem hydraulic architecture.

Embolism studies

Our cryo-SEM studies revealed that air only enters cut open vessels under normal post-harvest handling conditions (chapter 4.3), and that flower stalks which still have much air in the vessels after some hours of vase life show early leaf wilting (chapter 4.2). Wide vessels refill less or slower than narrow vessels (chapter 5.1). These cryo-SEM results sustain the theory of initial capillary rise and subsequent dissolution of air in xylem vessels, which both are stronger in narrow vessels. Most cryo-SEM studies could not be carried out with more than a few stems. However, all results are

in agreement with our theoretical explanation of induction and removal of air embolisms in cut chrysanthemum stems.

Cryo-planing has been optimised here to enable high resolution cryo-SEM on planed surfaces (chapter 3). Now most artefacts are due to the freezing procedure. The resolution of the acquired images depends mainly on the extent of ice crystals in the tissues. It is not sure what happens during the rapid freezing of chrysanthemum stems. Freezing a chrysanthemum stem takes several seconds, which means that redistribution of water cannot be excluded. Our recent experiments on longitudinally cryo-planed segments of recovering stems (not presented) sometimes show clearly a water film along the vessel wall over hundreds of micrometers. This means that along the vessel wall air dissolution can take place. From the cryo-SEM results it is not clear whether the water film flows or not.

Is the found water film in the vessels a result of redistribution of water during freezing? Recently, two contradicting papers appeared. In one paper (McCully *et al*., 2000), the cryo-SEM method to study embolisms was 'proved' to be reliable with a double-freezing experiment. In this experiment a part of an intact root was frozen under hydraulic tension, and a few seconds later a more distal part of the same root was frozen. The first part thus would have been frozen under tension, and the latter in absence of tension. It was found that the root segments contained the same percentage of embolisms. By this finding, the authors argued that freezing under tension will not cause additional embolisms. In the other paper (Cochard *et al*., 2000) it was argued that it is not sure whether the tension was absent in the latter frozen root segment of the double freezing experiment of McCully *et al*.. Directly after freezing the first part, the second, more distant part is indeed isolated from the transpiring plant. However, a water potential gradient between the xylem conduits and the soil will continue to exist until the hydraulic root capacitance is recharged. Thus, from the double freezing experiment cannot be claimed that the cryo-SEM method is reliable. Moreover, Cochard et al. found that the amount of embolisms found using cryo-SEM results only were consistent with other (indirect) measurement techniques if the hydraulic tension was removed prior to freezing. We must conclude from their findings that during freezing of a stem segment redistribution of water can take place in systems under tension. In our experiments no high hydraulic tensions were present and it is not to be expected that a part of the recorded emboli are freezing artifacts. It is however difficult to prove whether tiny water droplets or water films in vessels are present before freezing or that they are artifacts.

Several other problems still have to be solved. The air-water interface is still under debate, both in our work (chapter 4.1 and 5.1) and in literature (e.g. Zwieniecki and Holbrook, 2000). It is difficult to directly measure the contact angle of the air-water interface with the vessel wall. Refilling of cut open vessels depends heavily on the surrounding vessels. Are the neighbouring vessels transporting water, or are they embolised too? Further the longitudinal position and length and width of the neighbouring vessels are important factors. Some scenarios were worked out in this project. As far as we know no other researchers simulated the refilling processes in cut open vessels of cut flowers. This is partly due to the fact that the arrangement and shape of xylem vessels are largely unknown. Moreover, most embolism studies are carried out on intact xylem, in which cavitations (and the resulting embolisms) occur randomly through the wood (e.g. Yang and Tyree, 1992; Canny, 1997; Tyree *et*

al., 1999), and not in one plane as in cut flowers. And even in these studies the influence of the arrangement of neighbouring vessels has not been studied in detail. Again it appears necessary to have a good model of the three-dimensional architecture of the vessel network. Though, even with rather simple models nice results were obtained by simulating the refilling of vessels in several arrangements of neighbouring vessels (chapter 5.1). Besides the need for a better understanding of the anatomy of the vessel network, embolism studies still cope with many uncertainties. Do the surface properties of vessel walls change after a period of embolisation? Do the walls of older vessels differ from the walls of younger vessels? What is the role (of the shape) of intervessel pits during refilling of vessels? Are Zwieniecki and Holbrook (2000) right when they assume that refilling vessels keep their intervessel pits embolised till refilling of the vessel lumen has completed? This is particulary important for systems with a negative xylem pressure. What is the role of the living tissue along xylem vessels? Can neighbouring living cell actively refill the vessels as proposed by Canny (1995, 1997)?

Anatomy & vase life

We concluded in chapter 5 that chrysanthemums with wide and long xylem vessels are more vulnerable to early leaf wilting. Now the question arises how to grow chrysanthemums that possess a safe vessel system, consisting of many short and narrow vessels. Proper comparative anatomical research has to be carried out to answer this question. In literature some aspects are clear. Species occurring in regions with water stress or frost conditions tend to have more compact vessels as compared to species occurring in tropical regions without water stress (Zimmermann, 1983). Furthermore, if a plant is grown under conditions of water stress, the vessels are also more compact compared to the same genotype grown under mild conditions (Lovisolo and Schubert, 1998). From these general rules may be concluded that chrysanthemums that are grown under the best conditions (highest production) will have relatively wide and long vessels. Chrysanthemums grown under such high productive conditions may therefore be relatively sensitive to early leaf wilting. Some experiments (C. Slootweg, personal communication) point out that indeed chrysanthemums grown under so-called turbo-conditions are more sensitive to early leaf wilting than the same variety grown under more conventional (less productive) conditions. This possibly implies that improving the xylem anatomy (to prevent early leaf wilting) in chrysanthemum stems will cause a lower crop production rate.

The research presented here has not yielded the knowledge how anatomy is influenced by either genotype or environmental conditions. Hopefully this study may help to develop a better understanding of the formation of the vessel system. A thorough literature review on environmental influences on xylem anatomy of plants (which is still lacking) would help to design good experiments on the influence of the growing conditions on the stem xylem anatomy.

It must be considered that our research was focussed on air blockage in the cut open vessels in cut chrysanthemum. The water balance in cut flowers is also negatively affected by several other large problems as bacterial plugging, absence of stomatal closure, wound reactions at the cut surface and more (Van Doorn, 1997).

Other problems, not directly related to the water balance are *Botrytis* infections, shortage of carbohydrates, hormonal problems, and the ageing processes. For economical reasons the cultivation period needs to be as short as possible. A short growth period implies lower production costs, less chance for pests, and a faster reaction on the demands of the market. As already suggested, a fast growth period may cause wide and long vessels and thus a higher sensitivity to early leaf wilting. In short, a large number of inter-related aspects has to be dealt with. If one factor changes, all other factors may change too. Not only fundamental research is needed to reveal the underlying processes. Also thorough analysis of the sensitivity of the (related) aspects is needed to optimise both cultivation and vase life of cut flowers. As mentioned in the general introduction, the retailers and the consumers ask more and more for a guaranteed vase life. A good prediction of vase life depends on a good knowledge of the product, both of the internal characteristics, and of the history of the post-harvest handling. But even if everything is understood and known, the prediction of vase life of a certain bunch of cut flowers is probably limited. The climate and the weather are strong examples of systems that are predictable only to a certain extent. More than cut flowers, the weather is dependent on thoroughly studied processes. Nonetheless, the weather in The Netherlands can be predicted for only a few days with an acceptable degree of certainty. This is primarily not due to a low quality of the weather prediction models, but merely due to the fact that the weather has a chaotic nature (Tennekes, 1990). In a chaotic system the least changes in input variables can cause extremely high changes in the output variables. Whether the vase life of cut flowers also shows chaotic behaviour is an interesting point to study, for it is good to know the theoretical limits of the predictability of vase life.

The research presented in this thesis reveals several anatomical aspects of the chrysanthemum stem xylem. It has been pointed out that the vessel anatomy has great influence on the occurrence of early leaf wilting. Most new theoretical findings will be applicable for other cut flower species too, although it must be considered that the stem anatomy of other cut flower species can differ considerably from the stem anatomy of chrysanthemum. Other woody dicotyledonous plants will show the most comparable characteristics. The monocotyledonous species may show great differences. They only possess a primary vascular system and from practice is known that monocotyledonous cut flowers (e.g. tulip, lily and iris) do not suffer from early leaf wilting.

Conclusions from this thesis

The research presented in this thesis provides several new insights and new methods. The stem hydraulic architecture and the problem of air uptake after cutting of cut chrysanthemums was studied in detail. This research led to several novelties:

- A detailed description of the stem xylem vessel anatomy of cut chrysanthemum.
- The calculation of hydraulic conductivity with use of detailed digital image analysis of stem segments that were not uniform over the length.
- A theory that explains the regulation of the vessel length distribution in plants.

- A literature review on cryo-planing for cryo-SEM, completed with own results. The cryo-planing technique was used in this research to visualise xylem contents.
- A physical explanation of both the induction of and recovery from embolisms in cut open vessels in cut flowers (not only resulting from this thesis, but from the whole research project, as described in the general introduction).
- An attempt to compare and explain the hydraulic architecture of different stem segments.
- Some new insights in biology and particularly in plant anatomy.

As most research, this study yielded more questions than answers. Some aspects of the research project still need a thorough study:

- How are the anatomical characteristics of the xylem water transport system regulated during development of the plant?
- What is the influence of genetic variation and environmental factors on the development of the xylem water transport system?
- How to model the hydraulic architecture of the xylem water transport system, in order to simulate the transport of xylem sap and the dynamics of embolisms, and to compare the hydraulic architecture of plants with a different cultivation or genotype?

Other interesting matter

Stochatype

The common idea is that genotype plus environmental conditions make the phenotype. However, at least at the molecular level, but for sure also on higher levels, stochastic processes occur during the development of a phenotype. These stochastic processes are by definition not caused by environmental conditions. The genes only can regulate the freedom of the stochastic processes, but not their occurrence. Therefore it must be concluded that a phenotype is not only the result of the genotype and the environmental conditions, but also of stochastic processes.

Some differences between cut flowers and intact plants concerning water uptake

- The xylem is cut open. This enables the entrance of air and (living) particles.
- Embolisms via the cut surface in cut flowers are comparable to some extent to embolisms due to cavitation in intact plants, however, some clear differences are present. Cavitation in intact plants occurs at sub-atmospheric pressures, and not in a specific plane, but rather in wide and long vessels at random locations.
- After cutting, the xylem pressures at the cut surface change to the slightly higher than atmospheric pressure of the vase water.
- The quality of the xylem sap (as contents of solutes and gas) in cut flowers depends highly on the quality of the vase water. In intact plants the surrounding tissues, especially in the roots, define the quality of the xylem sap.
- Cut flowers lack roots. Signal chains in which roots play a role are disturbed.

Conductivity per pixel

The conductivity of a xylem vessel is often estimated by the calculation of the conductivity of its superimposed ellipse in a cross-section. This method was also used in chapter 2.2 and chapter 5.2. As explained there, a little error of the estimated axis of the ellipse causes a large error of the resulting calculated conductivity. It would be an improvement to be able to calculate the conductivity more precisely, thus avoiding (systematic) errors. It is probably possible to calculate the conductivity of each pixel in a cross-sectional digital image of the vessel. Does an algorithm for this calculation already exist, and if not, are there any mathematical restrictions to develop this algorithm?

A computer simulation of the origin of leaf arrangements

The possible leaf arrangements were explained already in the early 1900s. A graphic computer simulation would help investigators and students to understand the possible leaf arrangements and occurring abnormalities.

Do we need a detailed anatomical map of the xylem of an average chrysanthemum stem?

One of the possibilities to understand the hydraulic architecture of the (chrysanthemum) stem is to draw a detailed map of an average stem. As already pointed out in this discussion, locations and dimensions of xylem vessels may be non-determined at their formation. An anatomical map should therefore not describe absolute shapes and locations, but rather chances of occurrence and dimensions of vessels for each point. Or, viewed from the vessels themselves, a certain vessel at a certain location has a certain chance distribution for its dimensions and for the connectivity with other vessels. A map or model that just describes these chances would be a strong tool to simulate (the plasticity of) the hydraulic architecture of plants *in silico*.

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Summary

Cut flowers show a wide variance of keepability. The market demands more and more a guaranteed quality. Therefore, methods must be developed to predict vase life of cut flowers. Chrysanthemum (*Dendranthema x grandiflorum* Tzvelev) and some other cut flowers suffer from unpredicted early leaf wilting during vase life. Researchers from Wageningen University and from the Research Station for Floriculture and Glasshouse Vegetables started a joint project to investigate the problem of early leaf wilting and to come to a better prediction of vase life of cut flowers. Preliminary experiments pointed out that early leaf wilting is caused by a decrease of the water uptake due to embolisms that are induced at the cut surface. This thesis reflects a part of the project on early leaf wilting and is focussed on the anatomical aspects of the stem water transport system.

Chrysanthemums are propagated by stem cuttings, which grow in about 12 weeks to commercial maturity. Flowering is induced by a short day treatment. Cut chrysanthemums have an erect stem and the leaves are helically arranged along the stem. The primary vessel bundle network was elucidated, revealing that leaves have their direct water supply from different vascular bundles, which are positioned around nearly half of the circumference of the stem (2.1). The xylem water transport system consists of primary xylem and secondary xylem. The older the stem part (i.e. the lower in the stem) the higher the relative amount of secondary tissue.

Digital image analysis procedures were constructed to enable the quantification of large amounts of anatomical data. Xylem vessel characteristics along the chrysanthemum stems were thus quantified and a mathematical description of the vessel characteristics was developed (2.2). Hydraulic conductivity, amount of vessels, average diameter of vessels, and vessel length all showed a gradual exponential decrease from the base to higher up the stem. The hydraulic resistivity calculated from vessel lumina was 30% lower than the experimentally measured resistivity, irrespective of the position in the stem. The remaining 30% is at least partly caused by the resistance of the intervessel pits.

A new theory was developed to explain the regulation of vessel lengths in plants (2.3). Vessel length depends on the amount of fused tracheary elements. The only assumption in the theory is that each element has the same chance to be the end of the vessel during vessel formation. This results in an exponential vessel length distribution, which indeed is always found in our chrysanthemum stems. The plant can thus determine its vessel length distribution by just steering the chance factor. This theory provides the most simple mechanism that enables plants to regulate the

length of xylem vessels. Stochastic regulation of biological processes might be widely present in nature.

We reviewed and refined a method to obtain flat planes in all desired directions through frozen hydrated (biological) specimens (Chapter 3). A combination of proper sample preparation, trimming with a circular diamond saw, tight mounting using indium, and planing with a diamond knife proved to be a useful method to prepare stem xylem tissue for observation in a cryo-scanning electron microscope (cryo-SEM). This planing method is useful for a wide range of other applications of cryo-scanning electron microscopy.

We used cryo-SEM to study the dynamics of emboli in stem xylem vessels (Chapter 4). In accordance with our theoretical assumptions, wide vessels that were cut open at the cut surface appeared to embolise at a lower hydraulic tension than narrower vessels (4.1). The tension needed to embolise all vessels is easily reached in cut flowers, and it therefore can be assumed that under normal postharvest conditions all cut open vessels embolise. Our cryo-SEM results agree with the hypothesis that refilling of embolised vessels is needed to restore the fresh weight of cut chrysanthemums on vase after a dehydration treatment (4.2). Under normal post-harvest conditions air only enters cut open xylem vessels (4.3). The blockage of the xylem water flow due to emboli in cut chrysanthemums is therefore located in the base of the stem in the cut open vessels.

We developed a physical explanation of the mechanisms of induction and removal of emboli in cut open vessels of cut flowers (5.1). The refilling of embolised vessels after dehydration takes place in two phases: 1) initial rise of vase water into the vessels, resulting in redistribution and compression of the thus entrapped air, and 2) dissolution of the entrapped air into the surroundings. It was concluded that the anatomy of the xylem vessels plays an important role in rehydration capability of cut flowers after air aspiration. A compact vessel system (narrow, short and well connected vessels) restores better from emboli than a vessel system consisting of wide, long and loosely connected vessels.

Knowing that the stem anatomy is important with respect to embolism repair, we tried to find an accurate, but easy method to test the stem anatomy on its sensitivity to early leaf wilting (5.2). Within our experiments we found that plants with wider vessels were more sensitive to early leaf wilting, but no absolute thresholds were found at comparing over different experiments. It was confirmed that the anatomy of the stem water transport system is important with respect to vase life quality. However, the combination with several other factors ultimately determines the vase life quality. Xylem thickness and some other stem tissue dimensions seem to be good indicators of the quality of the xylem vessel system and its sensitivity to embolism. A more extensive study is needed to prove the practical value of the use

of these indicators. Breeders and growers may use our findings and optimise genotype and growing conditions to obtain chrysanthemums with narrower and shorter vessels at the cut surface in order to prevent the problem of early leaf wilting.

Samenvatting

Snijbloemen vertonen een grote verscheidenheid in houdbaarheid. De markt vraagt meer en meer om een gegarandeerde kwaliteit. Dat is de reden dat er methodes ontwikkeld moeten worden om het vaasleven van snijbloemen te kunnen voorspellen. Chrysant (*Dendranthema x grandiflorum* Tzvelev) en verscheidene andere snijbloemen kunnen op de vaas al heel snel en onvoorspelbaar slap blad krijgen. Onderzoekers van Wageningen Universiteit en van het Proefstation voor Bloemisterij en Glasgroenten hebben de krachten gebundeld om het probleem van de vroege bladverwelking te onderzoeken, met als doel te komen tot een betere voorspelling van het vaasleven van snijbloemen. Oriënterend onderzoek wees erop dat vroege bladverwelking wordt veroorzaakt door een verminderde wateropname. Deze verminderde wateropname wordt veroorzaakt door luchtverstopping van de houtvaten aan het snijvlak. In dit proefschrift is een deel van het onderzoek naar vroege bladverwelking beschreven. De nadruk ligt hier op de anatomische aspecten van het watertransportsysteem in de stengel.

Chrysanten worden vermeerderd door stek van de stengelscheuten, die in ongeveer twaalf weken uitgroeien tot het oogstbare stadium. De bloei wordt geïnduceerd door een 'korte-dag' behandeling. Snijchrysanten hebben een rechte stengel, met een spiraalsgewijze bladordening. Het primaire vaatbundelnetwerk werd in kaart gebracht en daaruit bleek dat bladeren hun directe watertoevoer krijgen uit verschillende vaatbundels, uit bijna de halve omtrek van de stengel (2.1). Het xyleem watertransportsysteem is opgebouwd uit primair en secundair xyleem. Hoe ouder het deel van de stengel (dus hoe lager in de stengel), hoe groter het aandeel van het secundaire xyleem is.

Digitale beeldanalyseprogramma's werden geschikt gemaakt om het kwantificeren van grote hoeveelheden anatomische data mogelijk te maken. Op deze wijze werden de anatomische eigenschappen van de xyleemvaten over de lengte van de stengel gekwantificeerd en vervolgens verwerkt tot een wiskundige beschrijving (2.2). De hydraulische geleidbaarheid, het aantal vaten, de gemiddelde vatdiameters en de vatlengtes nemen allemaal exponentieel af van beneden naar boven in de stengel. De hydraulische weerstand, berekend uit de afmetingen van de vatlumina was 30% lager dan de experimenteel gemeten weerstand, onafhankelijk van de positie in de stengel. De ontbrekende 30% wordt op z'n minst gedeeltelijk veroorzaakt door de weerstand van de hofstippels tussen de vaten.

Een nieuwe theorie werd ontwikkeld om de regulatie van vatlengten in planten te verklaren (2.3). De lengte van een xyleemvat wordt bepaald door het aantal gefuseerde vatelementen. De enige aanname in de theorie is dat elk vatelement

dezelfde kans heeft om tijdens de aanleg het terminale element van het vat te zijn. Dit resulteert in een exponentiële vatlengtedistributie, die inderdaad altijd te vinden is in onze chrysantenstengels. Op deze wijze kan een plant de vatlengtedistributie bepalen door slechts die ene terminatiekans te sturen. Deze theorie geeft het meest eenvoudige principe weer waarmee een plant de lengte van de xyleemvaten kan reguleren. Stochastische regulatie is mogelijk een veel voorkomend principe in biologische processen.

In hoofdstuk 3 worden de bestaande methoden beschreven en verfijnd voor het verkrijgen van vlakke oppervlakken in bevroren gehydreerde (biologische) preparaten. Een combinatie van een goede monstervoorbereiding, 'trimmen' met een diamant-cirkelzaag, bevestiging met gebruik van indiumfolie en polijsten met een diamantmes bleek een goed bruikbare methode om een dwarsdoorsnede van stengelweefsel klaar te maken voor observatie in een cryo-scanning electronen microscoop (cryo-SEM). De polijstmethode is bruikbaar voor vele toepassingen van cryo-scanning electronen microscopie.

Bij het bestuderen van de dynamiek van embolie in de xyleemvaten in de stengel gebruikten we de cryo-SEM methode (hoofdstuk 4). In overeenstemming met onze theoretische vooronderstellingen bleek dat wijde vaten die aangesneden zijn aan het snijvlak, al bij een lagere hydraulische zuigspanning lucht opnamen dan smallere vaten (4.1). De minimale zuigspanning die nodig is om alle vaten te emboliseren is laag en het kan daarom aangenomen worden dat onder normale naoogstcondities alle aangesneden vaten lucht opnemen. Onze cryo-SEM waarnemingen bevestigen de hypothese dat hervulling van (een deel van) de vaten nodig is om het initiële versgewicht te herkrijgen na een uitdroogbehandeling (4.2). Onder normale naoogstcondities kan lucht alleen de aangesneden xyleemvaten binnenkomen (4.3). De blokkade van het watertransport in xyleemvaten door embolie in snijbloemen is daarom gelocaliseerd onderin de stengel, in de aangesneden vaten.

Een fysische verklaring van het mechanisme van inductie en verwijdering van embolie in aangesneden vaten wordt uitgewerkt in 5.1. Hervulling van luchtgevulde vaten in snijbloemen na een uitdroogbehandeling gebeurt in twee fasen: 1) initiële opstijging van vaaswater in de vaten, resulterend in herverdeling en samenpersing van de ingesloten lucht en 2) het oplossen van de ingesloten lucht in de omliggende weefsels en de omgeving. De anatomie van de xyleemvaten bleek een belangrijke rol te spelen in de rehydratiecapaciteit van snijbloemen na opname van lucht via het snijvlak. Een compact vatsysteem (smalle en korte vaten met veel onderlinge verbindingen) herstelt beter van luchtopname dan een vatsysteem wat bestaat uit wijde, lange en weinig verbonden vaten.

Wetend dat de stengelanatomie van groot belang is voor het herstel van embolie, hebben we getracht een nauwkeurige, maar gemakkelijke methode te vinden om

stengelanatomie te testen op gevoeligheid voor vroege verwelking (5.2). In onze experimenten vonden we dat planten met wijdere vaten gevoeliger waren voor vroege verwelking, maar we vonden geen absolute drempelwaarden geldend voor alle experimenten. Zodoende was bevestigd dat de anatomie van het watertransportsysteem in de stengel van belang is voor de kwaliteit van het vaasleven, maar dat de combinatie met andere factoren de uiteindelijke kwaliteit van het vaasleven bepaalt. Xyleemdikte en enkele andere karakteristieken van de stengel op weefselniveau lijken goede indicatoren te zijn voor de kwaliteit van het watertransportsysteem in de stengel en de daarmee gerelateerde gevoeligheid voor embolie. Meer onderzoek is noodzakelijk om de praktische waarde van deze indicatoren te toetsen. Veredelaars en kwekers kunnen onze vindingen gebruiken om zowel genotypes als groeicondities te optimaliseren om chrysanten te verkrijgen met smallere en kortere vaten aan het snijvlak om zodoende de problemen van vroege bladverwelking te voorkomen.

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Curriculum Vitae

Jacob Nijsse werd op 4 maart 1974 geboren in het Zeeuwse Biezelinge. In 1992 behaalde hij zijn atheneum diploma aan de Voetiusscholengemeenschap (nu Calvijncollege) in Goes. In datzelfde jaar begon hij aan de studie Biologie aan de Landbouwuniversiteit in Wageningen. Binnen deze studie koos hij voor de specialisatie Cel. Het doctoraalexamen werd in januari 1997 behaald met een afstudeervak bij de vakgroep Plantencytologie en –morfologie (thans Plantencelbiologie) en een onderzoeksstage bij het “Electron Microscopy Unit, USDA-ARS”, Beltsville, MD, USA. Aansluitend daarop werd hij aangesteld als assistent in opleiding (aio) bij de leerstoelgroepen Tuinbouwproductieketens en Plantencelbiologie van Wageningen Universiteit. Het uitgevoerde onderzoek heeft geleid tot dit proefschrift. Per maart 2001 is hij als post-doc werkzaam bij het laboratorium voor Plantenfysiologie van Wageningen Universiteit aan een onderzoek naar imbibitie-gerelateerde kiemproblemen in zaden.

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