Factors affecting the vase life of Rosa cultivar 'Sonia': Microbiological and scanning electron microscopic investigations

Factoren die van invloed zijn op het vaasleven van Rosa cultivar 'Sonia': Microbiologisch en raster electronenmicroscopisch onderzoek

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
dr H. C. van der Plas,
in het openbaar te verdedigen
op woensdag 15 mei 1991
des namiddags te vier uur in de aula
van de Landbouwuniverstiteit te Wageningen.

Roos (foto Judith Tromp)

STELLINGEN

1. Bij onderzoek naar de invloed van planthormonen op het vaasleven van snijbloemen, dient men aseptische technieken te hanteren.
(dit proefschrift, hoofdstuk 1).

2. De microflora die zich ontwikkelt in het vaaswater van snijbloemen wordt bepaald door de ecologische omstandigheden in de vaas en niet door de microflora aanvankelijk aanwezig op de stengel.
(dit proefschrift, hoofdstuk 2).

3. De rol van microfungi in snijbloemenvaaswater verdient meer aandacht.
(dit proefschrift, hoofdstuk 1, 2 en 4).

4. Dode- of geïnactiveerde micro-organismen spelen een niet te onderschatten rol in het vaasleven van snijbloemen.
(dit proefschrift, hoofdstuk 1 en 5).

5. Een roos is een roos, is een roos, is een roos (Stein, E. 1931. In: Before the Flowers of Friendship Faded, Faded Friendship Faded).
Dit betekent ook:
Iedere roos heeft haar eigen identiteit, groeivermogen, beginbesmetting. Voor statistisch betrouwbaar onderzoek is het noodzakelijk zo homogeen mogelijk uitgangsmateriaal te gebruiken, in het bijzonder als voor die onderzoekingen binnen de gegeven tijdsduur van het vaasleven bloemen worden opgeofferd aan periodiek uit te voeren analyses.
(dit proefschrift, hoofdstuk 3-8).

6. De effecten van lage aantallen micro-organismen, sterk verdunde microbiële pectolytische enzymen, en lage concentraties microbiële metabolieten in vaaswater op de waterhuishouding, knopontplooiing en vaasleven van Sonia rozen, doen specifieke werkingsmechanismen vermoeden.
(dit proefschrift, hoofdstuk 5, 6 en 7;
Halverson, L.J. & Stacey, G. 1986. Microbiol. Rev., 50, 193-225).

7. Kunstbloemen verdienen voorrang in ziekenkamers van operatiepatiënten en intensive care afdelingen van ziekenhuizen.

8. Het begrip snijbloemvatverstopping door bacteriën wordt menigmaal ten onrechte gebruikt. Dit duidt dan op een verstoorde waterbalans waaraan andere oorzaken ten grondslag liGgen dan bacteriële verstopping van de watertransportbaan van de snijbloem .
(dit proefschrift, hoofdstuk 7;
Accati Garibaldi, A. 1983. Acta Horticulturae, 138, 255-260).

9. Microbiologisch onderzoek aan vaaswater en stengeldelen van snijbloemen, zoals rozen, na een vaasleven van een week of langer, geeft geen inzicht in de ontwikkeling van de microflora gedurende de daaraan voorafgaande dagen van het vaasleven, terwijl juist deze bepalend zijn voor het verloop van het vaasleven.
(Aarts, J.F.Th. 1957. Mededelingen van de LH Wageningen, 57(9)1-62).

10. De toepassing van weefselkweekmethoden voor snelle selectie en vermeerdering van siergewassen zowel als van land-, tuin- en bosbouwgewassen, dient bevorderd te worden.

11. - In de bijna een-en-negentig jarige geschiedenis van de Nobelprijs voor literatuur is vijf maal een vrouw bekroond.
Dit is geenszins een bewijs dat het literaire oeuvre van vrouwen van minder gehalte is dan dat van mannen.
- Anna Cramer (1873-1968) is niet de enige verzwegen vrouwelijke componist die onze vaderlandse muziekgeschiedenis rijk is.

12. Stat rosa pristina nomine, nomina nuda tonemus.
(Eco, U. 1983. Il nome della rosa)
Van de mens van weleer
blijft ons de naam.
De geur,
de klank,
de vorm verdwijnen.
Naakte namen
en
het geschreven woord
resten ons.

Stellingen behorend bij het proefschrift:
Factors affecting the vase life of Rosa cultivar 'Sonia':
Microbiological and scanning electron microscopic investigations.
Henriëtte M.C. Put
Deventer-Wageningen, mei 1991

Stat rosa pristina nomine,
nomina nuda tenemus.
De roos van weleer bestaat als naam,
naakte namen houden we over.

Uit: Il nome della rosa (Umberto Eco, 1983)

Voor:
Anton

'A rose is a rose is a rose is a rose' Gertruda Stein (1874-1946)

Coverplate: Margaretha Roosenboom (1843-1896) Still life with roses
Oil Paint On Canvas, 50 × 60 cm.

References: Hermes, N. 1989. Margaretha Roosenboom
In: Bloemen uit de kelder: negen kunstenaressen rond
de eeuwwisseling, 40-47
Redactie: Oele, A., Van Rijsingen, M., Van den Donk, H.
Uitgever: Waanders, Zwolle en Gemeentemuseum, Arnhem

De foto's bij de titelpagina en bij de titels van de hoofdstukken 1, 8 en 9 zijn van Judith Tromp.
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De foto's bij de titels van de hoofdstukken 2-7 zijn van de auteur.

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V O O R W O O R D

'Sonia' rooskleurige roos

In de ochtend van de 20° april 1983 reisde ik van Deventer naar Wageningen, bezocht het Sprenger Instituut aan de Haagsteeg, en had een gesprek met de directeur drs. G.J.H. Rijkenbarg. Een tweede gesprek volgde, dit keer met de afdelingshoofden en collega microbioloog Y. de Witte. Een derde gesprek zette puntjes op de overeenkomst die werd gesloten tussen de verpakkingsindustrie Thomassen & Drijver-Verblifa N.V. (TDV) te Deventer en het Sprenger Instituut (SI) te Wageningen.

In deze overeenkomst werd onder meer vastgelegd: "TDV detacheert Henriëtte "M.C. Put, adviseur microbiologie en -technologie voor maximaal 4 dagen per "week bij het SI voor het verrichten van onderzoek binnen het kader van de "doelstellingen, en in opdracht van het SI. Gemiddeld zal zij één dag per week "beschikbaar zijn voor haar TDV-functie. Het dienstverband tussen TDV en "Henriëtte blijft volledig van kracht."

De duur van deze overeenkomst strekte zich uit van 1 oktober 1983 tot 1 januari 1987, het bereiken van de SUM-gerechtigde leeftijd (Stichting Uittreden Metaalindustrie). Daaropaansluitend werd een persoonlijke overeenkomst gesloten tussen de directeur van het SI en mijzelve, teneinde publicatie te realiseren van de resultaten van de uitgevoerde reeksen onderzoekingen. Deze overeenkomst werd beëindigd op 1 september 1988.

Geen rozen op de (spoor)wegen tussen Deventer en Wageningen

Wel: lange wachttijden, vuile wachtkamers, tochtige perrons, in volle treinen publicaties lezend, in slaap sukkelen, kou vatten.. Doch ook onderhoudende autotochtjes met Natalio Gorin van SI Wageningen naar NS Arnhem, en de donderdagse maaltijden met Christie Berkholst bij Diedenoord's resto.

Door het SI werd gekozen voor onderzoek naar de relatie tussen micro-organismen in vaaswater en het vaasleven van snijbloemen, speciaal 'Sonia' rozen. De resultaten van dit onderzoek zijn vastgelegd in een 7-tal publicaties die de kern vormen van dit proefschrift. Het onderzoekplan kwam tot stand na circa 3 maanden literatuuroriëntatie. Het plan werd ter discussie gesteld en geaccepteerd door respectievelijk: de afdeling fysiologie van het SI (hoofd O.L. Staden), de adviescommissie 'Sierteeltprodukten' van het SI (voorzitter J.A.M. Brockhoff) en de contactcommissie houdbaarheid van snijbloemen (voorzitter H. Veen).

Schieten in de roos

Bestudering van literatuur over snijbloemen, vaaswater en micro-organismen, was aanleiding het onderzoekplan te richten op enkele nog weinig bestudeerde microbiologische factoren in het vaasleven van snijbloemen, en microbiologische methodieken te gebruiken die zelden zijn gebezigd bij onderzoek van snijbloemen en vaaswater. Dit betreft: 1. de initiële besmetting van snijbloemenstengels; 2. de rol van fungi in het vaasleven van snijbloemen, naast die van bacteriën; 3. het gebruik van gewassen reincultures en gezuiverde of zuivere microbiële metabolieten voor de bestudering van mechanismen die leiden tot een verkort vaasleven; 4. De toepassing van SEM-technieken (Scanning Electron Microscope). Immers, 'verstopping' van xyleemvaten van snijbloemen zou slechts bestaan in de geest van de onderzoeker, tenzij de onderzoeker kijkt op de juiste plaats, schrijven Parups en Molnar (1972), Rasmussen en Carpenter (1974).

Een roos op uw hoed

Iedere persoon die direct of indirect heeft bijgedragen tot de totstandkoming van dit proefschrift wordt een roos op de hoed gestoken. Dit zijn alle medewerkers van het voormalige Sprenger Instituut.

In het bijzonder noem ik: medewerkers van de afdeling CMK (Chemie, Microbiologie en Kwaliteit) waarbij ik was ingelijfd en die mij wegwijs maakten; de technische dienst, bibliotheek, fysiologie, statistiek, directie en personeelszaken.

Ik dank personen die teksten kritisch bekeken o.a. (B. Boekestein, U. van Meeteren en H. de Stigter); Muriel Rhodes-Roberts, die mijn Engels beproefde en bijschaafde; Ans van Hardeveld, die menige tekst menig maal invoerde en herinvoerde in de tekstverwerker; John Bosch (equipment engineering TDV) voor het vervaardigen van de vele figuren en grafieken; mijn co-auteurs: Bram Boekestein, Anke Clerkx, Lieke Jansen, Ton van der Meijden, Wim Klop en Frans Rombouts.

De wonderlijke wegen van een rozesteel

De uren doorgebracht achter de Jeol 35 C raster electronenmicroscoop zijn mij dierbaar geworden. Het geduld van SEM-specialiste Anke Clerkx, strekt mij tot voorbeeld. De gedeelde spanning en de vreugde bij het zien van 'mooie' beelden en scherpe opnamen, de discussies en keuzes maken voor publicaties, doch ook het wetenschappelijk natafelen veelal leidend tot nieuw nieuwsgierig makend onderzoek. Dit alles onder de bescheiden hoed van het TFDL (Stichting Technische en Fysische Dienst voor de Landbouw) en de EM voorman B. Boekestein, is een onlosmakelijk deel geworden van dit proefschrift.

Een roos is een roos, is een roos, is een roos

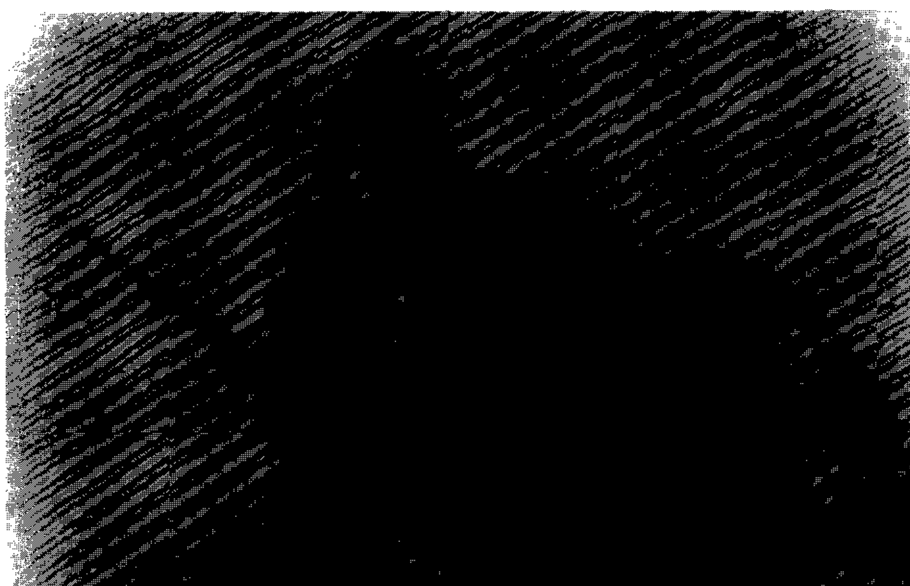
Vijftig rozen, vijftig signalen van vreugde en waardering voor mijn promotor en co-auteur professor dr ir Frans M. Rombouts, die mij stimuleerde en begeleidde op de weg van publiceren naar promoveren. Rozen ook voor Anton, wetenschapper, taalslijper en liefste vriend, die ik heel erg mis.

Deventer, januari 1991

CONTENTS

Chapter 1

Introduction



Rosa *(Judith Tromp)*

Chapter 1

1. INTRODUCTION

Prehistoric paintings and pottery record that cut flowers have long been used as ornaments and evidence for the expression of love, friendship, triumph and sadness. The long history of the art of painting comprises many still life studies of cut flowers, widely regarded as classic examples of perfect beauty.

Palladius (4th century a.c.) in his Opus Agriculturae "De Re Rustica" devoted a special section to the study of the keeping quality of cut roses "De rosis viridibus servandis" (Palladius, Ed. J.M. Gesner, 1781. See Appendix). In recorded mediëval mystical experiences, the rose also plays an important role; (1) as a symbol of love, beauty, happiness and silence; (2) as a symbol of virginity - the white rose; (3) as a symbol of martyrdom - the red rose (Lejeune, 1978).

The second part of the 20th century brought us: (1) the "flower power" wave; (2) the spring time flowering bulb fields of tulips, daffodils and hyacinths in the Netherlands; (3) the magnificent Keukenhof gardens near Amsterdam; (4) the folklore of the flower-corso; (5) the best-seller "The Name of The Rose" (1983) by the Italian writer Umberto Eco, and also linguistic expressions such as: be on a bed of roses; no rose without a thorn. The widespread use of flowers at weddings and funerals further reflects their ability to demonstrate otherwise unexpressible sentiments.

1.1. Economic importance

Since World War II the horticultural production of ornamental flowers has increased continuously in the Netherlands, and the supply of cut flowers to the flower auctions in the Netherlands is still showing an upward line (see Table 1).

Table 1: The turnover of cut flowers in The Netherlands

Year	Million guilders
1975	962,-
1980	1.648,-
1985	2.501,-
1987	2.689,-
1988	2.938,-
1989	3.036,-
1990	3.297,-

Source: CBOP, Commodity Board for Ornamental Plants; PVS, Productschap voor siergewassen, Den Haag

Table 2: The annual turnover of the most important cut flower species at the auctions in the Netherlands

Year	1986		1988		1989		1990	
Cultivar:	Annual turn-over (x 10^6)	Price/ flower (guilders)	Annual turn-over (x 10^6)	Price/ flower (guilders)	Annual turn-over (x 10^6)	Price/ flower (guilders)	Annual turn-over (x10^6)	Price/ flower (guilders)
Rosa large flowers	465,5	0,43	466,5	0,46	528,-	0,44	569,-	0,45
small flowering	1000,-	0,28	1231,-	0,30	1.333,5	0,29	1.418,-	0,29
polyanthus	23,-	0,38	51,5	0,43	72,5	0,40	85,-	0,43
Chrysanthemum: flocculate	33,5	0,58	28,5	0,70	30,5	0,67	34,-	0,72
polyanthus	779,-	0,52	902,-	0,54	1033,5	0,48	1.101,-	0,51
Tulip:	690,5	0,28	870,-	0,24	863,5	0,26	902,-	0,25
Freesia:	620,-	0,24	616,5	0,25	558,-	0,26	560,5	0,27
Carnation: standard	153,-	0,33	198,-	0,37	222,-	0,32	210,5	0,36
polyanthus	431,5	0,27	658,-	0,30	770,-	0,25	688,-	0,27
Gerbera	318,-	0,44	300,-	0,44	312,-	0,40	309,-	0,41

Source: CBOP, Commodity Board for Ornamental Plants; PVS, Productschap voor Siergewassen, Den Haag

Rose cultivars are at present the cut flower for which there is the greatest demand: this is followed by chrysanthemum, tulip, freesia, carnation and gerbera cultivars, as shown in Table 2.

1.2. The capability of cut flowers for a post-harvest vase life

Factors which may contribute greatly to the vase life of cut flowers are: (1) the ornamental value of the species or the cultivar; (2) the cultivation method; (3) the moment of harvesting; (4) the method of harvesting; and (5) post-harvest handling.

- *The ornamental value of the species or the cultivar*

Flower species and cultivars which posesses less ornamental value, as well as a short vase life, are of minor importance for the cut flower market (Halevy *et al.*, 1978; Halevy, 1989).

Fashion trends, however, may also play a significant role in the consumer's preference for a special cut flower cultivar. For the commercial launching of a new cut flower cultivar variety or hybrid of, for example tulip, gerbera, or rose, a 'popular' name given to that flower may induce the consumer to buy it. What is in a name? A rose with any other name may smell as sweet (Shakespeare, 1564-1616) perhaps, but not necessarily be chosen for purchase.

By modern hydroponic methods and laboratory tissue culture techniques, it is possible to cultivate and propagate new hybrids very rapidly in high numbers. Statistical evaluation of the ability of new cultivars or hybrids to survive well during vase life is therefore much easier and quicker to ascertain, speeding up introduction to the cut flower market (Van de Pol & Breukelaar, 1982; Pierik, 1985; Hickleton *et al.*, 1987; Mor & Zieslin, 1987; and De Jong, 1989).

- *The cultivation method*

In the Netherlands, most ornamental flowers are grown in greenhouses with precise regulation of temperature, humidity, irrigation, light-dark phases, or soil substrate constitutuents. Therefore many flower cultivar can be cultivated all the year round and difficult climatic conditions circumvented.

- *Premature harvesting*

As soon as the flower is cut and separated from the mother plant, it obviously no longer receives the dissolved nutritive elements from the mother plant via the mobile sap. The cut flower from then on is entirely dependent on its own reserves, especially those of sugars and starch, which may quickly be exhausted (Paulin *et al.*, 1981; Paulin, 1986).

Premature picking of roses may lead to a low petal starch reserve; this is most likely to be an indispensable source of energy for the cut flower to bloom maximally (Berkholst, 1989). With the rose 'Sonia'; insufficient amounts of petal starch were shown to be due to (1) premature picking, and (2) the breakdown of starch after cutting during any long storage periods preceding the vase life. Low storage temperature (2-5°C) did not arrest this starch breakdown after cutting, although storage at higher temperatures (10-15°C) caused petal starch to disappear much more rapidly (Berkholst & Navarro Gonzáles, 1989). Thus , practices such as immature cutting, long storage and exposure to high temperatures, are detrimental to the quality of roses and to their vase life.

- *Harvest methods*

The harvesting equipment, handling methods, and general standards of hygiene have a profound influence on the final keeping quality of the flowers.

Mechanical damage at the cut end of the stem due to rough handling and the application of blunt scissors may lead to blockage of the xylem vessels, thus disturbing the water flow to the flower.

Dirty equipment including buckets, hands, gloves and cloths, comprise the first exogenic source of microbiological contamination of the flower stem and its cut surface. Thereafter many opportunities for microbial contamination of the cut flower may occur.

- *Post-harvest handling: from the grower to the consumer's home*

During the post-harvest period, from the point of cutting to the vase in the consumer's home, the methods of handling, packaging, storage and transport throughout the whole commercial chain from grower to retailer markedly affects the final keeping quality of the cut flowers.

1.3. The fate of cut flowers

- The effects of cutting

The flower cutting operation is of paramount importance in sofar as it affects the life of the cut flower. For validly interpreting certain aspects of cut flower physiology, i.e. the water relations and water balance, it is essential to compare the behaviour of cut flowers with that of intact ones under the same conditions. The study of De Stigter (1980) shows that this is possible by means of experiments involving single-stem, self-rooted hydroponically-grown roses: simply removing the root system turns an intact rose plant into an otherwise identical cut flower.

By using intact-plant behaviour as a reference which shows the fate of the rooted rose, the specific effects of the cutting operation emerge more conclusively. Two aspects were considered by De Stigter (1980): (1) the short-term effects; i.e., the immediate effects of replacing water uptake through the roots by that occurring through the exposed xylem vessels. This involves the elimination of root resistance and the substitution of the original tracheal sap by vase water with an artificial chemical composition and mainly a higher osmotic (or solute) potential; ; (2) the long-term effects: a cut rose shoot with a mature flower bud is a relatively young plant part; it may reach this stage in a matter of six weeks from axillary bud break. The ensuing flowering of the cut rose then takes about two weeks essentially the same time as needed for the flowering of the intact hydroponically grown rose (De Stigter & Broekhuysen, 1986). Hydroponic production of cut flowers for post-harvest physiological and microbiological studies, have not been reported by other workers. Commercial trials, however, are still in progress (Hickleton *et al.*, 1987). Intact root-grafted roses on a shoot with cut roses from the same shoot were compared by Durkin & Kuc (1966). These authors observed that the intact flowers lasted at least twice as long (8-13 days) as the cut flowers (4-10 days) which were placed in vase water under the same climatic conditions as the rooted roses.

- Effects of transport and storage

Cut flowers are mainly transported in the dry state. During storage at low temperatures the flowers usually are held dry and piled up horizontally, or more rarely, they are placed upright in buckets or basins filled with water.

Water stress occurs during the period when cut flowers are held in the dry state. Thereafter, recovery of the natural water balance is necessary.

The water balance of cut flowers is a result of interrelated processes involving: (1) water uptake; (2) water transport; (3) water loss during day and night periods; (4) the capacity of the flower to retain its water, and (5) competition between flower and leaves. De Stigter (1980a) observed that the long-lasting constancy of water uptake by cut roses in consecutive dark periods sharply contrasted with the rapid decline in water content during the light periods, as shown in Figures 1 and 2. These observations may or may not be compatible with the concept of the causative agent of reduced water uptake, as proposed by many other authors (Rasmussen & Carpenter, 1974; Durkin, 1979). They claim reduced water uptake to be due to vascular occlusions occurring as massive and immobile plugs of solid materials filling xylem vessels. Water stress caused by xylem plugging is a constant non-reversible stagnation of the water transport in cut flowers which disturbs the water balance throughout both day and night.

Flowers like many gerbera and chrysanthemum cultivar show a cavity or hollowness in the center of the stem, which is formed during stem elongation: thus there are two possible pathways for water uptake, one directly through the xylem vessels at the cut surface, and the other indirectly from the cavity of the stem into the adjacent lateral vessels. The stem cavity shows a diameter 5-10 fold longer than the diameter of the xylem vessels, and is filled with water up to the level of the surrounding water in the flower container when trapped air can disappear from the cavity (see Figure 3, Van Meeteren, 1980). Consequently xylem vessel plugging occurring in the lower part of the vessels, below the level of the vase water, may not cause a total water stress and a total cessation of water uptake. Water uptake through the second lateral pathway from the inner cavity may occur and circumvent any xylem plugging, as long as transpiration rate is not too high, as is mostly the case with (leafless) Gerbera.

- *The role of "pulsing" and flower-holding-water solutions*

"Pulsing" is a procedure wherein flower tissues are infiltrated with certain chemicals directly after harvest, to protect the flower during transport and subsequent storage and obtain optimal longevity during the vase life. Pulsing solutions may consist of: sugars (energy sources and osmotic components); organic acids (pH regulators); germicides; an ethylene inhibitor; mineral salts (improving water uptake), and on occasion growth regulators (phytohormones).

In flower-holding-water solutions ethylene inhibitors are not applied. Depending upon the contact time of the pulsing solution and the cut flower, the concentrations of similar applied chemicals in the pulsing solution are much higher than in the cut flower holding water. In addition, in pulsing and holding water solutions a wetting agent is sometimes applied (Halevy & Mayak, 1981).

1.4 Factors which may influence the vase life of cut flowers

The vase life of cut flowers which are cultivated, harvested, transported and stored under controlled conditions may be influenced by: (a) physiological factors, e.g. cut surfaces and wounding of stem xylem vessels, vessel wall changes, xylem vessel cavitation, air embolisms, changed hormone activities depletion of cabohydrates (energy), and (b) microbiological factors, e.g. the composition of the vase water microflora, their multiplication abilities and interactions, the infiltration ability of the microorganisms into the flower xylem vessels and the toxicity of microbial metabolites.

1.4.1. Effects of physiological factors

Physiological factors affecting the vase life of flowers may be categorised as follows.

- *Harvesting injury of xylem vessels*

Injuries of the stem during the harvest may cause mechanical blockage of xylem vessels at the cut surface and higher up the stem, thus disturbing the water relations of the flower (Dixon & Peterson, 1989). Further material from injured cells may act as endogenous elicitors of phytoalexins, providing signals towards which recognition by the plant may induce biochemical activities acting as plant defence mechanisms (Darvill & Albersheim, 1984; Halverson & Stacey, 1986): (see also section 1.5.).

- *Cavition*

A disruption of water column continuity in the xylem vessels of an intact plant can be induced by water stress, usually as a consequence of the dehydration of the wood xylem vessels during the winter period.

Cavition in cut flower vessels however, can be caused by (1) air-drying during transport and dry storage, and also by (2) physical blockage at the cut surface and to approx. 200 mm up the stem.

The consequence of cavitation by air-drying is a decline in water conductance up the stem, induced by water stress, although no vessel occlusions are present (Dixon & Peterson, 1989). Water-stress-induced cavitation can also be caused by ageing of the flowers in the vase. Cavitation can be shown by infiltrating a fluorescent tracer (berberine sulfate) through the stems: cavitated vessels do not stain and show no occlusions. Cavitation can be localized and measured by ultrasonic acoustic emissions, and thus be distinguished from water stress caused by the breakage of cellulose fibers in the wood and other diverse forms of stem plugging such as tyloses (Tyree *et al.*, 1984; Tyree & Dixon, 1986, Dixon, 1987; Dixon *et al.*, 1988; Dixon & Peterson, 1989).

- *Xylem embolism*

Air bubbles and dissolved gases may occur in xylem vessels. As xylem pressure becomes increasingly negative the adhesion between water molecules is reduced and vaporization occurs. The maximum pressure difference (ΔP, in MPa) withstood by a meniscus at an intervessel pore can be calculated from the pore diameter (D, in μm) and xylem sap surface tension (T, in $N^2\ m^{-1}$) using the capillary equation $\Delta P = 4\ (T/D)$), which refers to the ΔP of a pit membrane pore at it's bubble pressure (Sperry & Tyree, 1988). When embolisms occur at pressures less negative than normal, other mechanisms sometimes initiate vaporization, e.g. mechanical shock with air coming out of vessel water, due to rapid pressure changes.

The air-seeding hypothesis is a third explanation of the cause of xylem embolisms, and this has the most experimental support (Zimmermann, 1983); viz: embolisms may be triggered by air aspirated into the xylem vessels via pores in the wall where it adjoin an air space. Once inside the xylem vessel, the air disrupts the cohesion of the water column which therefore retracts (cavitates), leaving a vessel filled with water, vapor, and air (Sperry & Tyree, 1988). Eventually, the vessel becomes completely air-filled as air comes out of solution from surrounding water, a phenomenon accelerated by higher temperatures, resulting in decreased stem or vessel conductance, as shown by Dixon & Peterson in cut roses (1989).

Embolism of adjoining xylem vessels is prevented as long as pressure differences across intervessel walls do not exceed the surface tension of the air-water interface at the pores in the wall. The largest pores are the most vulnerable to the penetration of air; such are the pores in the pit membranes which under normal circumstances facilitate water flow between vessels (see Figures 4 and 5).

After a long period of dry storage of cut flowers, rehydration and de-aeration of xylem vessels may be slow, resulting in sub-optimal conditions for a good vase life (De Stigter & Broekhuysen, 1989). Cavitation and air-embolism alone may not be harmful for the vase life of cut 'Sonia' roses, unless more than half of the xylem vessels are blocked (Van Doorn & Buis, 1985). Repair of the water relations of cut flowers affected by embolism and cavitation may be enhanced by using de-aerated vase water of low pH to which a wetting agent has been added (Durkin, 1979).

- *Other physiological factors*

affecting the vase life of flowers: e.g. carbohydrates and phytohormones are mentioned in sub. 1.2 and 1.5 (Table 3) respectively.

1.4.2. Effects of microbiological factors

- *Micro-organisms as particles in the vase fluid*

Apart from toxic and other effects towards cut flowers, micro-organisms may act by their mere physical presence and dimensions as particles, small enough to enter xylem vessels passively, along with the transpiration stream of water. If micro-organisms are taken up in sufficient quantity, they may plug the vessels especially at the intervessel pores and disturb the water uptake (De Stigter & Broekhuysen, 1986b; Van Doorn *et al.*, 1986). It is therefore important that after harvest cut flowers, are not confronted with deterious water by being placed in dirty buckets or basins with re-used water, containing dust, soil particles, plant hairs, plant fragments and dead cells, slime, and viable micro-organisms, which might use the dirty materials as a growth medium. Both micro-organisms and other non-identified particles may be taken up passively by the sap stream of the cut flowers, and plug the xylem vessels.

- *Active infiltration and adhesion of micro-organisms into xylem vessels*

Active infiltration of micro-organisms may be possible due to microbial motility, aided by chemotaxis. The bacterial flagellum does enable the bacterial cell to move actively; Brownian movement will cause only random cell rotation. Movement of flagellate bacteria into a capillary system such as xylem vessels may result in an active movement higher up into the vessels compared with non-motile bacteria which move up only passively in the sap stream.

Motility, taxis, and tropisms are of value in the natural environment of micro-organisms. They are complex systems which have evolved and been retained as a consequence of phenotypic responses of the organism to the environment (Carlile, 1980; Konings & Veldkamp, 1980). Micro-organisms show taxis or tropisms towards a wide variety of physical and chemical stimuli. A cell may respond to a stimulus when it occupies a physiologically suboptimal position within a concentration gradient. A cell capable of chemotaxis must be able to detect ('sense') changes in the concentration of a chemo-effector, transmit this sensory information to the locomotory organelles (i.e. flagella), and then make the appropriate response; it must also be able to adapt to the continued presence of the chemo-effector by ceasing to react when the concentration of the chemical reactor becomes uniform.

The infiltration of micro-organisms, passively or actively, into the xylem vessels of cut flowers may result in: (1) physical blockage of the water transport vessels (xylem) at the cut surface and in the open vessels; microorganisms may also block the pit membranes of these vessels, obstructing the water flow through the lateral pit membranes; (2) spontaneous reactions may occur in the vessel cells, disturbing the water relations of the vessel system; (3) the adhesion of infiltrating micro-organisms to the cut surface, the vessel walls, and to the pit membranes, may be followed by multiplication, which may seriously disturb the water transport and the water balance of the cut flower.

In nature, micro-organisms frequently bind specifically or non-specifically to a substratum or to other cells, by means of specialised microbial components (adhesins) or anchoring structures (fimbriae).Free-living micro-organisms in aquatic habitats often adhere to submerged surfaces such as particles of debris, other organisms, or cut flower stems - sometimes forming biofilms (Marshall, 1980 & 1980a). Adhesion may affect the activity of the adherent micro-organisms since the conditions at a submerged surface differ from those in the bulk aqueous phase: e.g. surfaces of cut flowers may adsorb or excrete (from the phloem) nutrients and stimulatory or inhibitory ions so that the solid-liquid interface may be a significantly more advantageous (rarely disadvantageous) habitat compared with the bulk liquid phase (Woltering, 1987). Furthermore the micro-organisms may be able to detach temporarily by producing an emulsifying agent (emulcyan) which induces cell-surface hydrophobicity, thus permitting detachment and colonization of fresh surfaces (Fletcher, 1979 & 1979a; Fletcher *et al.*, 1980).

1.5. Different views on the contribution of physiological and microbiological factors in the vase life of cut flowers

- *The work of Aarts (1957)*

Aarts (1957) did not confine himself to study the vase life of only one flower cultivar, but studied simultaneously the behaviour of at least 12 cut flower cultivars. He also investigated the effects of a great number of biocides, bacteriocides as well as fungicides on the vase life of cut flowers, and studied also the influence of a number of sugars and some other flower nutrients.

Aarts (1957) concluded that, (1) sugar together with a non-toxic concentration of a bacteriocide and a fungicide added to the vase fluid significantly increased the vase life of the flower placed in that fluid, to an extent dependent on the flower cultivar or race. (2) Bacteria in vase water were harmful towards the flower due to a direct plugging of the vessels with bacterial particles, but (3) non-filterable substances also induced plugging. (4) Even in the absence of bacteria in the vase water, vessel blockage occurred, probably as a consequence of substances secreted by disorganized vascular cells.

Vessel plugging was not always demonstrated microscopically; instead the water conductivity of the vessels was measured as parameter for plugging. The role of fungi was not investigated.

Unlike Aarts (1957) who studied many flower cultivars and many vase life influencing factors, later scientists mainly studied the vase life of one flower cultivar, and one or two variants of the vase fluid. The most studied cut flower cultivars are: *Rosa* hybrids and *Dianthus* (carnation). Vase life disturbance phenomena of cut roses are: bent neck, delayed bud development and premature fall of flower petals. *Dianthus* cultivars on the contrary are very sensitive towards exogenic and endogenic ethylene (see later).

- *The role of phytohormones*

Phytohormones or plant hormones are compounds produced by plants which regulate the plants' own metabolic processes such as cell division, seed germination, fruiting, flowering, senescence (Bruinsma, 1983). Such agents include abscisic acid, auxins, cytokinins, ethylene and gibberellins. The phytohormone ethylene probably plays the most important role in the vase life of cut flowers. However, other phytohormones are also involved in flower bud development and the senescence of cut flowers, although the mechanisms underlaying their activity are not always clear and there may be subtle interactions (Schröder, 1987; Borochov & Woodson, 1989; Zucconi & Bukovac, 1989).

Phytohormones or phytohormone-like compounds may also be formed by certain micro-organisms such as *Azotobacter*, *Agrobacterium* Pseudomonads and many fungi, as shown in Table 3.

TABLE 3 Role and physiological effects of plant hormones; their production by micro-organisms

Plant hormone	Chemical nature	Activities or role			Hormone production by micro-organisms	References
		In intact plant	Added to cut *Rosa* flower vase fluid	Other effects on (cut) roses		
ABA Abscicic acid	terpenoid	Growth & germination inhibitor accelerator of leaf abscission	- High transpiration: reduces water loss, prevents wilting, prolongs vase life. - Normal water balance: increases petal senescence, decreases vase life	Exogenous C_2H_4 increases endogenous ABA in petals	Fungus, *Cercospora rosicols*	1)
IAA and derivatives Auxins(s)	3-indole-acetic acid and - indole-derivatives	Plant growth regulator, can stimulate C_2H_4 production IAA's from phytopath. microbes cause --> galls, knotts and hypertrophic growth	Added or produced by vase flora, effect not yet known	Not yet clear	Wide spread among bacteria and fungi. Also among phyto-paths. e.g. *Pseudomonas*, *Agrobacterium*, *Xanthomonas* species	2)
BA cytokinins	benzyl-amino-purine Derivatives of adenine	Stimulate plant cell-division and (cell differentiation)	Promote vase-life, improving water balance or may be ineffective	Interacts with C_2H_4 and ABA in flower senescence, which need clarification	Algae, some phytopathogenic bacteria and *Rhizobium* species; many fungal phytopath	3)
Ethylene	C_2H_4 Intermediates in biosynthesis are: IAA (auxins) and ACC (1-amino-cyclopropane-1-carboxylic acid)	Regulates plant growth and development	Mechanism not yet studied.	Exogenic treatment, sensitivity varied per cultivar. Role unclear	Many filamentous soil fungi, and some bacteria. Some micro-organisms produce ethylene inhibitors: e.g. AOA, amino-oxy-acetic-acid	4)

Continuation Table 3

Gibberellins A1, A2, A3, etc.	A common tetracyclic structure	Regulation of stem elongation and seed germination	Role is not yet clear	Antibiotic activity, versus virus infections	*Algae*, *Gibberella fujikuroi* *Fusarium moniloforme*. *Arthrobacter*, *Azotobacter* species	5)

References: 1) Mayak & Halevy, 1972; Salisburg & Ross, 1985. 2) Yu & Yang, 1979; Fett *et al*., 1987; Kernan & Thornburg, 1989. 3) Halevy & Mayak, 1975; Morris, 1986; Jameson & Morris, 1989. 4) Lynch, 1974; Lieberman, 1979; Domsch *et al*., 1980; Yang, 1980; Apelbaum & Yang, 1981; Halevy & Mayak, 1981; Faragher & Mayak, 1984; Yang & Hoffman, 1984; Abelis, 1987; Kende, 1989; Lee, 1989; Reid, 1987 & 1989; Reid *et al*., 1989. 5) Dickinson & Lucas, 1982; Mor & Zieslin, 1987.

(continuation)

- *Ethylene*

Many micro-organism e.g. filamentous soil fungi and some bacteria may produce ethylene (Lynch, 1974; Domsch *et al.*, 1980; Lee, 1989); but other micro-organisms produce inhibitors of ethylene formation (Lieberman, 1979). The role of ethylene in the postharvest life of roses, is still unclear. Reid *et al.* (1989) studied the ethylene sensitivity of 27 different rose cultivars by exogenic treatment with ethylene, and observed that several cultivars were very sensitive (< 100 ppb C_2H_4 at 22°C). Sonia roses, the cultivar used in our studies, and also often used by European researchers, was found to respond favourably only to high concentrations (> 1000 ppb C_2H_4) at 22°C. The time course of ethylene emanation by rose petals resembles that of carnation and other flowers and is composed of three distinct phases: (1) a low steady state in ethylene production; (2) an accelerated rise to a maximum; and (3) a decline in its production (Halevy & Mayak, 1981). A rise in ethylene production was also evident in rose flowers during cold storage. Following cold-(stressed) storage, the ethylene production of stored roses was higher than that of fresh flowers and their longevity shorter (Faragher *et al.*, 1986). However, ethylene production by rose petals is low, at most 1-5% of that produced by carnation petals (Faragher & Mayak, 1984).

Inhibitors of ethylene synthesis failed to prolong the longevity of rose flowers despite the reduction in ethylene production. Inhibition of the response of flowers for ethylene by silver thiosulfate (STS) increased the longevity of cut rose flowers (Veen, 1983 & 1987; Faragher *et al.*, 1986a) and prevented the accelerated abscission of rose petals caused by exogenously applied ethylene (De Stigter & Broekhuysen, 1986a). STS shows also biocidal activity and may inactivate vase water micro-organisms (Reid et al., 1989a).

Stressing of plant tissues by chemicals, drought, cold, insect damage, mechanical wounding or cutting may result in increased ethylene production. Stress-induced ethylene may assist the plant or flower to cope with stress conditions. Under drought conditions, ethylene would cause leaf abscission and thereby reduce water loss (Apelbaum & Yang, 1981).

- *Wounding of plant tissue by cutting*

Wounding of plants can be caused by mechanical (wind) and physical (insect bites, cutting) damage, and a wounded plant may lose a part of its natural defence mechanism mediated by the epidermis, although contact between the wounded part of the plant and the non-damaged tissues remains. A cut flower, however, is fully separated from the mother plant and its rooted system. It therefore is not clear whether the wounding reactions of a cut flower are comparable or not with those observed in a rooted flowering plant after wounding. Wounding of plant tissue leads to several reactions in the plant, i.e. (1) genetical changes affecting phytoalexin synthesis; (2) biochemical reactions involving hormone activity; (3) morphological changes such as stem vessel wall degradation, which may all interact reciprocally.

- *Genetic response to wounding*

In plants, as in all organisms, the plasma membrane serves two main roles; viz: (1) the transport of solutes in and out of cells, and (2) sensory transduction, i.e. the sensing and initiation of the cellular responses to changing environmental conditions (Sussman & Harper, 1989; Siebertz *et al.*, 1989).

- *Hormone mediated genetic expression*

Wounding of plants is responsible for the regulation of a wide variety of genetic products in plants e.g. BAP, the cytokinin (benzylaminopurine); NAA, the auxin (α-naphtalene acetic acid); IAN, indole acetamide; IAA, the auxin (indole-3-acetic-acid). Auxin (IAA, indole-3-acetic-acid) levels regulated the expression of a wound-inducible proteinase inhibitor II,-chloramphenicol acetyl transferase (CAT) in gene fusion experiments, in vivo and in vitro (Kernan & Thornburg, 1989).

The wound inducible genetic system involves plant defences against microbes and herbivores (i.e. wounding). This is followed by the induction of two small gene families (proteinase inhibitor I & II) which encode quantitatively microbial or insect resistance factors; these are induced from a quiescent to an active state after wounding.

- *Phytoalexins and their elicitors*

Phytoalexins appear as the result of plant-microbe interactions. They are an important factor in plant disease resistance, being the response of the plant to an infection by pathogenic or nonpathogenic micro-organisms: bacteria (Billing, 1982) and fungi (Domsch et al., 1980). They are a chemically heterogeneous group of low-molecular-weight compounds with antimicrobial properties and are not found in healthy plant tissues; they appear only at the site of an infection. Phytoalexin synthesis can be induced by molecules of abiotic or biotic origin called elicitors. They include oligosaccharides, as shown in Table 4, and there are about 100 known phytoalexins. It is probable, that all plants have the ability to synthesize phytoalexins, which usually are structurally correlated with the plant family or species (Albersheim & Valent, 1978).

The presence of an endogenous elicitor in plant cell walls may be the mechanism by which abiotic molecules induce elicitor activity. Thus, plants have the capacity to recognize molecular signals originating from microbes or their own cell walls, and are thus triggered to elicit phytoalexins as part of their defense response.

The specificity of many plant-microbe interactions implies some mechanism of recognition, one of which may be the exchange of molecular signals between host and microbe. Such signal molecules can have either a positive or a negative effect on the plant microbe interaction. In general, recognition is the culmination of a complex series of events involving genetic and physiological changes. The response to inoculation of a virulent microbial strain may require 10 min or up to 2 days to produce immunity in the cells surrounding the inoculation site, and the defense may be equally effective against compatible or incompatible microbes (Sequeira 1973; Kuhn, 1987).

Initiation of some host plant defenses may be mediated by the recognition of a signal resulting from the degradation of the plant cell wall, rather than by the direct recognition of a component of the pathogen. For example: endopolygalacturonases digest the plant cell wall and release oligogalacturonides, which can induce phytoalexin synthesis. A systematic study is needed to establish a correlation between pathogenicity and the presence or absence of polygalacturonases. An incompatible race might induce the activity of elicitors with the initiation of a more dramatic defense response. Darvill and Albersheim (1984) suggested that abiotic elicitors might cause cell injury or death with the release of cell wall pectic fragments. These cell wall fragments could be the same, as the endogenous oligosaccharide elicitors. However, it is not known whether cell wall pectic fragments released by cutting injury of flower stems may act as endogenous biotic elicitors. It is also not clear whether biotic and abiotic elicitors in the vase water fluids of cut flowers e.g.: fungal cell wall fragments; microbial enzymes; heavy metals; or detergents may act as signal molecules which elicit phytoalexin synthesis, which may influence the flower development during the vase life (Collmer, 1987).

Elicitors probably act in concert with other mitigating factors. For example plant oligogalacturonide cell wall fragments and fungal β-glucans together act synergistically to induce phytoalexin accumulation in soybean (See later). Many signal molecules have been identified as oligosacharides (Halverson & Stacey, 1986; Nothnagel *et al.*, 1983).

Table 4: Elicitors of phytoalexin synthesis in plants

<u>BIOTIC ELICITORS</u>:	- peptides from fungal mycelium
a. Exogenous	- oligosaccharides (β-glucans) and chitosan from fungal cell walls or culture filtrates.
	- polysaccharides from microbial cell walls
	- pectic degrading microbial enzymes (endopolygalacturonases)
b. Endogenous	- small molecules from plant tissue (dead or injured cells)
	- plant cell-wall fatty acids
	- plant cell-wall (pectic) polysaccharides
	- plant enzymes (oligogalacturonide releasing enzymes; pectic degrading enzymes)
	- pectic plant cell-wall fragments released by microbial acitivty
	- ethylene
<u>ABIOTIC ELICITORS</u>:	- heavy metals
	- autoclaved ribonuclease
	- chloroform
	- detergents

- *Oligosaccharides and Oligasaccharins*

By wounding of plant leaves intravascular glycosidases are released which interact specifically with adjacent cell walls to liberate low mol.weight oligosaccharides. These oligosaccharides have been isolated and can be fed through the petiole of detached leaves to induce the accumulation of proteinase inhibitors I & II. The kinetics of the induction of both families of proteinase inhibitors involve a 4-6 h period following wounding before mRNA begins to accumulate. This long preinduction period is unusual. Other wound-inducible systems, such as those following the inoculation of a virulent microbe or virus, show a more rapid gene activation (Lawton & Lamb, 1987).

Naturally occurring plant cell wall carbohydrates exhibiting biological regulatory functions have been termed oligosaccharins. Thus β-glucans and oligogalacturonides would be classified as endogenous oligosaccharins. The mechanism by which the β-glucan enhances phytoalexin synthesis is not known. Some reactions are shown within 2-4 h after treatment of the plant (in vitro or in vivo) with the phytoalexin elicitor. Under assay conditions an active hapta-β-glucoside elicitor shows a half maximal response of phytoalexin synthesis after using only 0.6 ng: (10^{-8} - 10^{-10} M) of the glucoside (Sharp *et al.*, 1984). This means that very low concentrations of a certain phytoalexin elicitor may be needed to elicite a complete physiological reaction of the plant (Rickauer *et al.*, 1989).

- Gaps in our knowledge of the mechanisms leading to water stress and vascular blockage of cut flowers

A scientifically literate reader would have considerable difficulty when trying to relate general conclusions drawn from a biochemical study of a phytopathogenic bacterium to those drawn from a genetic study on a rust fungus (Gabriel, 1986). The same reader may have similar difficulties when comparing conclusions drawn from physiological studies on the post-harvest life of cut flowers with microbiological data. It is unclear whether (1) plant hormones (ethylene and indole-3-acetic acid) produced by micro-organisms in vase water or in cut flower vessels, influence the vase life of the cut flower, and (2) whether microbiological elicitors may initiate vessel wall reactions which reduce the vase life of the flowers. Furthermore, (3) the role of micro-organisms in, and the mechanisms of vascular blockage of cut flowers, are also controversial.

Since water stress is not always directly related to vascular blockage, other phenomena might be the consequence of a disturbance of the water relations of the cut flower. Microscopic examinations as well as scanning electron microscopic studies are scarce, and these studies only sometimes indicate vascular blockage by micro-organisms; however visualization of vascular blockage may easily be missed(Parups & Molnar, 1972; Sacalis, 1975).

- *Visualization of vascular blockage*

"Vascular blockage due to plugging with micro-organisms is only in the imagination of the scientist", concluded Rasmussen & Carpenter (1974). Some 15 years earlier (Aarts, 1957) as well as some 15 years later, visualization of vascular blockage due to micro-organisms is still lacking in many studies, notwithstanding conclusions that microbial plugging of xylem vessels of cut flowers is the cause of water stress and flower senescence (Burdett, 1970; Dansereau & Vines, 1975; Marousky, 1980; Van Meeteren, 1980).

The evidence implicating micro-organisms in the vase water as agents reducing the vase life of cut flowers by plugging of the vascular system, is not conclusive. Van Doorn *et al.* (1986) concluded that an adverse effect on rose flowers was only manifest when the initial bacterial number exceeded 10^9 per ml vase water. Zagory & Reid (1986) however, observed that microbial numbers of only 10^3-10^4 cfu (colony forming units) per ml vase water was deleterious, the vase life of carnations and roses being reduced from 14 and 6 days to 7.5 and 4.5 days, respectively. Similar numbers of micro-organisms also reduced the vase life of *Chrysanthemum morifolium Ramat* and cultivars of carnation, yet other microbial species isolated from vase water apparently had no effect when tested by the same method, although the number of cfu's at the end of the vase life did not exceed 10^5-10^7 per ml vase water.

It must be emphasized that the demonstration of the presence of micro-organisms inside stems and the location of the plug by agar plating methods involves both avoidance of contamination with exogenous micro-organisms, and the simultaneous application of methods for the enumeration of different bacterial and fungal species. However, the plating method does not give any information on colonization and effective plugging inside the vascular system (Van Doorn *et al.*, 1990).

- *Light microscopic observations*

Aarts (1957), reported plugging at the cut surface of *Dahlia* stem after 8 days of vase life, and plug formation by amorphic materials in the stem, about 4 cm above the cut surface. Likewise, Lineberger & Steponkus (1976) revealed vascular occlusions due to microbial growth and cell deposition in sections of stems of *Rosa* hybrida L. cv. Red American Beauty. Microbial occlusions were restricted to the basal 2.5 cm of the stem. In addition, gum deposition was observed, but this always occurred above the vase solution level. Micro-organisms reacted histochemically with the protein stain mercuric bromphenol blue, and the gums were identified with periodic acid-Schiff's reagent and GLC (gas liquid chromatographic) analysis of the resultant monomers after acid-hydrolysis of the occluding material.

Earlier published work of Gilman & Steponkus (1972) however, showed no wound responses of rose xylem after the excision of the cut flower. This is normally accompanied by lignin, tannin, or tylose formation in the cut vessels, and normally is detectable by histochemical-microscopy.

- *Histochemical microscopy*

Histochemical studies by Parups & Molnar (1972) of 5-day-old xylem-plugged rose stems showed that the material blocking the vessels of senescing rose stems contained carbohydrates, pectin-, lipid-, and protein-like compounds and some enzymes. Tannins, lignin, and callose were not encountered in the blocked vessels. However, it was not clear whether the plugging materials originated from micro-organisms, microbial particles, microbial metabolites, or from decomposition of the vascular cell walls (Molnar & Parups, 1977).

- *Scanning electron microscopy (SEM)*

Vascular blockage in the tracheids of cut maidenhair fern fronds was observed by light and electron microscopy and indicated the presence of numerous vascular occlusions in the xylem. No microbial particles were observed. Histochemical studies indicated pectinaceous occluding materials; occlusions were not present in the xylem of freshly-cut fronds or in fronds which had been maintained in vase water supplemented with Ag^+ ions. Tannins and lignin were not present. Even when only small parts of a tracheid had been removed, amorphous pectinaceous material was present in the vascular lumen, appearing to reflect a substantial degradation of the primary cell wall in the scalariform pits (Fujino *et al.*, 1983).

Ag^+ is biocidal and a strong inhibitor of pectinase and cellulase enzymes. The pectin sources are the primary cell wall, perforation plates, pit membranes and end walls of the vascular system. Wounding of many plant tissues causes increased production of ethylene, which is the hormone-mediated response to the injury. Since Ag^+ has been shown to be a highly specific inhibitor of ethylene action, it seems possible that the formation of vascular occlusions in fronds of maidenhair fern might be a response to ethylene produced by the damaged cells.

"Gum" formation in plants may occur in response to various physiological or pathological disturbances that induce a break-down of cell walls and cell contents, and stimulate the production of growth regulators and enzymes which might degrade the primary wall forming amorphous gel-like occlusions (VanderMolen *et al.*, 1977).

The SEM studies of cut rose stems by Rasmussen & Carpenter (1974) revealed several types of vascular occlusions after 5-10 days in either water or sucrose solutions at 18°C. They found that, (1) the number of tracheids which exhibited blocking at floral senescence was less than 4%; (2) the incidence of blocking was similar at the stem base, water-line and stem neck; (3) breakdown of secondary tissue in the xylem was observed more frequently than other types of occlusions, but this appeared only after 5 days of vase life; (4) occasionally, blocking materials were found protruding through the pits and end plates of tracheids; (5) some tracheids in stems held in a 2% sucrose solution developed occlusions with fungal and bacterial growth or slime plugs; (6) the number of occlusions was not influenced by sucrose in vase water, although the occlusions were much larger and observed after a much shorter vase life (3 days) compared with roses placed in water (5-10 days); (7) misleading clogging materials may occasionally come from the phloem at the time of harvest, or may be dried exudate from the cutting process.

A serial study involving SEM-examinations of the cut surface of hydroponically-grown *Rosa* flowers hybrid Tea-rose cv. Sonia (Sweet Promise) showed that the cut surface very quickly became the site of prolific bacterial and fungal growth (within 2-3 days of vase life), in spite of a low level of microbial contamination of the vase water (i.e. containing only 10^3-10^4 cfu of bacteria per ml). The water uptake was diminished compared with the uptake in biocide-containing vase water, but there was no total blockage, and the longevity of the rose flowers was not decreased dramatically (De Stigter & Broekhuysen, 1986b).

Concerning the mechanism of vascular plugging, Durkin & Kuc (1966) concluded from their experiments with cut rose flowers that, (1) the actual mechanism for plugging remains obscure; (2) water deficiency is induced by vascular blockage; (3) vascular blockage is not necessarily identical with plugging. In fact, in most instances the vascular blockage is not caused by the accumulations of micro-organisms in the vascular system, because experiments conducted under completely sterile conditions have not prevented blockage, nor have microorganisms been recovered from blocked stems. Durkin & Kuc (1966) further concluded that micro-organisms do effect the longevity of cut rose flowers, but such problems only occur after tissue breakdown when the blockage is well advanced. Moreover, it is not widely accepted that xylem conductivity is a good parameter for (1) estimating the water relations of the cut flower; (2) acting as a sensitive indicator for xylem blockage; or (3) indicative of microbial plugging of the vessels.

As already discussed, neither xylem blockage nor water stress need necessarily be caused by micro-organisms plugging the vessels (Accati, 1980 & 1983; Van Alfen *et al.*, 1983). For even when micro-organisms are virtually absent, vascular blockage and water stress may occur, causing a decreased vessel conductivity. Thus the causes and the consequences of the vascular plugging phenomena described remain unclear.

1.6. The selection of a greenhouse rose cultivar as material for post-harvest research

The selection of the rose as material for research is based not only upon the economic importance of rose cultivars in the horticultural production of ornamental plants in the Netherlands; biological scientific and artistic factors have also led to this choice, because:

- the rose possesses a high grade of widely-accepted beauty, which is evident during all the phases of development from the prime bud to mature flower;
- the seven phases of the bud development are well described in the literature and easily differentiated (Berkholst, 1980);
- the ideal vase life of roses placed in an environmentally-controlled room (temperature, relative humidity, day/night exposure to light, ethylene washing) can be the basis for the evaluation of vase life; the influence of various factors can easily be recorded by parameters suitable for statistical processing;
- cut rose hybrids cultivated in the Netherlands form very low amounts of endogenic ethylene (C_2H_4) a plant hormone that regulates the flower senescence. Moreover, the cut rose exhibits a low sensitivity to exogenously- and endogenously- produced ethylene (Woltering & Van Doorn, 1988; Reid *et al.*, 1989a).
- the cut rose is very sensitive towards microbiological activity in variously-modified vase fluids (Put, 1986);
- the starch content of the outer petals of roses gives reliable information concerning undesirable premature picking, unduly long storage periods, or too high storage temperatures, as shown by Berkholst & Navarro Gonzáles (1989);
- the vase life of a 'Sonia' rose cut at phase 1-2 placed in a controlled room at 20°C, is normally 8-10 days. Therefore it is possible to finish one test cycle within 8 days, and carry out new tests weekly;
- fresh roses, of the same quality can be obtained nearly the whole year around;
- the initial microbiological contamination of hygienically picked roses is usually very low, so that with careful handling of the

flowers, activities of the initial microbial flora will not interfere with other factors to be studied (Put, 1990); at the same time the addition of a secondary interfering biocide can be avoided.

- Preliminary investigations

These investigations were designed to ascertain the influence of the stem microflora of *Rosa* 'Sonia', *Gerbera* 'Fleur' and *Chrysanthemum* 'Spider' and their metabolites on the vase life of these three cultivars, by cross-testing the respective microbiological materials obtained from shake cultures of stem segments, as shown in the scheme of Figure 6, using vase cultures in suspensions containing about 10^7 cells per ml vase water. The results may be itemised as follows:

i *Rosa* flowers were much more susceptible to a reduction in water uptake caused by micro-organisms or their metabolites than were *Gerbera*- and *Chrysanthemum* flowers.

ii The effect of micro-organisms (10^7 ml^{-1} vase water) tended to be greater than that of their metabolites.

iii No significant differences in water uptake by the respective cultivars tested were observed, when the effect of adding viable versus heat-inactivated microbial cells to the vase fluid were compared.

iv When comparing the influence of the various metabolic components of the stem microflora, however, it was shown that the *Chrysanthemum* microflora containing the highest number of fungi, caused the most serious disturbance of the water relations and the greatest reduction in the ornamental value of the three cultivars tested (Put, 1986).

- Methods used

Plant material was used immediately, or stored at 4°C for 24-48 h. Flowers were cut with a stem length of 45 cm, the stems were wiped with a paper tissue soaked in ethanol (98% v/v) to minimize contamination, and put singly into cylindrical vases containing resp. 100 ml of (1) sterile tap water, or (2) a sterile 1% (w/v) sucrose solution in tap water, (3) a suspension in sterile tap water of the micro-organism to be tested, (4) a known concentration of a pectic enzyme, (5) $Al_2(SO_4)_3$ solution, (6) the microbial EPS-containing retentate obtained after filtration, (7) the low Mol.wt compound contained in such filtrates. The vases were held at 20°C, 60% r.h. (relative humidity) and with 10.25 W/m^2 photoradiation at flower height during a 12 h light/dark photoperiod each 24 h (Zieslin, 1989).

Vase life measurements were estimated as follows: every 24 h the water uptake (ml), the flowering condition or stage of flowering, and the ornamental value up to a maximum of 8 d of vase life was measured. After 2, (3, 5) and 7 d of vase life the microbiological state of the vase water was assessed. SEM preparations were made after 24-48 h of vase life, a deliberately short contact time chosen, to exclude microbial colonization and to detect any early response of the host vascular system towards the infiltrating micro-organism, pectic enzyme or microbial EPS.

Water conductivity was measured in 3 consecutive stem segments of 5 cm each, after 24 en 48 h of vase life, and microbial EPS was obtained by molecular filtration of the supernatant of a shaken microbial culture. The microbes were removed by centrifuging and pressure filtration. Vascular plugging was also tested by estimating acid fuchsin solution uptake, using 0.5% w/v acid fuchsin in 50% v/v ethanol. A beetroot tissue cube test was devised to demonstrate the ability of micro-organisms to form cell membrane- or cell wall-damaging products. Finally the results were evaluated statistically: analyses of variance were applied to all the measurements and observations of the water uptake, water conductivity, vase life and flower opening of the 'Sonia' roses tested. All test objects involved three replicates, and a few tests were repeated 2-3 times.

- *Microbiological factors investigated*

The initial numbers and types of micro-organisms on stems of freshly harvested cut flower cultivars (*Rosa*, *Gerbera*, *Chrysanthemum*) were determined, and the multiplication ability of the initial stem microflora in vase water was assessed. Further studies assessed the infiltration ability into xylem vessels of pure cultures of stem micro-organisms. The influence of $Al_2(SO_4)_3$ in vase fluid on the infiltration of bacteria up the flower stems was determined by means of scanning electron microscopy, and the influence on the vase life and the xylem morphology of microbial factors in vase water was also investigated. This involved vase experiments with suspensions of known numbers of cells in pure cultures, purified microbial pectic enzymes, and microbial exopolysaccharides.

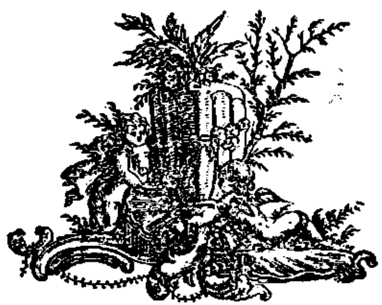
1.7. Vase life experiments

The purpose of the microbiological investigations executed by the author was: (1) to clear-up the uncertain role of micro-organisms on the post-harvest physiology of *Rosa* flowers; and (2) to describe the phenomena concerned therein.

APPENDIX I

PALLADII
RVTIL. TAVRI AEMILIANI
DE
RE RVSTICA

CVRANTE
Jo. MATTHIA GESNERO



MANNHEMII,
Cura & Sumptibus Societatis literatae
MDCCLXXXI.

PALLADII RVT. TAVRI AEMIL.
VIRI ILLVSTRIS,
DE
R E R V S T I C A
LIBER PRIMVS

TITVLVS I.
De praeceptis rei rusticae.

PARS est prima prudentiae, ipsam, cui praecepturus sis, aestimare personam. Neque enim formator agricolae debet artibus & eloquentia rhetores aemulari, quod a plerisque factum est: qui dum diserte loquuntur rusticis, affecuti sunt, ut eorum doctrina nec a disertissimis possit intelligi. Sed nos recidamus praefationis moram, ne, quos reprehendimus, imitemur. Dicendum autem nobis est (si divina faverint) de omni agricultura, & pascuis, & aedificiis rusticis, secundum fabricandi magistros, & aquae inventionibus, & omni genere eorum, quae vel facere vel nutrire oportet agricolam ratione voluptatis & fructus, suis tamen temporibus per universa distinctis. Sane in primis hoc servare constitui, ut eo mense, quo ponenda

A 3

De fodiendis, palandis & ligandis vineis, vel laetandis arboribus, & plantis circumfodiendis.

Tit. XX. Nunc locis maritimis & calidis fodiendae funt vites: vel (fi haec provinciae confuetudo eft) exarandae, & in eifdem locis palandae, aut ligandae funt vineae prius quam gemma procedat, cujus concuffione vel attritu incurritur grande difpendium. Nunc oleae caeteraeque arbores laetamen accipiunt decrefcente luna. Sufficiet autem majori arbori vehes una; minori media; ita ut fubducta a radicibus terra & fimo permifta, revocetur. Tempore hoc, fi quae funt in feminariis plantae, circumfodiendae funt, & amputandi eis rami fuperflui, vel radiculae, quas circa in fuperiore parte miferunt.

De rofis, liliis, croco, violis conferendis.

Tit. XXI. Hoc menfe rofaria conferemus, quae fulco breviffimo aut fcrobibus ponenda funt, vel virgultis, vel etiam femine. Semina autem rofarum non putemus medios flofculos effe aurei coloris, quae rofae fuerunt, fed baccas nutriunt, quas in breviffimi piri fimilitudinem plenas feminibus poft vindemiam reddunt maturas, quarum tamen maturitas ex colore fufco & mollicie poterit

aeftimari. Si qua etiam funt antiqua rofaria, hoc tempore circumfodiuntur farculis vel dolabris, & ariditas univerfa reciditur. Nunc & quae rara funt, poffunt ducta virgarum propagine reparari. Si rofam temperius habere volueris, duobus palmis ab ea [in] gyrum fodies, & aqua calida bis rigabis in die. Nunc & liliorum bulbos ponemus, vel lilia ante habita farriemus fumma diligentia, ne oculos circa radicem nafcentes, & minores bulbulos fauciemus, qui a matre fubtracti, atque in alios digefti ordines, nova lilieta formabunt. Item violarum plantae, & croci bulbi ferendi funt, vel fubtiliter (fi fuerant ante) fodiendi.

De lini femine ferendo.

Tit. XXII. Hoc menfe aliqui lini femen laeto folo in jugerum x modios fpargunt, & lina confequuntur exilia.

De canneis & afparagis & plantis falicum vel geneftae, & feminariis myrti & lauri.

Tit. XXIII. Tempore hoc canneta ponenda funt factis breviffimis fcrobibus, & oculis cannarum per fingulas fcrobes obrutis, qui femipedis fpatio inter fe diftare debebunt. Si calidae & ficcae provinciae ftudemus, valles humidas vel irriguas opus eft deputare

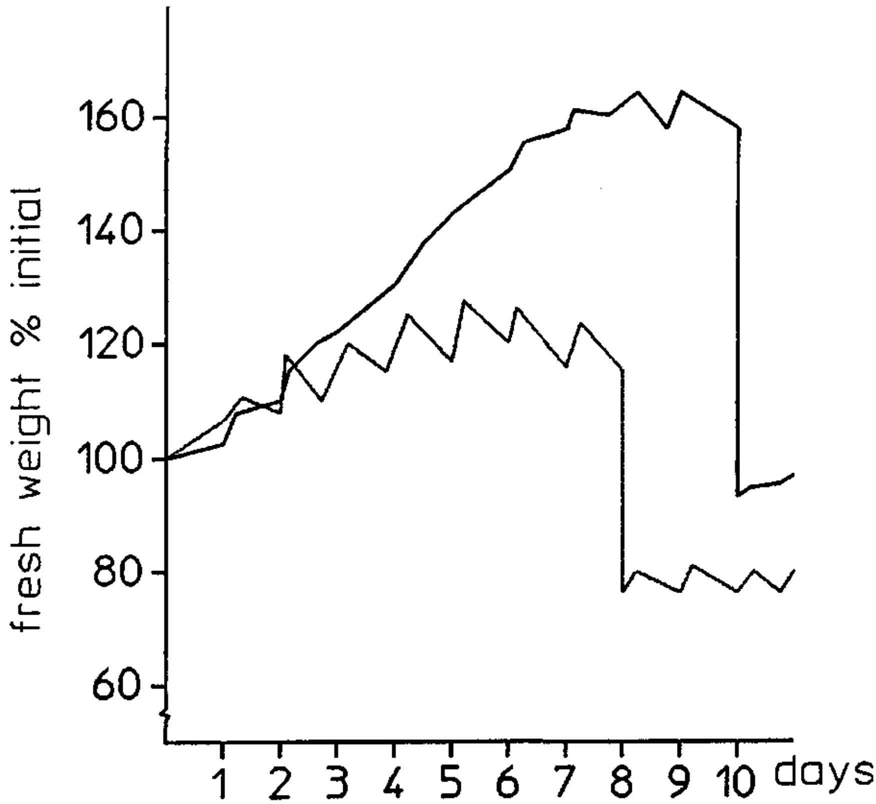


Fig. 1. Fresh weight of cut (broken lines) and intact roses (straight lines) at the end of the 14 h and 10 h light: dark periods, expressed as a percentage of the initial weight. On day 8 and 10 respectively, petals showed symptoms of senescence, and were removed (De Stigter, 1980).

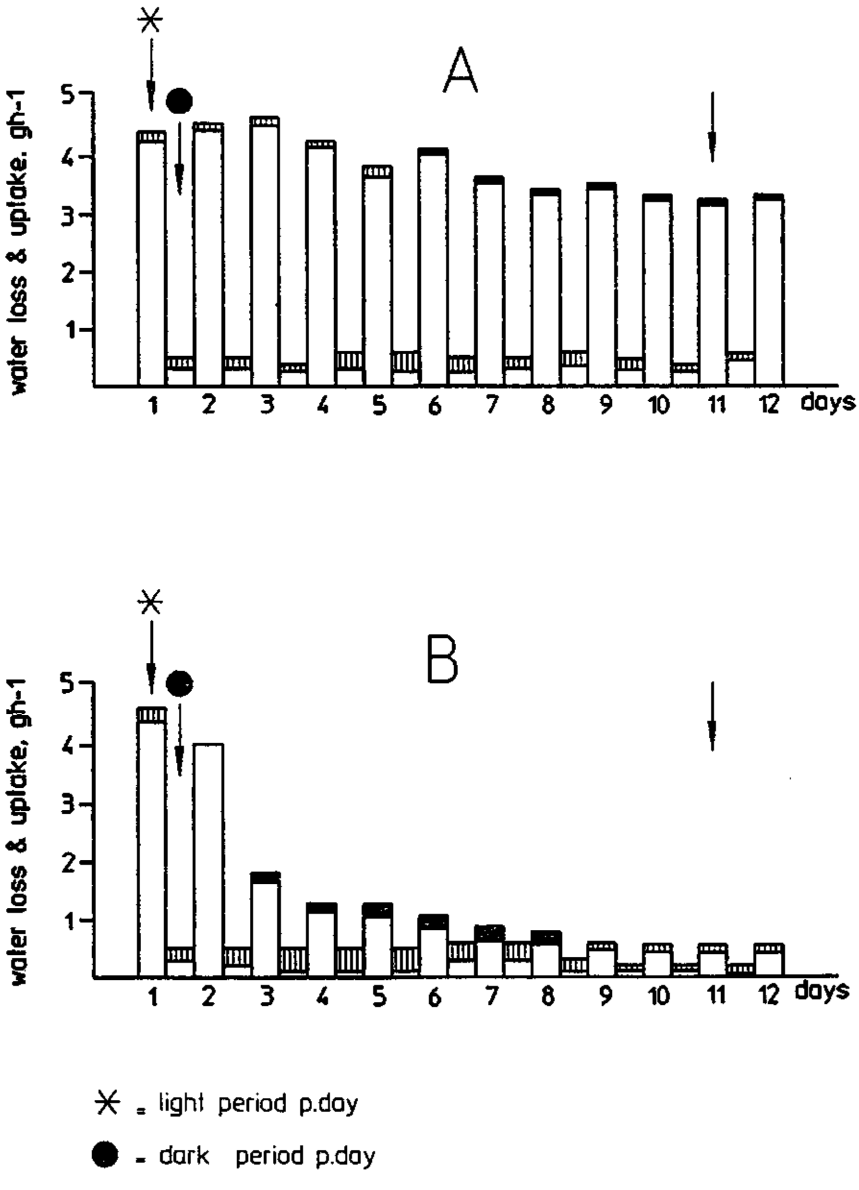


Fig. 2. Water loss and uptake of intact (A) and cut (B) roses, per h in light and dark. Surpluses are vertically hatched, deficits are solid black. Column width corresponds to the duration of light and dark periods: 14 h and 10 h respectively. Arrows indicate time of petal removal, of Fig. 1 (De Stigter, 1980).

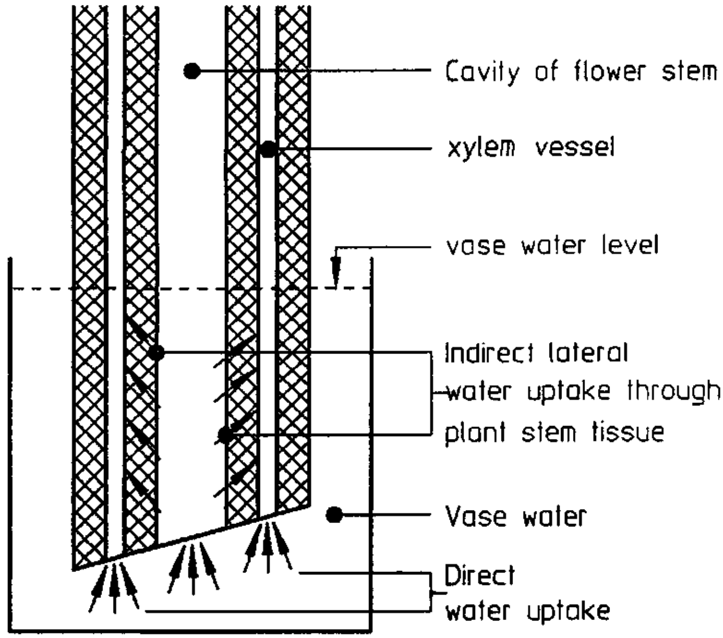


Fig. 3. The two routes of water uptake in a cut flower with a hollow stem (Van Meeteren, 1980).

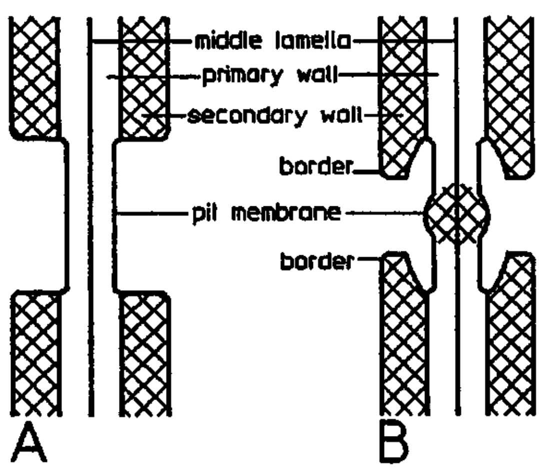


Fig. 4. A diagram to illustrate differences in the structure of pits in plant xylem vessels. A, a normal pit, B, a bordered pit (from Sperry & Tyree, 1988).

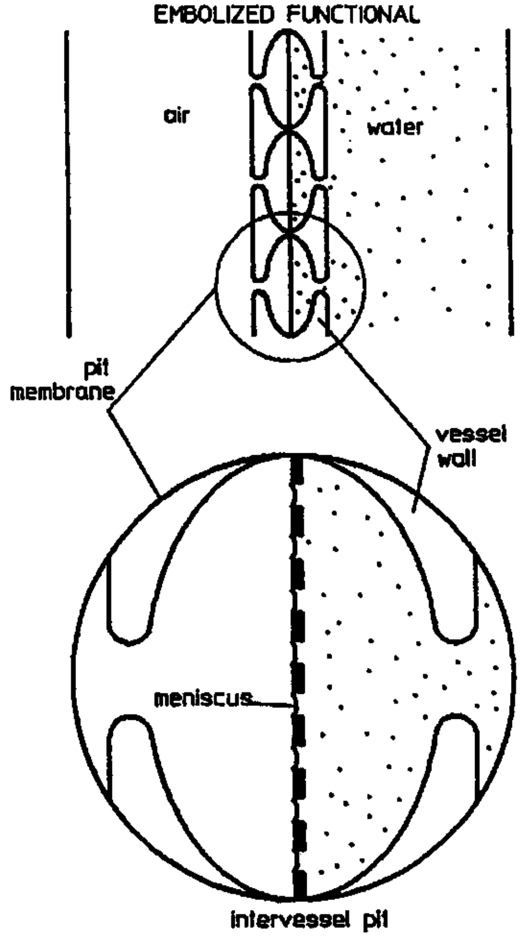


Fig. 5. Diagram of wall structure between adjacent xylem vessels showing intervessel pit structure. The porous pit membrane develops from primary cell walls of the two vessels and middle lamella; it is overarched by thick secondary walls to form a pit chamber that opens to the vessel lumen via a pit aperture. When a vessel is embolized, air is prevented from spreading to adjacent functional vessels by the capillary force of the air-water meniscus spanning pit membrane pores. If the pressure difference across this meniscus exceeds the force holding it there (which is dependent on pore diameter and sap surface tension), air is aspirated into the functional vessel and it becomes embolized, this is the air seeding mechanism of water stress-induced embolism (from Sperry & Tyree, 1988).

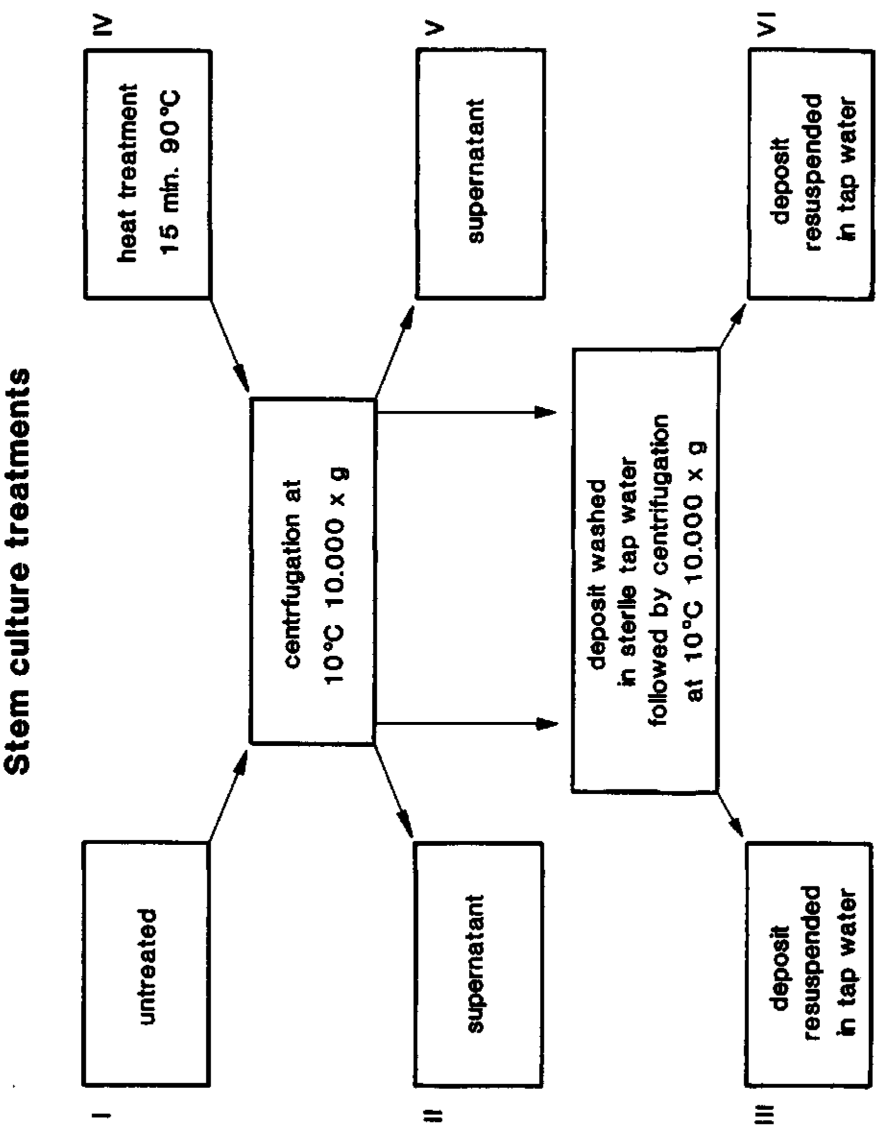


Fig. 6. A summary of the various experimental procedures used to assess the influence of microbial stem cultures on the vase life of cut flowers (Put, 1986).

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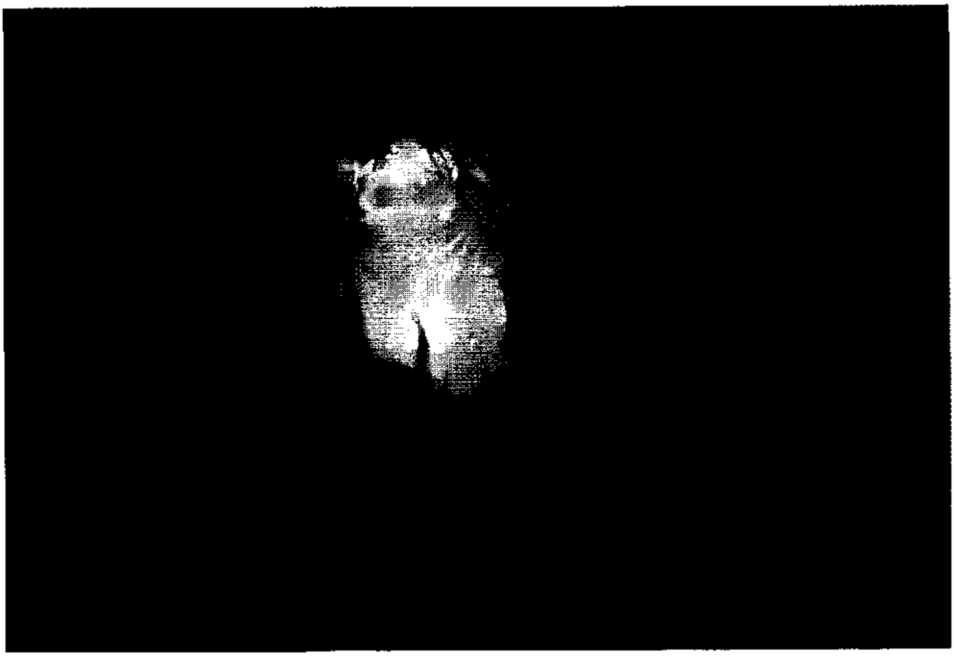
Chapter 2

Micro-organisms isolated from freshly harvested cut flower stems and developing during the vase life of: *Chrysanthemum*, *Gerbera* and *Rosa* cultivars.



Rosa cv. 'Sonia' : stage 2 of flower opening (Berkholst, 1980)

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Micro-organisms from freshly harvested cut flower stems and developing during the vase life of chrysanthemum, gerbera and rose cultivars

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ABSTRACT

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A wide variety of micro-organisms, bacteria and fungi, was isolated from freshly harvested cut flower stems and vase contents of *Chrysanthemum (Dendranthema grandiflora)* cultivar 'Spider', *Gerbera* cultivars 'Appelbloesem' and 'Fleur' and *Rosa* cultivar 'Sonia'. Fungal spp. were isolated much more frequently than by other authors. Bacterial genera, present on the stems, were mainly also present in the corresponding vase water. The dominant initial stem microflora, *Enterobacter, Bacillus* spp. and fungi, lost their dominance in the vase water, which after 3 days of vase life showed a predominance of *Pseudomonas* spp. The longer the vase life, the greater were the changes in the microflora of the vase water, which later again showed a predominance of *Enterobacter* spp. and often also of *Bacillus* spp. After ≥10 days of vase life, fungal growth increased dramatically in *Chrysanthemum* and *Gerbera* vase water. The unique ecological conditions in the vase fluid and, to a lesser extent, the antagonistic activities of many of the microbial species of the mixed vase flora will have led to the initial predominance of *Pseudomonas* spp. and to typical changes in the dominant flora during the course of the flowers' vase life. The microbial load on stems of cut *Rosa* was found to be much lower than those on *Chrysanthemum* and *Gerbera* stems. The end of the vase life of the *Rosa* flowers was characterized by 'normal' senescence symptoms or by weak wilting of leaves and flowers. In *Chrysanthemum* and *Gerbera* however an extensive water stress developed. Further studies are required to evaluate the effects of pure cultures of stem micro-organisms on the vase life of cut flowers. For the prevention of microbial activity in cut-flower vase water, which leads to a shorter flower vase life, good commercial practices are desirable.

Keywords: cut flower; *Chrysanthemum Dendranthema*; flower stem microflora; flower vase microflora; *Gerbera; Rosa*.

Abbreviations: API = Appareils et Procédés d'Identification; CFU = colony-forming units; M.A. = malt extract agar; McK.A. = MacConkey agar; My.A. = mycophyl agar; P.C.A. = plate count agar; Ps.A. (F.P.) = *Pseudomonas* selective agar (Fluorescens and Pyocyanin).

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INTRODUCTION

Bacterial plugging of xylem vessel elements has been claimed to be the major cause of rapid senescence of cut flowers (Aarts, 1957). However this phenomenon has seldom been clearly demonstrated, for in many such studies bacterial numbers have not been adequately assessed and bacterial species causing senescence of cut flowers have rarely been identified (Ford et al., 1961; McClary and Layne, 1977; van Meeteren, 1980; Zagory and Reid, 1986; Put and Jansen, 1989).

Fungal plugging of xylem vessel elements is, on the contrary, reported to be rare (De Stigter and Broekhuysen, 1986).

It has also been suggested that some (usually unidentified) microbial products excreted into vase water by pure or mixed (usually unidentified) microbial species can cause vascular plugging (Gentile and Accati, 1981; Accati-Garibaldi, 1983; Put, 1986; Zagory and Reid, 1986).

In addition, it is not clear whether cut-flower vase micro-organisms originate from the initial stem flora, or may have been introduced accidentally into the vases. There is also little information available on the potential of the microflora for multiplication in vase water.

The present paper gives the types and numbers of micro-organisms isolated from freshly harvested cut flowers. In addition, attempts were made to identify the dominant microflora in their vase water.

MATERIALS AND METHODS

Plant material. – The following plants were studied: *Chrysanthemum morifolium* cultivar 'Spider' (*Dendranthema grandiflora*, Anderson, 1987), *Gerbera* cultivars 'Appelbloesem' and 'Fleur', and *Rosa* hybrid cultivar 'Sonia'.

Harvest of flower cultivars. – The flowers were grown in commercial glasshouses and harvested between February and October. Cut flowers were sampled 2–4 times and each cultivar was harvested from two different glasshouse growers. The harvested flowers were packed into parchment paper, transported to the laboratory within 2–3 h and stored at 2–5°C for <48 h. *Chrysanthemum* and *Gerbera* were cut at <10 cm above the soil. The length of the stem varied between 65 and 75 cm. *Rosa* flowers were harvested by cutting the stems ~1.50 m from the soil at a length of ~65 cm.

Preparation of stem samples for plate counts. – From each cultivar, 10 flowers per test were used. Each test was repeated 2–4 times. The intervals between the tests were ~1 month. From each of the 10 cut flower stems tested, five segments of 4-cm length were cut off from the lower part of the stem and added to 20 ml of sterilized tap water with 0.05% v/v of Tween 80 (Difco) in a sterile screw-capped bottle. The bottles were shaken for 30 min and supernatants, containing only the microflora washed off from the corresponding flower stem, were stored in sterile bottles at 10°C for a maximum of 2–4 h, whereafter microbial analyses of the supernatants were carried out.

Preparation of samples for estimation of the dominant vase water microflora. – Ten flowers of each of the cultivars were further cut off to a stem length of 45 cm and put singly into cylindrical vases containing 100 ml sterile tap water, or a sterile 1% w/v sucrose solution in tap water (Aarts, 1957; Chin and Sacalis, 1977). The effect of 1% w/v sucrose in vase water on the development of the stem microflora was also included in the studies, as 1% w/v sucrose is frequently added to vase water to supplement the energy needed for flowering and to positively affect the balance between solution uptake and transpiration. The depth of the stems immersed in water was ~ 18 cm. The height of the water column in the vases was held between 8 and 18 cm by the addition of sterilized tap water. Protection against contamination was achieved by sealing a plastic film on the top of each vase (Parafilm M; American Can Co.). The vases were held at 20±2°C in an atmosphere of 60% relative humidity and lighting at 10.25 W m^{-2} photoradiation at flower height during alternating 12 h light/dark periods. Samples from the vases were taken aseptically each third day of vase life until flowering senescence. Microbiological analyses were carried out within 2–4 h after sampling.

Media. – The media used were nutrient agar (Difco, 1984; 0001) or plate count agar (Difco, 1984; 0479 at 28°C), and/or both agar media to which 100 μg ml^{-1} of pimaricin (Mycopharm, Delft, The Netherlands) were added to suppress fungal growth. Enterobacteriaceae were counted at 37°C on MacConkey agar (Difco, 1984; 0075), *Pseudomonas* species were grown on *Pseudomonas* selective agar (Oxoid, 1983; CM 457) at 28°C, as well as on *Pseudomonas* F (fluorescens) and P (pyocyanin) medium (Difco, 1984; 0448 and 0449). Fungi were enumerated at 25°C on malt extract agar (Difco, 1984; 0112) or mycophyl agar (BBL, Division of bioquest; 11452, Becton Dickinson and Co., Cockeysville, MD 21030, U.S.A.) to which 50 μg ml^{-1} of globenicol Na succinate (Mycopharm, Delft) and 10 μg ml^{-1} of polymyxin B-sulphate (Pfizer, SA, Brussels) were added to suppress bacterial growth (Smith et al., 1952; Kirchman et al., 1982; Reasoner and Geldreich, 1985; Gould et al., 1985).

Microbial counts of stem flora and vase water flora. – One millilitre or decimal dilutions to a maximum of 10^{-6} ml of the supernatants of the shaken stem samples and vase water samples were pour plated using the different plate count and (selective) agar media. Surface spread plates were also made from the same dilutions using a Spiral Plate Count Machine (Model C, Don Whitley Scientific Ltd., Shipley, West Yorkshire). MacConkey agar plates were incubated at 37 ± 1 °C. The remaining bacterial plates were incubated at 28 ± 2 °C for 2–3 days. This incubation temperature was used to obtain an almost representative and rapid colony formation of psychrotrophic as well as mesophilic flora. For fungal growth, plates were incubated aerobically at 25 ± 2 °C for a maximum of 10 days, whereafter the number of colony-forming units (CFU) was assessed and the numbers of bacteria and fungi per 10-cm stem and per millilitre of vase water were calculated (Tan et al., 1983; Taylor et al., 1983).

Isolation and purification of micro-organisms. – Colonies were examined visually and representatives of all the different colonial types were randomly isolated from both the selective and non-selective media. The randomly selected colonies were examined microscopically. Cultures were then purified prior to further classification.

Identification of micro-organisms. – The final pure cultures, ~350 strains, were grouped on the basis of a preliminary identification by conventional methods (Buchanan and Gibbons, 1974), using the recommended time/temperature conditions.

After allocation to the main groups (bacteria, moulds or yeasts), the bacteria were further characterized using Gram staining, tests for motility and the presence of oxidase, oxygen requirement as revealed by the API M-medium (5012) and fermentative activity as revealed by the API OF medium (5011) (API system S.A. La Balme les Grottes 38390, Montalieu Vercieu, France).

Further identification was achieved using commercially available identification systems (Fung and Cox, 1981; Fung et al., 1984). The OXI/FERM tube II (F. Hoffmann-La Roche and Co. Ltd., Diagnostica, CH-4002 Basle, Switzerland) was used for the identification of oxidase-positive Gram-negative rods (Stanier et al., 1966). The presence of *Pseudomonas aeruginosa* was confirmed by growth at 42.5 °C. Enterobacteriaceae were identified by the Enterotube II method of F. Hoffmann-La Roche (Leclerc, 1962; Ewing and Fife, 1972; Dietsch, 1981; Cox et al., 1983). The presence of *Escherichia coli* (and pathogenic Enterobacteriaceae) was confirmed by growth at 42.5 °C; selective biochemical and/or serological tests were also carried out. When inconclusive results were obtained, the API systems 20 NE or 20 B (API-system SA, La Balme les Grottes 38390, Montalieu, Versieu, France) were applied (Lampe and van der Reyden, 1984). For *Bacillus* strains, the Enterotube II (La Roche) and API 50 CH systems were used, in combination with classical tests for the hydrolysis or decomposition of casein, gelatin and lecithin (Smith et al., 1952; Gordon et al., 1973; Berkeley and Goodfellow, 1981; Gil et al., 1986). The formation of bacterial polysaccharides, dextran and levan was detected on API sucrose agar code 5013 (Facklam, 1977). The identification of most of the *Bacillus* strains was cross-tested by the API Research Laboratories at Montalieu using a combination of API 50 CH and 20 E (Logan and Berkeley, 1981, 1984).

Fungal strains were identified at the Centraal Bureau voor Schimmelcultures (C.B.S.), Baarn, The Netherlands (Alexopoulos and Mims, 1979; Domsch et al., 1980; Kreger-van Rij, 1984).

Additional tests were applied to several strains of some of the bacterial and fungal species, which owing to their ability to multiply in vase water, might have had a significant role in the reduction of the vase life of cut flowers. These tests were the decomposition of pectin by the method of Wieringa (1956) or Perombelon and Hyman (1986), the hydrolysis of carboxy-methylcellulose (CMC) as described by Skerman (1959) and Robson and Chambliss (1984), the formation of exopolysaccharides on sucrose agar (API) and the minimum temperature for growth at 5–25°C in sucrose (0.1% w/v) flower stem extract broth (0.1% w/v).

Microscopic observations of the vase water. – Thin smears of the vase water samples incubated at 20°C were fixed and Gram stained. The fungi-containing samples were also examined using cotton blue lactophenol. In addition, preparations from vase water samples taken during the course of the incubation were studied by phase-contrast microscopy.

RESULTS

Microbial counts on stems. – The number of viable bacteria and fungi on freshly cut flower stems ranged from $< 10–10^4$ CFU per 10-cm length of stem (Table 1). For *Chrysanthemum*, 60 and 30% of the 40 flower stems tested contained $\geqslant 10^2$ viable bacteria and fungi, respectively, per 10 cm of stem, whereas 45 and 52% of the 40 *Gerbera* flower stems tested contained $\geqslant 10^2$ viable bacteria and fungi, respectively, per 10 cm of stem. However only 4 and 20% of the 50 *Rosa* stems tested contained $> 10^2$ viable bacteria and fungi, respectively, per 10 cm of stem. Moreover, it was observed that for 86 and 56% of the 50 *Rosa* stems tested, $\leqslant 10$ viable bacteria and fungi, respectively, were isolated per 10-cm length of stem. Thus the microbial load on stems of cut *Rosa* was much lower than that on the stems of the *Chrysanthemum* and *Gerbera* cultivars tested.

Microbial counts of vase water. – (1) *Chrysanthemum* and *Gerbera* cultivars. During the first 6 days of vase life, bacterial numbers exceeded a level of 10^6 CFU ml^{-1} vase water several times. Maximum numbers of CFU counted were $\geqslant 10^8$ bacteria and $> 10^7$ fungi ml^{-1} vase water (Table 2). (2) *Rosa* 'Sonia'. During the first 6 days of vase life, bacterial numbers remained $< 10^6$ CFU

TABLE 1

Estimation of the numbers of micro-organisms on 20 or 50 stems of freshly harvested cut flowers. For bacteria on plate count agar, incubated for 2–3 days at 28 °C; for fungi on malt extract agar incubated for 6–10 days at 25 °C

Flower cultivar	Micro-organisms	Percentages of stem segments of 10 cm length showing plate count numbers of:					Actual no. of stems
		0	1–10	10^1–10^2	10^2–10^3	10^3–10^4	
Chrysanthemum							
'Spider'–white	Bacteria	10	10	10	50	20	20
	Fungi	20	20	30	30	<5	20
Chrysanthemum							
'Spider'–yellow	Bacteria	20	10	20	50	<5	20
	Fungi	10	30	30	20	10	20
Gerbera							
'Appelbloesem'	Bacteria	10	20	20	50	<5	20
	Fungi	10	10	10	60	10	20
Gerbera							
'Fleur'	Bacteria	10	25	25	40	<5	20
	Fungi	15	20	30	30	5	20
Rosa							
'Sonia'	Bacteria	16	70	10	4	<2	50
	Fungi	8	48	24	12	8	50

ml^{-1} vase water (Table 2). During the prime senescence phase of the 'Sonia' flowers, the number of CFU counted seldom exceeded 10^6 ml^{-1} and fungal counts remained $< 5 \times 10^4$ ml^{-1} vase water.

Identification of stem microflora (Table 3). – The predominant bacteria isolated from freshly harvested cut flower stems were species of the genera *Enterobacter (E. agglomerans* and *E. cloacae*) and *Bacillus (B. subtilis, B. licheniformis, B. cereus). Pseudomonas* spp. were isolated occasionally. The predominant mould genera were *Cladosporium, Fusarium, Penicillium, Mucor* and *Rhizopus.*

It was furthermore observed that low initial numbers of micro-organisms on an individual stem mainly consisted of a wide variety of the flora.

Identification of vase water microflora. – During the first 3–6 days of vase life, *Pseudomonas cepacia, Pseudomonas fluorescens* and *Pseudomonas putida* replaced the initially dominant *Enterobacter* and *Bacillus* stem flora. However after > 6 days of vase life, *Enterobacter* spp. again became more dominant

TABLE 2

Enumeration of selected micro-organisms in vase water of three vases per cultivar, after 3–15 days of vase life (CFU ml^{-1} vase water (sterilized tap water))

		Micro-organisms					
		Fungi	Bacteria	Enterobacteriaceae	*Pseudomonas*	Other[1] Gram-negative bacteria	Gram-positive bacteria (*Bacillus*)
Medium used:		M.A./My.A.	P.C.A.	McK.A.	Ps.A. (F.P.)	P.C.A.	P.C.A.
Incubation temp.:		25°C	25°C	37°C	28°C	28°C	28°C
Flower cultivar	Vase life (days)	Min. Max.	Min. Max.	Min. Max.	Min. Max.	Average	Average
Chrysanthemum	3	15 -5×10^3	8×10^3–5×10^5	10^2–2×10^4	2×10^4–4×10^5	3×10^4	4×10^3
'Spider'	6	8×10^2–5×10^4	6×10^4–8×10^6	2×10^3–5×10^4	5×10^5–8×10^6	5×10^6	10^4
	9	2×10^4–6×10^6	5×10^6–$>10^8$	10^6–$\leqslant10^8$	10^7	$<10^6$	$\geqslant10^6$
	12*	$\geqslant10^7$	$\geqslant10^8$	$\geqslant10^4$–10^8	$>10^6$–$\geqslant10^7$	$>10^6$–$<10^7$	$\geqslant10^7$
	15**	$\geqslant10^7$	$>10^7$	$\geqslant10^8$	$>10^6$–$<10^7$	$>10^6$–10^7	10^8
Gerbera	3	5×10^2–8×10^3	2×10^3–4×10^5	2×10^2–4×10^3	5×10^3–5×10^5	8×10^3	6×10^2
'Appelbloesem'	6	4×10^2–10^5	8×10^4–$\geqslant10^6$	5×10^2–6×10^4	4×10^5–5×10^6	5×10^4	2×10^5
and 'Fleur'	9	2×10^5–3×10^5	$\geqslant10^7$–$\leqslant10^8$	2×10^6–10^8	8×10^6–$\leqslant10^7$	$\leqslant10^6$	$>10^6$
	12*	$>10^7$	$\geqslant10^8$	$\geqslant10^8$	$\geqslant10^6$–$\leqslant10^7$	$\geqslant10^6$–$<10^7$	$>10^6$
	15**	$>10^7$	$\geqslant10^8$	$\geqslant10^8$	$\geqslant10^6$–$\leqslant10^7$	$\geqslant10^6$–$<10^7$	10^8
Rosa	3	<10–25	55–2×10^2	<10	3×10^2	<10	<10
'Sonia'	6	<10–2×10^3	5×10^2–2×10^5	2×10^2–5×10^3	5×10^2–5×10^5	4×10^2	6×10^2
	7–9***	5×10^2–3×10^4	6×10^3–4×10^6	3×10^5–5×10^6	4×10^4–6×10^5	2×10^5	$\leqslant10^6$

[1]With the exception of Enterobacteriaceae and *Pseudomonas* spp.
*Wilting and leaf chlorosis; **wilting and stem curving; ***end of *Rosa* vase life, flower senescence.
M.A. = malt extract agar; My.A. = mycophyl agar; P.C.A. = plate count agar; McK.A. = MacConkey agar; Ps.A. (F.P.) = pseudomonas selective agar (Fluorescens and Pyocyanin).

TABLE 3

Identification of the microflora from freshly harvested cut flower stems (s) and present in the vase water (v) after 3–12 days of vase life

Micro-organism	Flower cultivar					
	Chrysanthemum white/yellow 'Spider'		*Gerbera* 'Appelbloesem' and 'Fleur'		*Rosa* 'Sonia'	
	s	v	s	v	s	v
Gram-negative rods						
Acinetobacter **sp.***	–	+	–	–	–	–
Achromobacter sp.*	–	+	–	–	–	–
Alcaligenes sp.*	–	–	+	+	–	+
Chromobacterium violaceum	+	–	–	–	–	–
Citrobacter freundii	–	–	–	–	+	+
C. var. *amalonaticus*	–	–	–	–	–	+
Enterobacter						
E. agglomerans	+	+	+	+	+	+
E. cloacae	–	+	+	+	+	+
E. gergovinae	–	–	–	+	–	–
E. sakazaki	+	–	+	–	–	–
Enterobacter sp.*	+	–	–	–	–	+
Erwinia						
*Er. amylovorum**	+	–	+	–	–	–
*Er. herbicola**	+	–	+	–	–	–
Flavobacterium sp.*	+	–	–	–	–	+
Pseudomonas						
P. aeruginosa	–	+	–	–	–	–
P. cepacia	–	+	–	–	–	–
P. fluorescens	–	–	+	+	+	–
P. maltophilia	–	+	–	–	–	+
P. putida	–	+	+	+	–	+
P. putrefaciens	–	–	–	–	–	+
P. stutzeri	–	–	+	–	–	–
P. vesicularis	–	+	–	–	–	–
Pseudomonas sp.*	+	–	+	–	+	+
Gram-positive rods						
Bacillus						
B. cereus (lecithinase –)	+	–	+	+	+	+
B. cereus (lecithinase +)	–	+	–	–	–	+
B. circulans	–	–	–	+	–	–
B. licheniformis	+	–	+	–	–	+
B. mycoides	–	+	–	+	–	–
B. polymyxa	+	+	+	+	–	+
B. subtilis	+	+	–	+	–	+
B. subtilis var. *niger*	+	+	+	+	+	–
B. thiaminolyticus	–	–	–	+	–	–

TABLE 3 *(continued)*

Micro-organism	Flower cultivar					
	Chrysanthemum white/yellow 'Spider'		*Gerbera* 'Appelbloesem' and 'Fleur'		*Rosa* 'Sonia'	
	s	v	s	v	s	v
Corynebacteria*	–	–	–	–	–	+
Gram-positive cocci						
Streptococcus						
S. lactis group*	–	+	–	+	–	–
Fungi						
Acremonium alternatum Link: Fr.	+	–	+	–	–	–
Aureobasidium pullulans (de Bary) Arnoud	+	–	–	+	–	–
A. pullulans var.*	–	–	–	+	–	–
Botrytis cinerea Pers.: Fr.	–	–	–	+	+	–
Botrytis sp.*	–	–	–	–	–	+
Cladosporium cladosporides (Fres.) de Vries	+	–	+	–	+	–
C. sphaerospermum Penz.	+	–	–	–	–	–
Drechslera sorokiniana (Sacc.) de Vries	–	–	–	–	+	–
Fusarium solani	–	+	+	–	+	–
F. tabacinum var. *plectosphaerella cucumerina* (Lindfors) W. Gans	+	–	–	–	–	–
*F. oxysporum**	–	–	+	+	–	+
Mucor hiemalis (Wehmer)	–	+	+	+	–	+
M. plumbeus Bon	–	–	+	–	–	–
M. racemosus	–	–	–	+	–	–
Penicillium brevicompactum (Dierckx)	+	–	+	–	+	–
P. brevicompactum atyp. I*	+	–	+	–	–	–
P. brevicompactum atyp. II*	+	–	+	+	+	–
P. purpurogenum Stoll	–	–	+	–	+	–
P. variable	+	–	–	–	–	–
Penicillium sp.*	+	–	–	–	–	–
Rhizopus stolonifer (Ehrenb. ex Link) & Vuill	+	+	+	+	+	–
Rhizopus sp.*	+	–	+	–	–	+
Trichoderma pseudokoningii Rifai	–	+	–	–	+	–

TABLE 3 (*continued*)

Micro-organism	Flower cultivar					
	Chrysanthemum white/yellow 'Spider'		*Gerbera* 'Appelbloesem' and 'Fleur'		*Rosa* 'Sonia'	
	s	v	s	v	s	v
Verticillium albo-atrum (Reinke and Berthold)	–	–	+	–	–	–
V. brevicompactum	+	–	–	–	–	+
V. psalliotae Treschow	–	–	+	–	–	–
Yeasts						
Candida albicans	–	+	–	+	–	–
C. famata	–	+	–	–	–	–
Candida sp.*	–	+	–	+	+	+
Kluyveromyces marxianus	–	–	–	+	–	–
Rhodotorula mucilaginosa	–	+	–	–	–	–
R. rubra var. I*	–	+	–	+	–	+
R. rubra var. II*	–	–	–	+	–	–
*Saccharomyces**	+	–	–	–	–	+

*Not further identified, some characteristics differ from the official descriptions of the characteristics of that genus or species.

(*E. agglomerans* and *E. cloacae*). Furthermore, *Bacillus* spp., *Erwinia* and also non-fermenting Gram-negative bacteria predominated later.

Fungal activity in vase water was low until after >6 days of vase life of *Chrysanthemum* and *Gerbera* cultivars; *Cladosporium, Fusarium, Penicillium* spp. and also some yeast genera were most frequently isolated (*Candida* and *Rhodotorula*).

Sucrose (1% w/v) added to the vase water initially induced a certain increase (about a factor of 10–50) in microbial multiplication rates and probably also a shift in the predominance of carbohydrate-dependent microbial species; after ~6 days of vase life these differences became less marked.

Additional tests on the identified (dominant) flora. – The results of the additional tests indicated the following.

(1) Some *Bacillus* spp., some *Pseudomonas* spp., *Kluyveromyces* strains and all the isolated mould strains hydrolysed pectin.

(2) Adhesive capsular slime was formed by *Bacillus* spp., *Pseudomonas* spp., some *Enterobacter* spp. and the *Rhodotorula* strains.

(3) Some *Bacillus* spp., some *Pseudomonas* spp. and some fungus spp. formed exopolysaccharides.

(4) The isolated *Fusarium oxysporum* and *Botrytis cinerea* strains belong to phytopathogenic genera (Domsch et al., 1980); they were shown to produce plant cell membrane-injuring compounds (beetroot test, Put and Klop, 1990).

(5) Cellulase was of minor importance due to the slow production of this enzyme.

DISCUSSION

Enumeration and identification of cut-flower stem microflora. – Most of the microbial species isolated from the cut flower stems are normal inhabitants of the upper layer of agricultural soil (Do Carmo-Sousa, 1969; Buchanan and Gibbons, 1974; Domsch et al., 1980) and the non-soil micro-organisms were probably introduced by other sources of exogenous contamination (Ford et al., 1961; McClary and Layne, 1977; Henis and Bashan, 1986). Some of the microbial species isolated, e.g. *Enterobacter agglomerans* and *Erwinia amylovora*, are commonly associated with plants (Lasko and Starr, 1970; Verdonck et al., 1987). *Pseudomonas cepacia* and *Bacillus polymyxa* are phytophagens, but the majority are common soil saprophytes or epiphytic constituents of the phyllosphere. In the phyllosphere of herbaceous plants, yeasts as well as filamentous fungi are usually found, although in lower numbers compared with bacteria (Last and Price, 1969; Buchanan and Gibbons, 1974; Davenport, 1976; Domsch et al., 1980; Kreger-van Rij, 1984).

The numbers of micro-organisms on *Rosa* stems were found to be much lower than those on *Chrysanthemum* and *Gerbera* stems (Table 1), which probably reflects their distance from the soil. Furthermore, the hairy epidermis of *Gerbera* (van Meeteren, 1980) and the numerous tiny side leaves of the *Chrysanthemum* stem (Henis and Bashan, 1986) probably facilitated the adherence of soil micro-organisms to the stems. Comparison of data presented here with data reported by other authors could not be made because published data concerning cut-flower stem microflora are not available.

Enumeration of vase water microflora. – Multiplication of micro-organisms in the vase water containing the cut flowers was relatively slow (Table 2). The microflora initially present on the stem of freshly harvested cut flowers may germinate (spores) and multiply in vase water, but exhibits a comparatively long generation time due to the nutrient-poor vase water growth medium. The addition of 1% w/v sucrose initially induced an increase in microbial multiplication rates and a shift in the predominance of carbohydrate-dependent microbial species, which later became less marked, probably due to the leakage of growth-promoting compounds such as carbohydrates and proteins from phloem vessels and injured plant cells (Kooy et al., 1982; Woltering, 1987). The competition between the different microbial genera and species might then have been of greater influence than the ability of the individual microbial cell to multiply in tap water or sucrose–tap water, enriched with compounds leaking out of cut flower vessels.

Identification of vase water microflora. – Most of the microbial genera present on the stems were also present in the vase water, but some of them were not. Equally, many of the microbial species developing in the vase water were not always detected on the corresponding stems.

These findings indicate: (1) the distribution of the micro-organisms on the stems is not homogeneous, so the microbial species present on 10 cm of the lower parts of the stems, used for plate counts and identification of the stem flora, will not have been true replicates of the microflora on the upper parts of the stems, washed off by the vase fluid; (2) the selectivity of the isolation method used may have failed, comparing single populations of micro-organisms adhering to a flower stem and a competitive mixed flora of the vase fluids.

The fact that *Pseudomonas* spp. were more frequently isolated from cut-flower vase waters than *Enterobacter* spp. may be because most *Pseudomonas* spp. do not require organic growth factors and can multiply readily in diluted aqueous media (Kooy et al., 1982). Enterobacteriaceae often have more demanding growth requirements so their multiplication may have accelerated after the leakage of carbohydrates (glucose, fructose, sucrose), proteins and growth factors from the disrupted plant tissues (Konings and Veldkamp, 1980; Zimmermann, 1983; Woltering, 1987).

E. agglomerans (Erwinia herbicola) showed yellow-pigmented colonies on plate count agar and may have been confused with other yellow-pigmented Gram-negative organisms (e.g. *Pseudomonas, Flavobacterium*) by other authors, and thus may not always have been recognized (Leclerc, 1962; Graham, 1964; Graham and Hopkins, 1967; Lampe and van der Reyden, 1984; Verdonck et al., 1987).

The predominance and shift in predominance of certain microbial genera in vase water, e.g. *Bacillus, Enterobacter, Pseudomonas* and fungi, during the course of vase life, which were mainly not correlated with the specific host plant, indicate that micro-organisms developing in the cut-flower vase fluid are more a function of the unique ecological conditions in the vase than of the micro-organisms initially present on the stems.

The effects of the vase microflora on the vase life of the flowers. – Many of the stem micro-organisms might cause a decreased vase life and a rapid senescence of cut flowers, especially if they are able to multiply in vase water to $>10^6$ ml^{-1} because: (1) microbial propagules may then be able to cause physical plugging of the xylem vessels and disturbance of water uptake (Put and Jansen, 1989); (2) toxic microbial compounds may be excreted into the vase water and accelerate senescence (Lasko and Starr, 1970; Accati-Garibaldi, 1983; Put, 1986; Zagory and Reid, 1986; Leary et al., 1986; Put and Klop, 1990; Put and Rombouts, 1989); (3) it is uncertain whether or not sound cut flowers can be affected pathologically by fungi during their fairly short vase life; although *Fusarium oxysporum*, which multiplied in the vase water, induced wilt symptoms in pot-grown carnations within 4 weeks after soil, root or stem inoculation (Demmink et al., 1987; Baayen et al., 1988).

Published data concerning vase water flora. – It was shown that the materials and methods used were mainly different and hardly comparable, as shown by the following examples.

(1) Dansereau and Vines (1975) reported on the vase water and xylem fluid flora of snapdragon cultivar 'Tampica'; Marousky (1980), in his bacteriological in vivo and in vitro vase water experiments, used snapdragon cultivar 'Oklahoma' and gladiolus cultivar 'White Friendship'.

(2) Gardner et al. (1982) identified the xylem-residing bacteria in roots of Florida citrus trees.

(3) Accati-Garibaldi (1983) analysed vase water of carnation cultivar 'Siva' flowers.

(4) Samples were mainly taken from vase water of senesced flowers (Aarts, 1957; Dansereau and Vines, 1975; Accati-Garibaldi, 1983; Zagory and Reid, 1986).

(5) Only small numbers of representative colonies (25–70) were isolated and identified by Ford et al. (1961), McClary and Layne (1974), Zagory and Reid (1986) and de Witte and van Doorn (1988).

(6) Gardner et al. (1982) isolated randomly 850 colonies and identified 550 pure cultures (see item (2) above).

(7) Numbers of isolated colonies were not given by Aarts (1957) and Accati-Garibaldi (1983).

(8) Human pathogenic micro-organisms were not found in the present study. Authors who reported the isolation of such bacteria (Ford et al., 1961; McClary and Layne, 1977) isolated them from cut-flower vase water or the flower containers, which were not treated aseptically.

(9) Plate counts of fungi were only reported by Zagory and Reid (1986) and data on the initial microflora of cut flower stems were not reported previously. In the present study, low numbers of fungi were found on the stems, many different species were identified and their distribution was not correlated with the specific host plant.

Further research. – The results presented here are very preliminary from a horticultural standpoint. Further research is needed to reveal the role which micro-organisms may play in the mechanisms leading to a shorter flower vase life. Therefore much more data need to be available on the initial microflora on stems of freshly harvested cut flower cultivars, the ability of pure cultures of the main stem and vase water microflora to multiply in vase water needs to be assessed as well as the effects of pure cultures of these micro-organisms on the water uptake and vase life of the cut flower cultivars concerned. These effects may consist of mechanical plugging of xylem vessels, physiological changes of the water relationships of the cut flower, morphological changes of the xylem vessel structure following elicitor or enzymatic activities and (phyto)toxicity of the microbial vase water culture.

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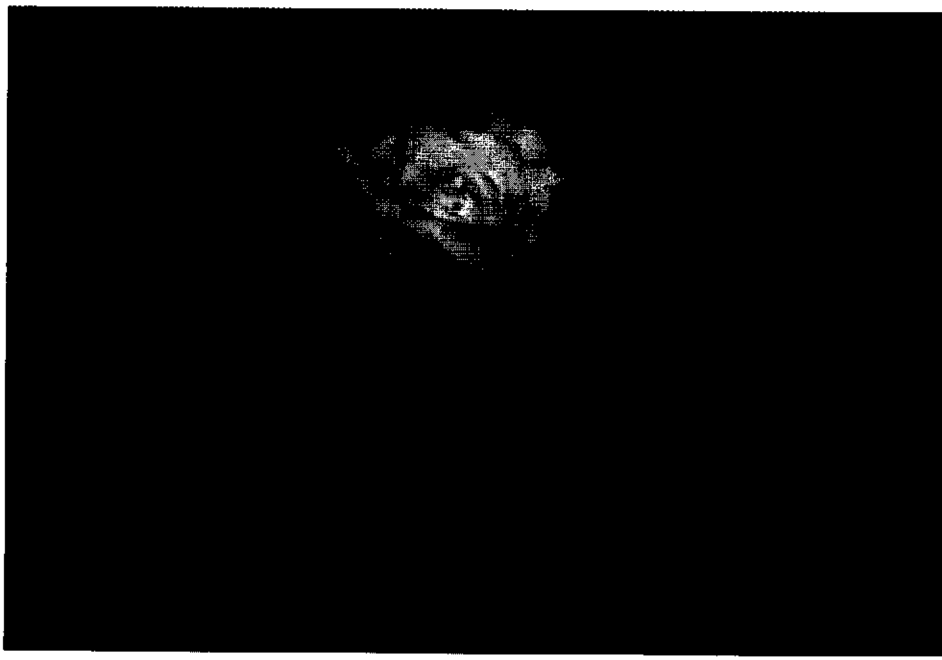
Chapter 3

Infiltration of *Pseudomonas putida* cells, strain 48, into xylem vessels of cut *Rosa* cv. 'Sonia'.



Rosa cv. 'Sonia': stage 3 of flower opening (Berkholst, 1980)

Infiltration of *Pseudomonas putida* cells, strain 48, into xylem vessels of cut *Rosa* cv. 'Sonia'

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Pseudomonas putida cells were unable to pass the inter-vessel pit membranes of the xylem system of cut roses (*Rosa hybrida* cv. 'Sonia'). It was further shown that (1) the number of bacteria which infiltrated into the xylem vessels decreased with increased distance between the cutting point and sampling point; (2) the number of bacteria which infiltrated into the open xylem vessels increased with time and with increasing numbers of pseudomonas cells; (3) only a minor part of the pseudomonas cells homogeneously suspended in the vase solution was able to infiltrate into the xylem vessels of the cut roses up to a distance from the cutting point of >1 cm; and (4) even low levels of infiltrated pseudomonas cells could be demonstrated by measurements of the water conductivity of stem segments. More research is needed to reveal which mechanisms (e.g. gumnosis) might have contributed, directly or indirectly, to the prevention of further infiltration of bacterial particles into the cut open vascular system of the *Rosa* cultivar.

Good keeping quality of harvested cut flowers is essential for increasing production of The Netherlands flower industry. Roses are one of the most important economic flower crops, but their post-harvest life is limited by several factors (Aarts 1957). For example, cut flowers have a shorter life than uncut flowers even when the conditions are optimal (Stigter 1980). Rose life may be further shortened by bacteria and their products which may infiltrate from vase water into the cut flower xylem, causing loss of water flow, wilting and flower senescence (Put 1986). The xylem consists of numerous short vessels (2·5–50 μm wide and 2–250 mm long) with inter-vessel pit membranes containing capillary holes of about 50 nm, which are too small for bacteria to pass through (Van Alfen *et al.* 1983; Zimmermann 1983; Stigter & Broekhuysen 1986). It is not known if micro-organisms can penetrate into the xylem system using the inter-vessel membrane pathway of cut flowers like roses and this paper reports on such an investigation.

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Materials and Methods

PLANT MATERIAL

Plants of *Rosa hybrida* cv. 'Sonia' were supplied by local glasshouse growers. The roses were harvested between 15 February and 15 March 1986 at flowering stage 1–2 (Berkholst 1980). They were wrapped in parchment-like paper, transported to the laboratory, stored at 4°C for 24–48 h and the experiments were then carried out immediately.

MICRO-ORGANISMS

Pseudomonas putida strain 48 was isolated from the vase water of cut roses and identified by the Oxy-ferm Tube II Test method (F. Hoffmann-La Roche & Co. A.G., Diagnostica, CH-40002, Basel, Switzerland). This strain does not form phytotoxic compounds or produce pectinolytic or cellulolytic enzymes.

VASE-LIFE EVALUATION OF *Rosa* CV. 'SONIA'

The rose stems were cut aseptically to a standard length of 45 cm and each was placed in a cylindrical vase calibrated up to 100 ml. All leaves except the upper three were removed from the rose stems. The vases contained sterile tap water (control) or a freshly prepared water-washed suspension of *Pseudomonas putida* strain 48 containing a known number of viable bacteria per ml. Protection against contamination was obtained by placing a plastic film on the top of each vase (Parafilm 'M', American Can Co). Each test consisted of comparable triplicates and was repeated three times.

Vase-life was evaluated in a room maintained at a temperature of 20° $\pm$ 1°C, a relative humidity (RH) of 60 $\pm$ 2% and with a 4000 lux light (10 W/m^2) with a 12 h photoperiod during each 24 h.

At the conclusion of each experiment the sterility of the control vase solutions was tested as well as the purity and the number of *Ps. putida* cells in the test vase suspensions. In addition, the water conductivity of stems of roses held in a vase of water which initially contained 10^4–10^8 *Ps. putida* cells/ml was measured after 24 h of vase-life. The water uptake was measured at 24 h intervals by reading the water column of the calibrated cylindrical vases in ml. To exclude any microbial contamination the flowers were not taken out of their vases for manipulations, such as measurement of the water evaporation of the flower.

The apparatus for measuring water conductivity consisted of an overhead 10 l bottle filled with glass-distilled water and connected by medical plastic tubing to a glass manifold of 24 pieces. A Mariotte tube was inserted through a rubber stopper into the 10 l bottle to maintain constant water pressure. The distance or height of the water column measured from the base of the Mariotte tube to the upper end of the stem segment was 60 cm. A piece of soft plastic tubing was attached to each T-piece for connection to a single stem segment (Mariotte, E. Dyon 1620-Paris 1684).

Triplicate samples of roses were taken from the vase water and three consecutive stem sections of 5 cm each were cut immediately under water from the basal end upwards and inserted onto the manifold in an upside-down position. The water solution was then allowed to flow through the segments for 60 min to purge air in the lines and to obtain an equilibrated water flow. Thereafter the lower end of each stem segment was connected to a plastic tube of known weight. The water flow through the segments was assessed by weighing of the tubes after 30, 60 and 90 min of water flow respectively. The average water conduction per 30 min was then calculated (Durkin 1979). Conductivity was defined as the reciprocal value to resistivity, i.e. resistance to flow, expressed per transverse-sectional area of 5 cm stem length (Fig. 1; Gilman & Steponkus 1972; Chin & Sacalis 1977).

Observations were made of the condition, the ornamental value and the flowering stage of the roses (Fig. 2; Berkholst 1980) at 24 h intervals.

SAMPLING OF STEM SEGMENTS OF ROSES FOR BACTERIAL COUNTS

Roses were removed from their vases and the outer part of the stem was carefully disinfected with ethanol (98%, v/v). They were then cut into four segments, each 5 cm long. From points 0, 5, 10 and 15 cm respectively, 1 cm was cut and the epidermis and cortex of the stem particles were peeled off to avoid possible introduction of micro-organisms attached to the outer epidermis of the stem. The xylem part of each stem sample was placed in a disposable pre-sterilized screw-capped polystyrene tube, 16 $\times$ 125 mm (Falcon Bioquest, Los Angeles, USA), containing 5 ml of sterile tap water plus 0·05% v/v of Tween-80 (BDH). After vortex mixing for 1 min, decimal dilutions were made in sterile tap water. A laminar flow cabinet was used for aseptic manipulations.

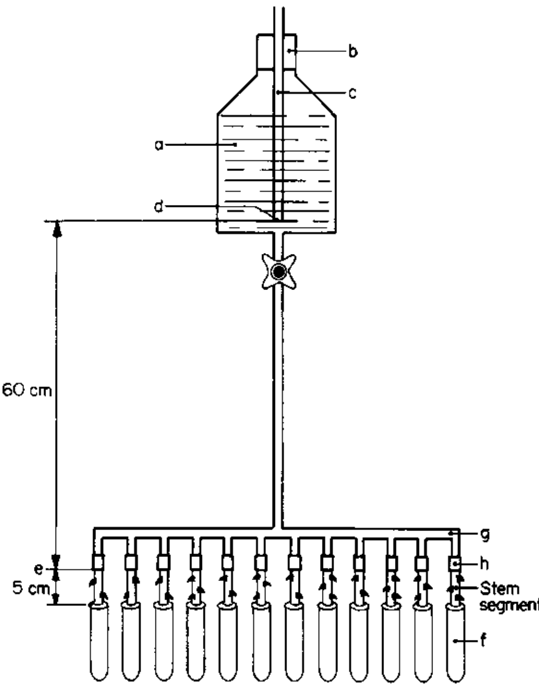

Fig. 1. Apparatus designed to measure xylem conductivity (Chin & Sacalis 1977). a, Bottle containing 10 l sterile glass-distilled water; b, rubber stopper; c, Mariotte tube; d, base of the Mariotte tube; e, upper end of the upside-down-inserted stem segment of 5 cm length; f, plastic tube of known weight to collect water flowing through the stem segment; g, glass manifold with 12 connectors; h, connector of soft medical plastic tubing.

SPIRAL PLATE COUNT METHOD TO ASSESS THE NUMBER OF BACTERIA INFILTRATED INTO XYLEM VESSELS OF ROSES

Viable counts were made on Plate Count Agar (Difco), using a Spiral Plate Count Machine (model C, Don Whitley, Shipley, West Yorkshire, UK). The plates were incubated at 28°C for 48 h. In the calculation of the number of bacteria in the sample tested, plates with between 20 and 500 colonies were used (Silley 1985). The water capacity of 1 cm of stem xylem vessel of roses was $0{\cdot}05 \pm 0{\cdot}003$ g. Thus the ratio of water capacity of stem segment (1 cm) to vase water (1 ml) is 1 : 0·05.

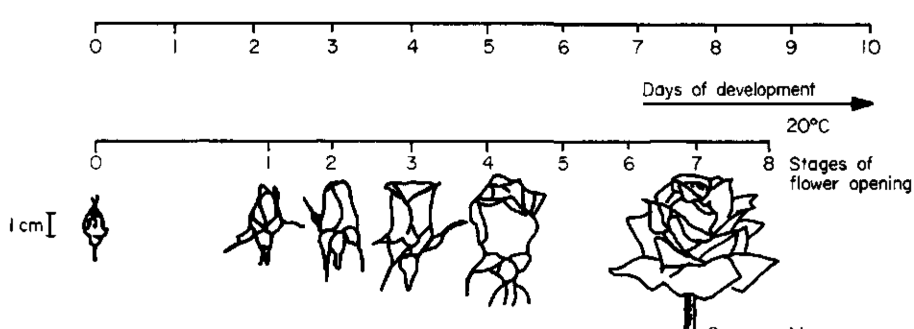

Fig. 2. Stages in the flower opening and development of ornamental value of *Rosa* cultivars (Berkholst 1980). The ornamental value is negative when the acceptability of the flower in the vase is visually decreased to <50%.

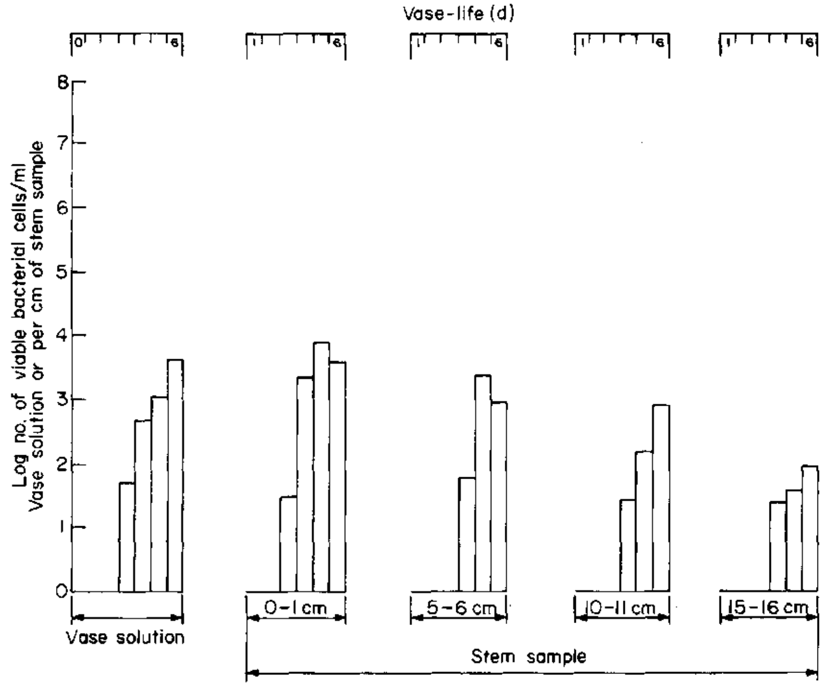

Fig. 3. Bacterial infiltration of xylem vessels of roses held in sterilized tap water during 1–6 d of vase-life. The average water capacity of xylem vessel segments of 1 cm stem length was 0·05 ml ± 0·003 ml.

STATISTICAL EVALUATION

Results of water uptake, water conduction, vase-life and flower opening were subjected to an analysis of variance. In addition, Least Significant Difference values were calculated. A probability level of significance of $P < 0{\cdot}05$ was used.

Results and Discussion

MULTIPLICATION OF *Ps. putida* CELLS IN VASE WATER

Plate counts of vase water after 1–6 d of vase-life showed: (1) no significant multiplication of *Ps. putida* cells at the highest initial cell concentrations of $>10^7$/ml of vase water and 2–4 d of vase-life; (2) that at the lowest initial pseudomonas numbers tested, 5×10^5/ml of vase water, multiplication yielded *ca* 10^7/ml after 6 d of vase-life; and (3) that in non-inoculated control vases, bacterial counts were up to 5×10^3/ml of vase water after 6 d of vase-life (Fig. 3).

INFILTRATION OF *Ps. putida* CELLS INTO THE XYLEM SYSTEM OF CUT ROSES

After 24 h of vase-life

The numbers of pseudomonas cells infiltrated into the first cm of the xylem vessels, with a water-holding capacity of *ca* 0·05 ml, after 24 h of vase-life was a maximum of five times higher than the numbers of pseudomonas cells initially present in the vase water. The infiltration into the xylem vessels decreased progressively as the distance from the cutting point to the sampling point increased (Fig. 4).

In control vases initially filled with sterile tap water the numbers of cfu on plate count agar were <1/cm after 24 h of vase-life. Further up the stems plate counts were also <1 cfu/cm of stem (see Fig. 3).

After >1–6 d of vase-life

During the vase-life of the roses, i.e. as long as the ornamental value of the roses remained acceptable, the numbers of pseudomonas cells infiltrated into the xylem vessels increased with

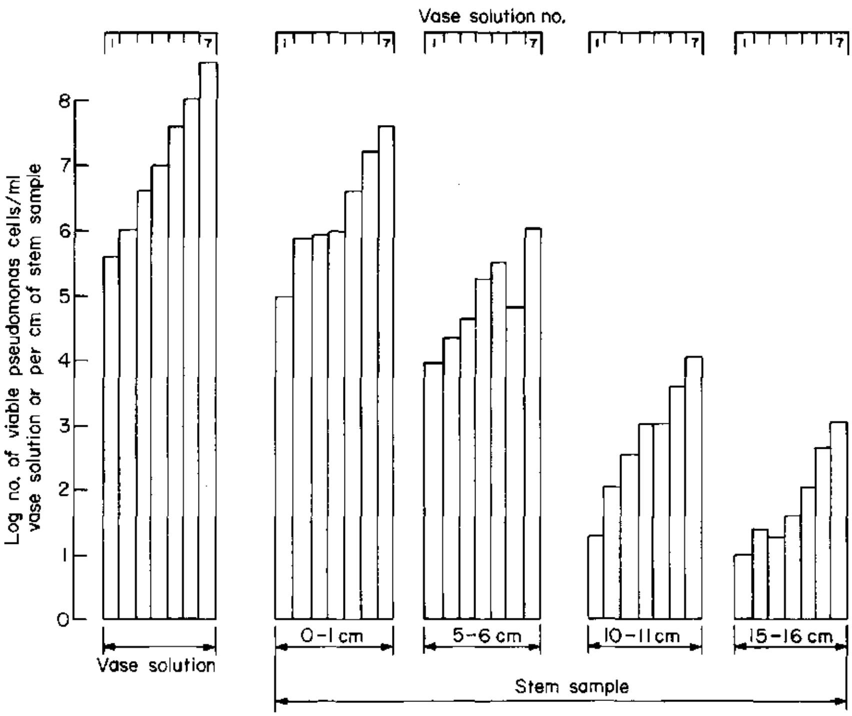

Fig. 4. The number of infiltrated cells of *Pseudomonas putida* strain 48 at four different levels of the xylem vessel system of cut roses after 24 h of vase-life when the initial number of *Ps. putida* cells was min. 5×10^5 and max. 5×10^8 cells/ml of vase water. The average water capacity of xylem vessel segments of 1 cm length was $0{\cdot}05 \pm 0{\cdot}003$ ml.

time and with increasing numbers of pseudomonas cells initially suspended in the vase water.

When the number of pseudomonas cells initially suspended in the vase water was *ca* 5×10^7/ml, the density of pseudomonas cells infiltrated into xylem vessels of the rose was *ca* 5×10^3 cfu/cm at 15 cm stem height, at the end of the vase-life of 3 d (Table 1).

The lower the initial cell numbers in the vase water tested, the longer the vase-life and also the longer the time taken for micro-organisms to infiltrate further up the stem. Vase water initially containing *ca* 10^5 pseudomonas cells/ml after a vase-life of 6 d showed infiltration at 15 cm stem height up to a maximum of 10^2 pseudomonas cells/cm of stem.

In control vases, initially filled with sterile tap water, the number of microbial cells (cfu) infiltrated from the vase water into the xylem vessels after 6 d of vase-life was a maximum of 10^2/cm of stem at a stem height of 15–16 cm (Fig. 3).

Table 1. Infiltration of *Pseudomonas putida* cells into xylem vessels of roses during 1–3 d of vase-life and an initial number of 5×10^7 pseudomonas cells/ml of vase water

Stem sample (cm)*	*Ps. putida* (cfu) per cm of stem sample — Vase-life (d) 1	3
0–1	10^6	10^7
5–6	10^5	10^5
10–11	10^3	10^4
15–16	10^2	5×10^3

* Water holding capacity of 1 cm stem: $0{\cdot}05 \pm 0{\cdot}003$ ml.

WATER RELATIONS AND VASE-LIFE OF CUT 'SONIA' ROSE HELD IN VASE WATER CONTAINING *Ps. putida* CELLS

Increasing initial numbers of *Ps. putida* cells per ml of vase water showed: (1) decreasing water uptake; (2) decreasing vase-life; (3) decreasing

Table 2. The influence of the number of *Pseudomonas putida* cells/ml vase water on the water uptake (ml/d) of *Rosa* cv. 'Sonia'

Log no. of *Ps. putida* cells/ml vase water	Vase-life (d) 1	2	3	4	5	6	7	8
	Water uptake (ml/d)							
8	7·0*	3·5*	2					
7	12·5	8·0	4·5*	3*				
6	15·5	13	8	6·5*	3*			
5	18·5	20·5	16·5	11·5	8·5	5*		
4	20·5	21·5	23	19	16	8	6*	*
0 (Control)	18·5	19·5	29·5	20	15	10	8	*

* End of the vase-life. Mean values of three triplicate tests.

flower opening; and (4) decreasing water conductivity of the respective roses.

WATER UPTAKE, VASE-LIFE AND FLOWER OPENING

The amount of water taken up by roses in a sterile vase solution increased during the first few days and gradually decreased each succeeding day until the end of the vase-life at full flower opening.

The amount of water absorbed by roses in vase water containing *Ps. putida* cells at 10^5–10^8/ml decreased abruptly to significantly lower levels 1–2 d before the end of the vase-life (Table 2). No significant differences in the daily uptake of vase water were noticed, however, in the period preceding the abrupt drop of water uptake.

The end of the vase-life of the roses was characterized by wilting of leaves and petals followed by 'bent-neck' of the flower stalk (Table 3).

Initial numbers of $>10^6$ *Ps. putida* cells/ml of vase water caused a vase-life reduction of $>50\%$ when compared with control roses held in sterile tap water. The flower opening also remained $<50\%$ of the normal development of the flower (Berkholst 1980).

An initial number of 10^4 *Ps. putida* cells/ml of vase solution, however, caused only a slight reduction in the vase-life, and a normal course of water uptake and flower development.

WATER CONDUCTIVITY

Water flow through three consecutive isolated 5 cm stem segments of 'Sonia' roses showed: (1) a decreased water flow capacity at increasing initial numbers of *Ps. putida* cells per ml of vase water; (2) the highest water flow reduction in stem segments cut from the base of the stem; (3) the same tendency for water flow reduction, although to a lower extent, as the base segment in stem segments cut at 5 and 10 cm stem height respectively; and (4) an initial number as low as 10^4 *Ps. putida* cells/ml of vase water resulted in a significantly lower water conductivity at three levels of the stem when compared with stem segments of roses held in sterile tap water (Table 4).

Table 3. The influence of the initial number of *Pseudomonas putida* cells per ml of vase water on the vase-life of *Rosa* cv. 'Sonia'*

Log no. of *Ps. putida* cells/ml vase water	Average vase-life (d)*
Control	8
5	7
5·5	5–6
6	5
6·5	3–4
7	3
7·5	1–2
8	1

* The end of the vase-life is the day at which the ornamental value of the *Rosa* flower in the vase is visually decreased to $<50\%$, compared with the normal bud development shown in Fig. 2 (Berkholst 1980).

SCANNING ELECTRON MICROSCOPE OBSERVATIONS

Scanning electron microscope observations of the cut surface and longitudinal sections of the stem of 'Sonia' roses held for 24 h in vase water containing 10^6 *Ps. putida* cells/ml showed a markedly decreasing number of pseudomonas

Table 4. The influence of the initial number of *Pseudomonas putida* cells/ml of vase solution on the water conductivity of stems of *Rosa* cv. 'Sonia' after 24 h vase life*

Log no. of *Ps. putida* cells/ml vase water	Water conductivity/30 min (ml)* Stem segments (cm) 0–5	5–10	10–15
8	0·01^{a}	0·36^{a}	0·41^{a}
7	0·30^{ab}	0·39^{ab}	0·31^{ab}
6	0·31^{ab}	0·41^{ab}	0·475^{abc}
5	0·58^{b}	0·95^{abc}	1·10^{bcd}
4	1·37^{c}	1·29^{c}	1·57^{d}
0 (Control)	2·34^{d}	2·08^{d}	2·32^{e}
Average	0·815	0·915	1·03
LSD value	0·54	0·63	0·50

* Mean values in a column followed by different letters are significantly different, $P < 0{\cdot}05$.

LSD, Least significant difference.

cells at increasing stem height up to 8 cm (Figs 5a, 5b, 6a and 6b).

XYLEM VESSEL LENGTH DISTRIBUTION

Xylem vessels of stems can be considered as parallel lines of different lengths (Fig. 7; Zimmermann 1983). A dextran blue solution in water (mol. wt 10^6) was used by Stigter & Broekhuysen (1986) to show that the minimum xylem vessel length of a cut rose is 1–2 mm while the maximum varies between 15 and 30 cm. The greater the distance from the cutting point to the point of the stem tested, the lower the number of open vessels and the less opportunity there is for bacteria to infiltrate into the xylem vessel system. The number of open vessels in the cutting surface of 'Sonia' roses is about 1500.

The curved shape of the xylem vessels and the spreading of the diameter of the individual vessels are factors that influence the 'normal' water flow resistance.

Microbial particles flushing up into the xylem vessels by the uptake of water may be attached to the vessel wall and initiate a complex defence mechanism. This may result in a decreased water conductivity.

The limit of the ascent of pseudomonas cells into the xylem vessels was shown to be lower than the maximum length of open vessels (Peresse 1974).

VASCULAR PLUGGING BY *Ps. putida* CELLS

Scanning electron microscope observations showed no vascular plugging when vase solutions contained *ca* 10^6 *Ps. putida* cells/ml (Figs 5 and 6).

Vase water containing $>10^6$ *Ps. putida* cells/ml showed wilting and 'bent-neck' before vascular occlusions by clotting or colonization of infiltrated pseudomonas cells might have occurred except at the base of the stem as shown in Fig. 4.

The extent of decreased conductivity (Table 4) of rose stem segments at increasing initial numbers of pseudomonas cells in vase water, after 24 h of vase-life, cannot have been the sole result of vacular plugging by *Ps. putida* cells. Therefore the number of infiltrated pseudomonas cells was too low, and the time (24 h) was too short, for mass colonization of infiltrated cells. Some other factors must have been involved.

The extent of decreased conductivity of stems of 'Sonia' roses when the vase solution contained 10^4 *Ps. putida* cells/ml indicated, moreover, that the vascular water transport system is very sensitive, complicated, and may involve several as yet unknown factors.

Consequently other factors should have played an additional role in vascular plugging of *Rosa* xylem vessels, and the decreased water conductivity (Table 4).

(1) The micro-climate of the cutting surface of

a)

b)

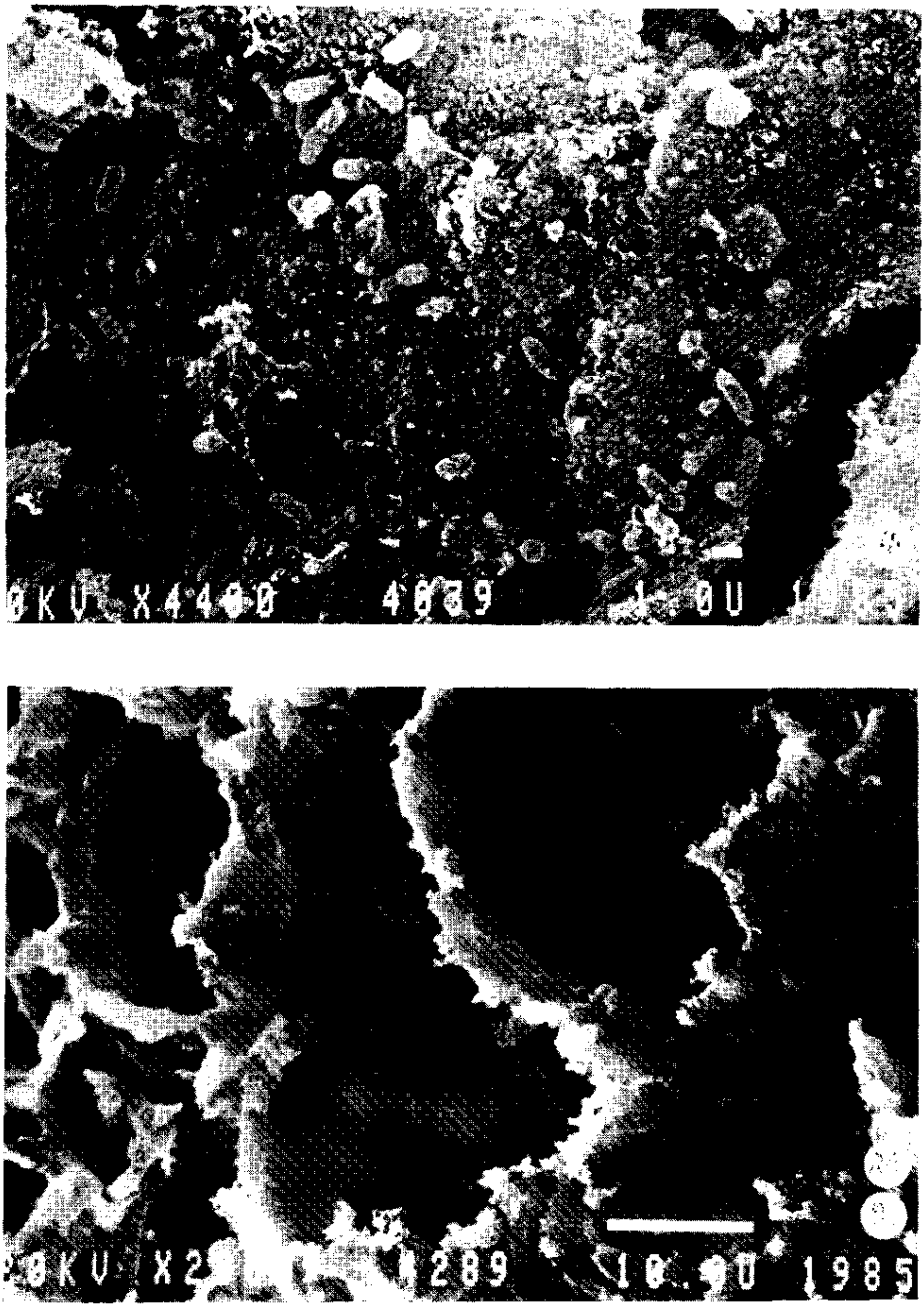

Fig. 5. a, Scanning electron micrograph (SEM) of the cut surface of *Rosa* cv. 'Sonia' held for 24 h in a vase solution containing *ca* 10^6 *Pseudomonas putida* cells/ml. The cut surface shows many bacterial rod-forming cells, entering the xylem vessels. Mucoid and amorphic materials are also shown. Bar marker represents 1 μm; b, SEM of the cut surface of *Rosa* cv. 'Sonia' held for 24 h in sterilized vase water. The cut surface and vessel entrance are clean. No bacterial cells or mucoid materials are shown. Bar marker represents 10 μm.

a)

b)

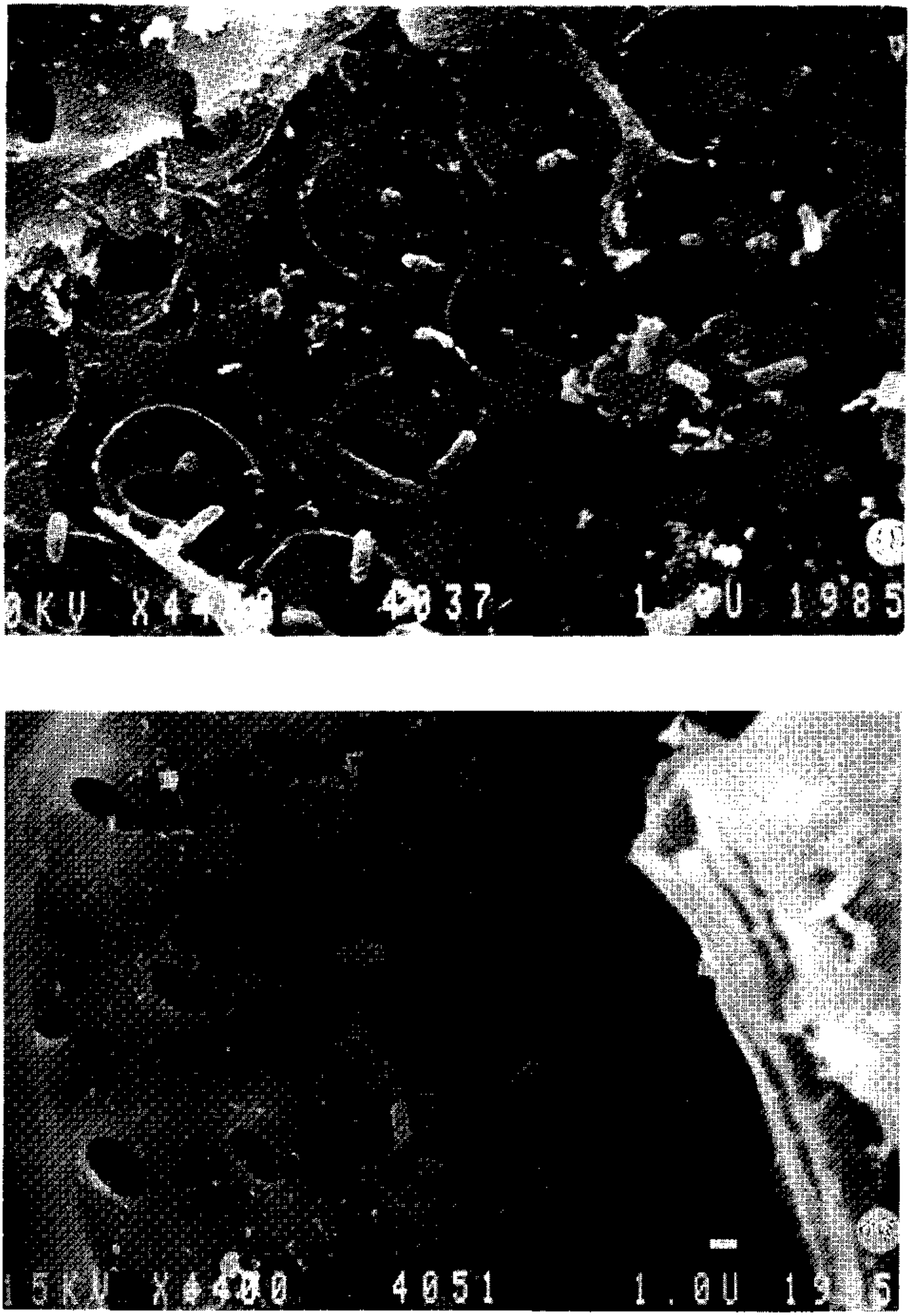

Fig. 6. a, Scanning electron micrograph (SEM) of a longitudinal section of a xylem vessel of *Rosa* cv. 'Sonia' at 2 cm from the cutting point, after 24 h of vase-life in vase water containing *ca* 10^6 *Pseudomonas putida* cells/ml. Many bacterial rods and some mucoid or amorphic materials are shown in the xylem vessel and around the vessel pits. The number of bacterial cells observed in the vessel is much lower than observed on the cutting surface of the roses. Bar marker represents 1 μm; b, SEM of a longitudinal section of a xylem vessel of *Rosa* cv. 'Sonia' at 8 cm from the cutting point, after 24 h of vase-life in vase water containing *ca* 10^6 *Ps. putida* cells/ml. A few rod-forming bacterial cells and some mucoid or amorphic material are observed in the vessel and around the vascular pits. Bar marker represents 1 μm.

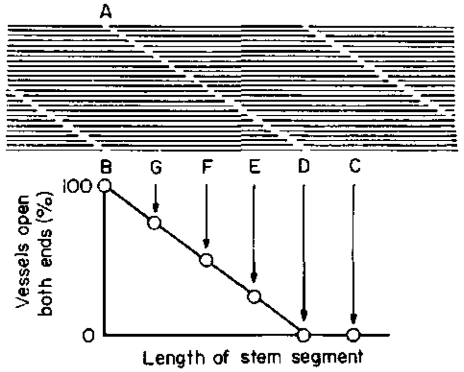

Fig. 7. Diagram of xylem vessel length distribution (Zimmermann 1983). As successive segments are cut off at B, C, D, E, F and G more and more vessels are cut open at both ends. The number of open vessels increases as the stem segment is shortened.

the roses may have stimulated the rapid multiplication of bacteria and blockage of the vessels on the cutting surface of roses placed in vase water with high initial numbers of pseudomonas cells ($>10^6$/ml, see Fig. 4).

(2) Pseudomonas cells, flushing up into the xylem vessels by the uptake of vase water, may become attached to the vessel wall and initiate the release, directly or indirectly, of plugging compounds (gums), resulting in a markedly decreased water conductivity after 24 h of vase-life, as shown in Table 4 (Parups & Molnar 1972; Van Alfen *et al.* 1983; Vandermolen 1983).

(3) The production of plugging compounds, directly or indirectly, by multiplying pseudomonas cells should be taken into account (Anderson 1984).

(4) Low mol. wt bacterial or vascular cell wall compounds flushing upward with the sap stream might act as elicitors, initiating the release of compounds which might have contributed to the defence of bacterial infiltration as well as to the rapid senescence of the cut roses (Albersheim & Anderson-Prouty 1975; Fujno *et al.* 1983; Vandermolen *et al.* 1983; Albersheim & Darvill 1985).

The *Ps. putida* strain used in the experiments is not phytopathogenic and does not produce pectin-degrading enzymes. *Rosa* cultivars are not sensitive to the plant hormone ethylene. Consequently the elicitor function of plant cell wall oligosaccharins could not have led to defence against the infiltration of bacterial cells into the vascular system, or to ethylene-induced rapid senescence of the *Rosa* flowers (Zagory & Reid 1986).

Moreover, vascular plugging by pectinaceous gels cannot have been caused by enzyme activity of the *Ps. putida* strain. The decreased water conductivity, however, measured up to ≥ 15 cm from the cutting point, might have been caused by occluding materials produced directly or indirectly by xylem cells. Histochemical studies are needed to detect such significant xylem element occlusions.

Much more research is needed to reveal which currently unknown mechanisms, other than gumnosis, could have led to the prevention of the spontaneous flushing up of bacterial cells with the vase water into the cut, open xylem vessels.

Henriëtte M.C. Put is on detachment at the Sprenger Institute, Wageningen. Ton van der Meyden is a trainee from the Horticulture College at Utrecht. Scanning electron micrographs were prepared by the Technical and Physical Research Service, Wageningen (Dr. A. Boekestein & Anke Clerkx).

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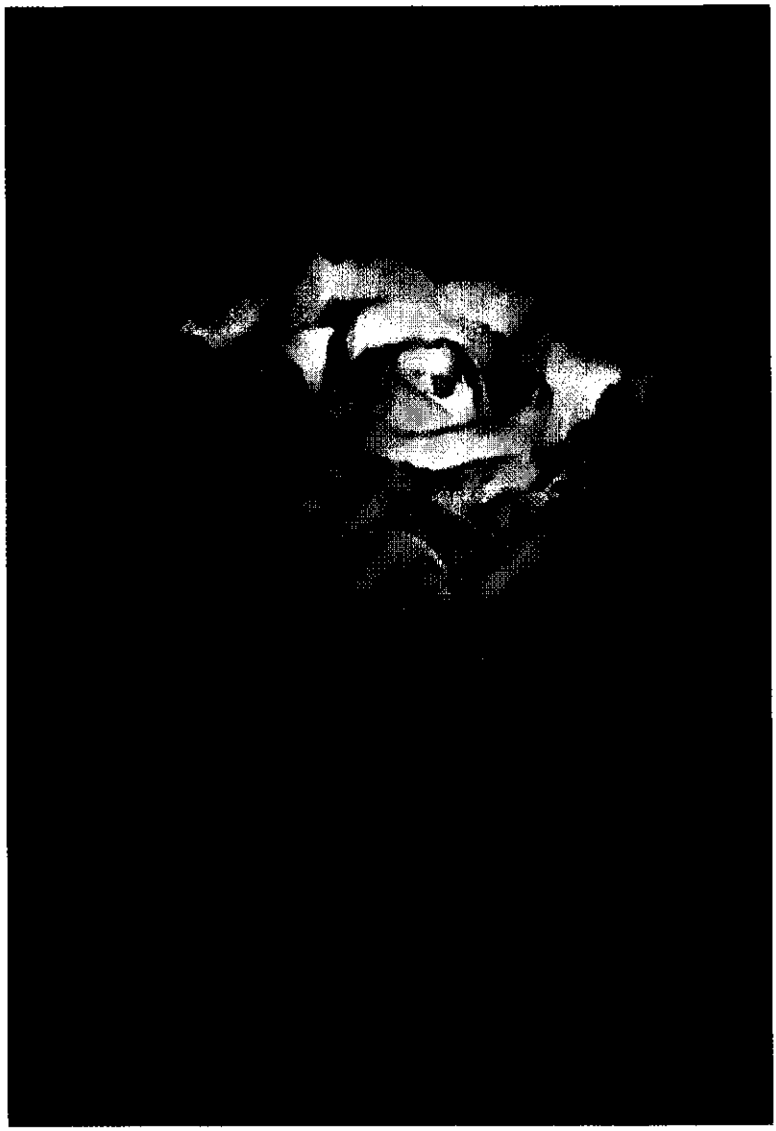
Chapter 4

The infiltration ability of micro-organisms ***Bacillus, Fusarium, Kluyveromyces*** and *Pseudomonas* spp. into xylem vessels of *Gerbera* cv. 'Fleur' and *Rosa* cv. 'Sonia' cut flowers: a scanning electron microscope study.



Rosa cv. 'Sonia': stage 4 of flower opening (Berkholst, 1980)

The infiltration ability of micro-organisms *Bacillus*, *Fusarium*, *Kluyveromyces* and *Pseudomonas* spp. into xylem vessels of *Gerbera* cv. 'Fleur' and *Rosa* cv. 'Sonia' cut flowers: a scanning electron microscope study

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Scanning electron microscope (SEM) observations have been made of transverse and longitudinal sections of xylem vessels of cut flowers of *Gerbera* cv. 'Fleur' and cut *Rosa* cv. 'Sonia' after a maximum of 24 h vase life. Vase waters used were: sterile tap water; sterile tap water plus suspensions of single pure cultures of *Bacillus polymyxa, B. subtilis, Fusarium oxysporum* microspores, segments of young mycelium of *F. oxysporum, Kluyveromyces marxianus,* or *Pseudomonas putida,* up to a maximum of 10^6 and 5×10^7 cells/ml of vase water.

The results of the SEM observations showed that only a small fraction of the microbial cells entered into the vascular system with the normal intake of vase water; most microbial cells remained attached to the submerged cut surface while a small fraction of the initially attached microbes were sometimes liberated into the surrounding vase water. The SEM figures give the impression that adhesion on to the cut stem surfaces of the bacterial cells (*Bacillus* and *Pseudomonas* spp.) by their capsular materials may have occurred, although the cut surface possibly acted as a filter, trapping cells because of the upward pressure of the transpiration stream into the vessels. Few attached yeast cells were found, but *Fusarium* microconidia formed cell clusters on the cut surface, partially blocking the xylem vessel entrance; *Fusarium* mycelium covered the cut surfaces as another filter layer through which only small mycelial particles could enter the vascular system.

The extent of infiltration of microbial cells into the xylem vessel system depended upon the number of microbial cells/ml of vase water (up to a certain maximal number) and on the shape and size of the individual cells and the width of the xylem vessels. The end of the vase life of *Gerbera* and *Rosa* flowers was marked by an extensive water stress in the xylem vessels so that the water intake necessary for a normal flower development was insufficient and wilting ensued. The water deficit may have been enhanced by secondary biochemical and physiological interactions between the microbes and the plant tissues, which may also have affected the infiltration of micro-organisms.

In a previously published paper (Put & Van der Meijden 1988) concerning the infiltration of *Pseudomonas putida* cells strain 48 into xylem vessels of cut *Rosa* cv. 'Sonia', it was shown that: (i) in roses *Ps. putida* cells were unable to pass the pit membranes between the xylem vessels. (ii) The number of *Pseudomonas* cells which infiltrated into xylem vessels decreased

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with increasing distance between the cutting point and the sampling point. This may have been partially due to the decreasing number of open vessels at increasing stem height. (iii) The infiltration of *Pseudomonas* cells increased when contact time and the number of *Pseudomonas* cells/ml of vase water increased, although only a minor fraction of the pseudomonads suspended in the vase water was able to infiltrate into the xylem vessels of the roses up to a distance of > 10 mm from the cutting point.

It is not yet known, however, whether vase micro-organisms enter the xylem system of cut flowers with the water flow like any other biological inert particle of the same shape and size, or whether biological activities complicate the mechanism of infiltration of micro-organisms from cut flower vase water into xylem vessels (Burdett 1970; Rasmussen & Carpenter 1974).

In a study of the effects on the vase life of cut flowers of *Rosa* cv. 'Sonia' of suspensions of *Bacillus*, *Enterobacter*, or *Pseudomonas* spp. added to the vase water, Put & Jansen (1988) found that: (i) the vase life of the roses was markedly decreased when decimal dilutions (10^8–10^4/ml) of water-washed viable or heat-killed cells of each of the separate bacterial species were added to the vase water; (ii) the initial numbers of either viable or heat-killed bacterial cells added/ml of vase water influenced the vase life; (iii) different bacterial strains exerted very similar effects, notwithstanding their morphological and biochemical differences. (iv) vascular plugging caused by infiltrating bacterial cells (e.g. *Pseudomonas*) could not alone have lead to the decreased water conductivity of *Rosa* stem segments as measured up to a distance of 10–15 cm from the cut surface, because the number of bacterial cells infiltrated into the xylem vessel was too low (Put & Van der Meijden 1988).

Phenomena which might be responsible for interactions between the vascular system of the cut roses and the viable or heat-killed cells are still unknown; they might be typical for cut roses, or for cut flowers in general when they are in vase water containing certain critical numbers of bacteria.

To gather more background cytological information on the relationships between the numbers of viable or heat-killed bacterial cells added to the vase water of *Rosa* flowers and the resultant decreased water conductivity of their stem segments measured after only 24 h of vase life, SEM was applied to study the infiltration ability into xylem vessels of micro-organisms (bacteria and fungi) added to the vase water.

Further investigations concerning the influence of microbial cells on the water relations of flower cultivars other than roses were desirable to assess host-specific phenomena, so comparable SEM investigations have also been made of the xylem vessels of *Gerbera* cv. 'Fleur', for comparison with roses; the *Gerbera* cultivar was chosen because of: (i) the biological and anatomical differences between the hollow-stemmed *Gerbera* and solid-stemmed *Rosa*, and (ii) the popularity of *Gerbera* as an ornamental cut flower in practice.

Factors which may affect the infiltration of micro-organisms into a vascular system are: (i) movement effected by bacterial flagella; (ii) the day (light) – night (dark) period; (iii) orthokinesis due to chemicals, e.g. *Escherichia coli* shows a positive chemotaxis towards oxygen, amino acids and sugars, but fatty acids are a signal for cells to escape 'overcrowded' conditions; (iv) microbial interactions such as antagonism, competition and synergism among the natural stem microflora; (v) small sub-communities formed during the vase life on the stem as well as on the cut surface (Ewing & Fife 1972; Bowen 1980; Carlile 1980; Halverson & Stacey 1986; Stigter & Broekhuysen 1986).

Little is known about the environmental ecology of the parent plant compared with the ecology of the vase life of cut flowers. The phenotypic responses of micro-organisms to the environmental change from living aerially on a rooted plant stem compared with living on an individual submerged cut flower stem are unknown, as are the interactions of the natural microbes in their new environment (Konings & Veldkamp 1980). The microbial strategies with respect to competitive utilization of various energy sources and the concentrations thereof have rarely been studied.

In this paper the results are given of a series of investigations into movements of micro-organisms into the stem xylem vessels of two cultivars of cut flowers: *Gerbera* cv. 'Fleur' and *Rosa* cv. 'Sonia' by SEM observations. To exclude any competitive activity due to vase water micro-organisms, pure cultures were studied using aseptic techniques throughout as far as possible.

Materials and Methods

PLANT MATERIAL

Gerbera cv. 'Fleur' and *Rosa hybrida* cv. 'Sonia' were supplied by local glasshouse growers. The roses were harvested at flowering stage 1–2 (Berkholst 1980) and the gerberas were taken from the plant when the stamens of about two circles of bisexual disc florets were ripe. The harvested flowers were wrapped in parchment paper, transported to the laboratory, stored for 24–48 h at 4°C, before the experiments were carried out.

MICRO-ORGANISMS

The micro-organisms used were *Bacillus polymyxa*–218; *Bacillus subtilis*–207; *Pseudomonas putida*–48; *Fusarium oxysporum*–7; *Kluyveromyces marxianus*–120. These five test strains were originally isolated from stems of cut flowers. Identification of the bacteria was done by API-20E, 50CH or 20NE systems (API systems, La Balme Les Grottes 38390, Montalieu, France). Mould and yeast strains were identified by the CBS (Centraal Bureau voor Schimmelcultures) at Baarn and Delft, The Netherlands. The five strains showed their characteristic differences in morphology as well as in their pathogenic and metabolic properties.

PREPARATIONS OF VASE WATER SUSPENSIONS

Bacteria

One to three isolated colonies of a 24 h old streak plate culture on Nutrient agar (Difco) were suspended in 1 ml of sterile tap water, mixed on a Vortex mixer at maximum speed and *ca* 0·2 ml was inoculated on Nutrient agar (Difco) in 500 ml Erlenmeyer flasks. The cultures were incubated at 28°C for 24 h. The growth was then washed off with sterile 0·05% Tween 80 (Difco) in tap water, centrifuged at 10°C and 10·000 **g** (Beckman J-21 C), resuspended in sterile tap water, Vortex-mixed, recentrifuged and diluted in sterile tap water to give *ca* 5×10^6/ml of *Ps. putida* and *B. subtilis* and *ca* 5×10^7/ml for *B. polymyxa* cells. Cell concentrations were assessed microscopically, followed by plate counting of decimal dilutions on Plate Count agar (Difco).

Yeast: Kluyveromyces marxianus

The same procedure was used as for bacteria except that the plating medium was Mycophyl agar (BBL) and incubation was at 25°C for 2–3 days.

Mould: Fusarium oxysporum

A mixture of spores and mycelium segments was inoculated on Potato Dextrose agar (Difco) in Erlenmeyer flasks. They were incubated at 25°C for 3 d (harvest of mycelium) and for 10 d (harvest of microconidia). The spores or mycelium were washed off into sterile 0·05% Tween 80 solution in tap water by gentle shaking, followed by Vortex mixing, centrifuging, washing (twice) and dilution in sterile tap water to give *ca* 5×10^6 microspores or mycelium particles per ml. Storage was at 2–5 °C. Mycelium particles and conidial numbers were assessed microscopically followed by plate counting of decimal dilutions of the mould suspensions on Potato Dextrose agar (Difco) and incubation at 25°C for 2–4 d.

VASE LIFE

Flowers were cut with a stem length of 45 cm, the stems were wiped with a paper tissue soaked in ethanol 98% v/v to minimize airborne contamination, and put singly into cylindrical vases containing either 100 ml of sterile tap water, a sterile 1% (w/v) sucrose solution in tap water, or a suspension in sterile tap water of the microorganism to be tested. Each variable tested consisted of triplicates.

The vases were held at 20°C, 60% r.h. (relative humidity) and 10·25 W/m^2 photoradiation at flower height during a 12 h light/dark photoperiod each 24 h. Every 24 h of the vase life, observations were made of: the water uptake (ml), the flowering condition of the gerberas (Van Meeteren 1980), the flowering stage of the roses (Berkholst 1980) and the ornamental value up to a maximum of 7 d of vase life. After 2, 3, 5 and 7 d of the vase life the microbiological state of the vase water was assessed by the streaking of vase water samples on agar plates. Scanning electron microscope preparations were made after a maximum of 24 h vase life, a deliberately short contact time, to exclude colonization and to detect early responses of the host vascular system towards the infiltrating micro-organisms (Peresse 1974; Van Alfen *et al.* 1983).

PREPARATION OF STEM SEGMENTS FOR SEM OBSERVATIONS

Pieces of the stem about 10 mm long were freeze-fractured and subsequently fixed overnight in 3% glutaraldehyde (in cacodylate buffer 0·1 mol/l; pH = 7·2), washed twice in buffer and then dehydrated through a graded series of ethanol/water (10% v/v to 100%) leaving the specimens in each solution for 10 min. The specimens were then critical-point dried and mounted with aluminium adhesive tape and silver paint on aluminium specimen tables. Finally, the specimens were coated with gold and examined in a scanning electron microsope (Jeol 35C) at 15 kV accelerating voltage.

Results

SEM OBSERVATIONS

Flowers in sterile vase water

No micro-organisms were observed in SEM preparations of the cut surfaces and the xylem vessels of surface-disinfected stems from *Gerbera* cv. 'Fleur' and *Rosa* cv. 'Sonia' up to a maximum of 300 mm stem height (Figs 1 and 2, 11 and 12).

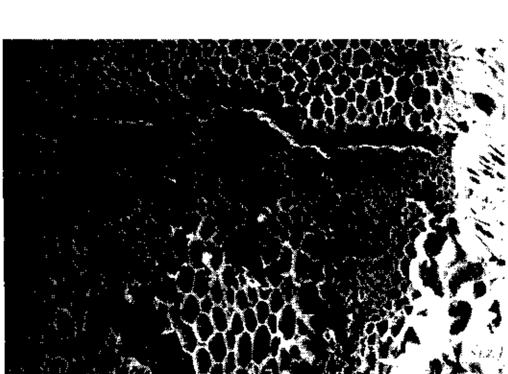

Fig. 1. *Gerbera* cv. 'Fleur', held in sterile vase solution, 0 h. Transverse section of cutting surface. Bar represents 100 μm.

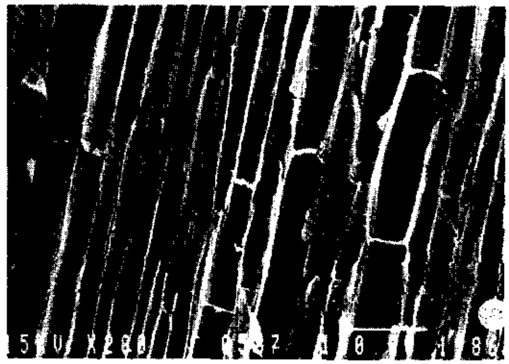

Fig. 2. *Gerbera* cv. 'Fleur', held in sterile vase solution. Longitudinal section near cutting point. Bar represents 100 μm.

Xylem vessel diameter

Scanning electron microscope examinations showed that the xylem vessel diameter of *Gerbera* cv. 'Fleur' was wider (varying between 20–100 μm) than the xylem vessel diameter of *Rosa* cv. 'Sonia', which varied between 2·5–35 μm (compare Figs 1 and 11).

Flowers in vase water to which suspensions of pure cultures of micro-organisms were added

All the SEM observations are summarized in Table 1(a) *Gerbera* and (b) *Rosa* and Figs 1–18 have been selected to illustrate some of the results obtained.

It was predictable that infiltration of micro-organisms into the wider xylem vessels of *Gerbera* might have been easier compared with roses. More microbial cells were indeed observed at longer distances from the cutting point of the *Gerbera* stems than were observed in SEM preparations of stem samples of roses (see Table 1a and b).

Bacillus subtilis-214 *cells* (ca 10^6*/ml) added to the vase water of* Gerbera *cv. 'Fleur'*

Blockage of cut xylem vessels by *B. subtilis* cells was not shown. Most *Bacillus* cells infiltrated into the xylem vessels were single ones, but some small cell clusters were observed. A few single cells and cell clusters infiltrated up to a distance of 50 mm from the cutting point. Around the cell clusters amorphous mucoid

Table 1. (a) A summary of the scanning electron microscope observations of xylem vessels of *Gerbera* cv. 'Fleur'

Micro-organisms added to vase water	Vase life (h)	Stem section	Distance from cutting point to sampling point (mm)	SEM observations	SEM fig. no.
None	0	T	0	No micro-organisms	1
			10		
			50		
	24	T	0		
			10		
			50		
	0	L	0		
			10		
			50		
	24	L	0		2
			10		
			50		
Bacillus subtilis–214 *ca* 5×10^6/ml	24	T	0	Free *Bacillus* cells observed; blockage of xylem vessels by *Bacillus* cells was rare	3
	24	T	10	Relatively high numbers of *Bacillus* cells	
	24	T	50	Relatively high numbers of *Bacillus* cells	
	24	L	0	*Bacillus* cells in open xylem vessels	
	24	L	10	Relatively high numbers of infiltrated *Bacillus* cells	
	24	L	50	Some vessels with relatively high numbers of *Bacillus* cells; other vessels few or no cells	4
Fusarium oxysporum–7 micro-conidia *ca* 10^6/ml	24	T	0	Clusters of conidia blocking (some) vessels	5
		T	10	Some vessels blocked by clusters of conidia	
		T	50	A few conidia infiltrated	
		L	0	Some vessels blocked by conidia	6
		L	10	Relatively high numbers of infiltrated conidia	
		L	50*	Relative low number of infiltrated conidia	7
Fusarium oxysporum–7 mycelium particles *ca* 3×10^6/ml		T	0	Mycelium partly covering the cut surface	8
		T	10	Few mycelium particles infiltrating into the vessel system up to 100 mm height	
			50		

(continued)

Table 1. *(continued)*

Micro-organisms added to vase water	Vase life (h)	Stem section	Distance from cutting point to sampling point (mm)	SEM observations	SEM fig. no.
		L	0	Infiltration of mycelium particles predominant in the widest non-blocked vessels; relatively high numbers of small mycelium particles up to 10 mm height	
			10		
(Deposit formed after 24 h)		L	50*	Few small mycelium particles	
Kluyveromyces marxianus–120 *ca* 5 × 10^6/ml	24	T	0	Clusters of yeast cells, partly blocking the open vessel ends	
		T	10	Low numbers of yeast cells	
		T	50	A few yeast cells	
		L	0	Large numbers of yeast cells infiltrated into cut-open vessels	9
		L	10	Low numbers of infiltrated yeast cells	10
(Deposit formed after 24 h)		L	50*	Few or no yeast cells	

T, transverse section; L, longitudinal section.
* No micro-organisms were observed at sampling points 150 and 300 mm from the cutting point.

Table 1. (b) A summary of the scanning electron microscope observations of xylem vessels of *Rosa* cv. 'Sonia'

Micro-organisms added to vase water	Vase life (h)	Stem section	Distance from cutting point to sampling point (mm)	SEM observations	SEM fig. no.
None	0	T	0–80	No micro-organisms	11
	4	T	0		
	8	T	0		
	16	T	0		
	24	T	0		
	4	L	10	No micro-organisms	
	4	L	20		
	4	L	80		
	24	L	50		12
	24	L	50*		

Table 1 (b) (*continued*)

Micro-organisms added to vase water	Vase life (h)	Stem section	Distance from cutting point to sampling point (mm)	SEM observations	SEM fig. no.
Pseudomonas putida–48	4	T	0	Cut surface half covered with bacteria	
ca 10^6/ml	8	T	0		
	16	T	0		
	24	T	0	Slightly more bacteria	
	4	T	10	Many fewer bacteria than at sampling point 0	
	4	T	20		
	4	T	80		
	24	T	10	Slightly more bacteria than after 4 h	
	24	T	20		
	24	T	80		
	4	L	10	A few bacterial cells *ca* 20/0·01 mm^2	
	4	L	20	*ca* 10/0·01 mm^2	
	4	L	80		
	24	L	10	Slightly more bacteria than after 4 h;	
	24	L	20	*ca* 100/0·01 mm^2	13
	24	L	80*	*ca* 30/0·01 mm^2	
Bacillus polymyxa–218	4	T	0	Surface almost covered with *Bacillus* cells; blockage of open vessels	14
ca 5 × 10^7/ml	24	T	0	Surface almost covered with *Bacillus* cells; blockage of open vessels	
	4	L	10	Numerous infiltrated *Bacillus* cells although lower numbers than observed at point 0	15
	24	L	10†	Slightly more infiltrated *Bacillus* cells	
Fusarium oxysporum–7 micro-condia	4	T	0	Xylem surface partly covered with conidia	
ca 10^6/ml	24	T	0	Slightly more conidia; some conidia clusters	
	4 or 24	L	5	Relatively numerous conidia in the vessel system	16
	4	L	10	Few conidia in the vessel system	
	24	L	5	Relatively numerous conidia in the vessel system	
	24	L	10	A few conidia present, *ca* 25/0·05 mm^2	
	24	L	50†	A few conidia present, *ca* 5/0·05 mm^2	

(*continued*)

Table 1 (b) (*continued*)

Micro-organisms added to vase water	Vase life (h)	Stem section	Distance from cutting point to sampling point (mm)	SEM observations	SEM fig. no.
Fusarium oxysporum-7 mycelium particles	4	T	0	Mycelium on cutting surface partly blocking open vessels	
ca 3 × 10⁶/ml	24	T	0	Thicker mycelium on cutting surface	17
	4	L	10	A few small mycelial particles	
	24	L	10	Slightly more small mycelium particles	
	24	L	50; 100	A few mycelium particles	
(Deposit formed in vase after 24 h)	24	L	150*	One mycelium particle only (artefact?)	
Kluyveromyces marxianus 120 *ca* 5 × 10⁶/ml	4	T	0	Surface partly covered with yeast cells, blocking of open xylem vessels	18
	24	T	0	As at 4 h	
	4	T/L	5	Yeast cells fewer than on the cutting surface	
	24		5	Yeast numbers slightly increased	
	24		10	A few yeast cells	
(Deposit formed in vase after 24 h)	24		50†	Sporadic yeast cells	

T, transverse section; L, longitudinal section.
* No micro-organisms observed at sampling points >150 mm.
† No micro-organisms observed at sampling points >100 mm.

Fig. 3. *Gerbera* cv. 'Fleur', held in *Bacillus subtilis* suspension, 24 h. Transverse section, cutting surface. Bar represents 1 μm.

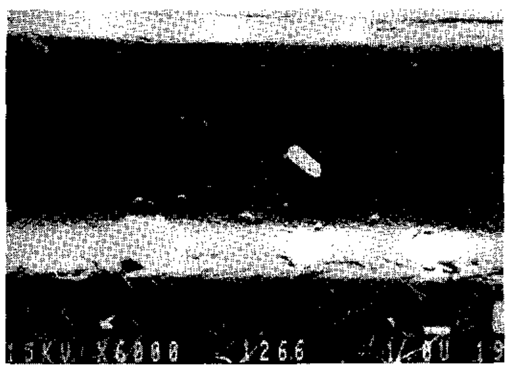

Fig. 4. *Gerbera* cv. 'Fleur', held in *Bacillus subtilis* suspension, 24 h. Longitudinal section, 50 mm from cutting point. Bar represents 1 μm.

Fig. 5. *Gerbera* cv. 'Fleur', held in ***Fusarium oxysporum*** conidia suspension, 24 h. Transverse section, cutting surface. Bar represents 10 μm.

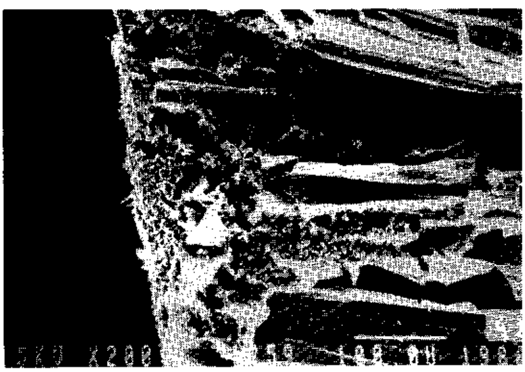

Fig. 6. *Gerbera* cv. 'Fleur', held in ***Fusarium oxysporum*** conidia suspension, 24 h. Longitudinal section, near cutting point. Bar represents 100 μm.

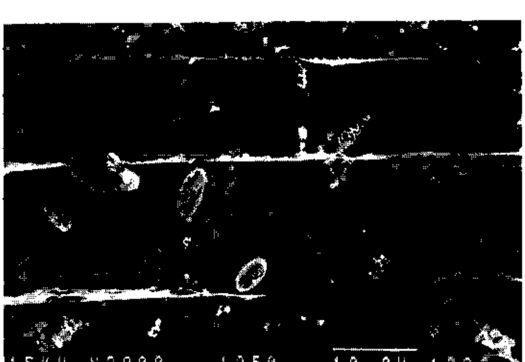

Fig. 7. *Gerbera* cv. 'Fleur', held in ***Fusarium oxysporum*** conidia suspension, 24 h. Longitudinal section, 50 mm from cutting point. Bar represents 10 μm.

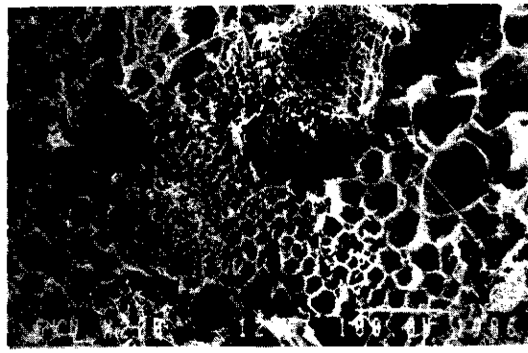

Fig. 8. *Gerbera* cv. 'Fleur', held in ***Fusarium oxysporum*** mycelium, 24 h. Transverse section, cutting surface. Bar represents 10 μm.

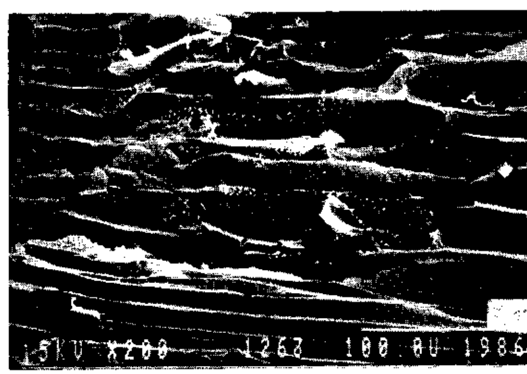

Fig. 9. *Gerbera* cv. 'Fleur', held in *Kluyveromyces marxianus* suspension, 24 h. Longtiudinal section, near cutting point. Bar represents 100 μm.

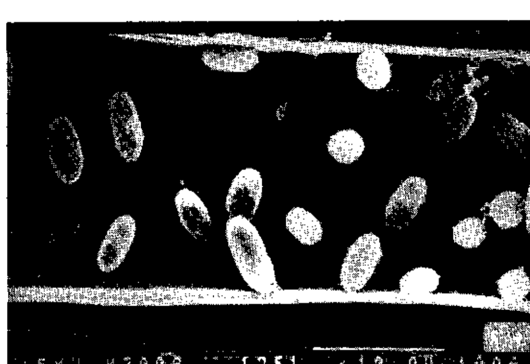

Fig. 10. *Gerbera* cv. 'Fleur', held in *Kluyveromyces marxianus* suspension, 24 h. Longitudinal section, 10 mm from cutting point. Bar represents 10 μm.

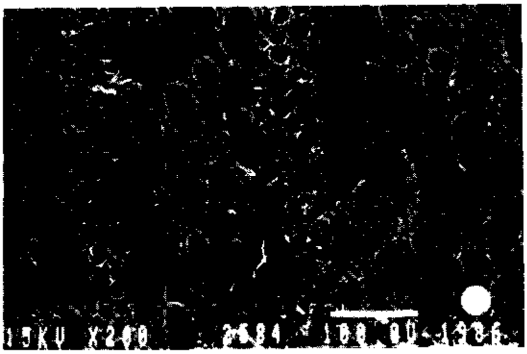

Fig. 11. *Rosa* cv. 'Sonia', held in sterile tap water, 24 h. Transverse section, 10 mm from cutting point. Bar represents 100 μm.

Fig. 12. *Rosa* cv. 'Sonia', held in sterile tap water, 24 h. Longitudinal section, 50 mm from cutting point. Bar represents 100 μm.

Fig. 13. *Rosa* cv. 'Sonia' held in *Pseudomonas putida* suspension, 24 h. Longitudinal section, 20 mm from cutting point. Bar represents 1 μm (Put & Van der Meijden 1988).

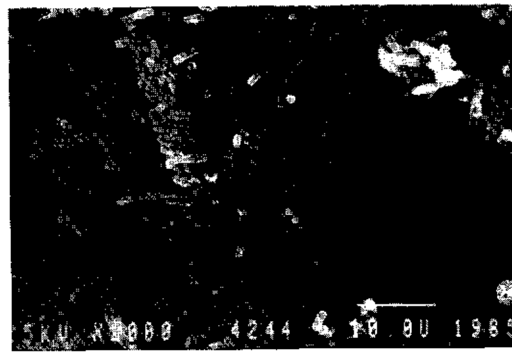

Fig. 14. *Rosa* cv. 'Sonia', held in *Bacillus polymyxa* suspension, 4 h. Transverse section, cutting surface. Bar represents 10 μm.

Fig. 15. *Rosa* cv. 'Sonia', held in *Bacillus polymyxa* suspension, 4 h. Longitudinal section, 10 mm from cutting point. Bar represents 1 μm.

Fig. 16. *Rosa* cv. 'Sonia', held in *Fusarium oxysporum* conidia suspension, 24 h. Longitudinal section, near cutting point. Bar represents 10 μm.

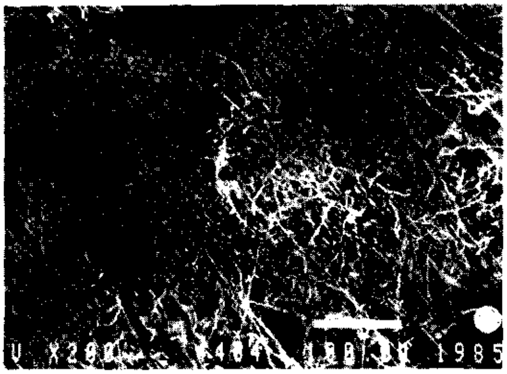

Fig. 17. *Rosa* cv. 'Sonia', held in *Fusarium oxysporum* mycelium suspension, 24 h. Transverse section, cutting surface. Bar represents 100 μm.

Fig. 18. *Rosa* cv. 'Sonia', held in *Kluyveromyces marxianus* suspension, 24 h. Transverse section, cutting surface. Bar represents 10 μm.

material was shown and probably aided the attachment of these cell clusters to the xylem walls.

Bacillus polymyxa-218 *cells* (ca 5 × 10^7*ml*) *added to the vase water of* Rosa *cv. 'Sonia'*

Blockage of a relatively large fraction of the xylem vessels of the cut surface was shown. Relatively large numbers of predominantly single rods were observed at *ca* 10 mm distance from the cutting point, after 4 h of vase life. The number of infiltrated *Bacillus* cells was slightly increased between 4–24 h of vase life. In addition, some small clusters were observed, they were surrounded by amorphous mucoid material, and were attached to the vessel wall. At *ca* 50 mm distance from the cutting point an occasional single rod-shaped cell was observed. The roses showed wilting symptoms after 24 h of vase life.

Fusarium oxysporum–7 *microconidia* (ca 10^6/*ml*) *added to the vase water of* Gerbera *cv. 'Fleur' and* Rosa *cv. 'Sonia'*

The cut surface of both cultivars was partly covered with small chains of conidia, attached together as well as to the cut surface, locally blocking the xylem vessel entrance and causing wilting of the *Rosa* flowers within 24 h. A relatively large number of microconidia infiltrated into the xylem vessels also caused vessel blockage. Attachment of conidia to the xylem vessel walls was not clearly shown, nor was slime formation evident around the conidial cells. Some of the microconidia germinated, and germ tube penetration into the vessel wall or pit membranes, may have occurred. Up to a distance of 50 mm from the cutting point of the *Gerbera*, many infiltrated conidia were observed, while at the same distance from the cutting point of the *Rosa* flowers only a few microconidia were evident.

Fusarium oxysporum–7 *mycelium particles* (ca 3 × 10^6/*ml*) *added to the vase water of* Gerbera *cv. 'Fleur' and* Rosa *cv. 'Sonia'*

Much of the cut surface of the stems was covered with a network of mycelium after 4 h of vase life. The initially thin mycelial layer became thicker during the course of the vase life, causing increased blockage of the xylem vessels; this enabled only the smallest mycelial particles to infiltrate into the widest non-blocked vessels up to maximum of 150 mm stem height. Attachment of mycelium particles to the vessel walls was not clearly shown. After 24 h of vase life weak wilting of the roses was observed.

Kluyveromyces marxianus–120 *cells* (ca 5 × 10^6/*ml*) *added to the vase water of* Gerbera *cv. 'Fleur' and* Rosa *cv. 'Sonia'*

The cutting surface of both flower cultivars was partly covered with yeast cells loosely attached to the surface and they blocked the xylem vessels. Infiltration of the xylem vessels by several single yeast cells was clearly observed. Complete blockage of the infiltrated *Rosa* vessels was evident, but the wider *Gerbera* vessels were not totally blocked by infiltrating yeast cells after 24 h of vase life. The number of yeast cells in the xylem vessels decreased at increasing distance from the cutting point. Single infiltrated yeast cells were occasionally observed, not attached to the xylem vessels up to a maximum of 50 mm stem height. Vascular attachment of yeast cells was not shown. After 24 h of vase life, weak wilting of the roses was observed.

Pseudomonas putida–48 *cells* (ca 10^6*/ml*) *added to the vase water of* Rosa *cv. 'Sonia'*

On the cut surface of the rose stems a thin layer of bacterial cells had developed after 4 h of vase life. The cells were surrounded by granular mucoid material which probably facilitated the attachment of the bacteria to the transverse surface structures of the cut stems.

After 24 h of vase life more cells were attached to the cut surface and they blocked a small fraction of the open vessels. Infiltration of bacterial cells into the xylem vessels adjacent to the cut surface was shown, but these vessels were not blocked. The greater the distance from the cutting and sampling points, the lower was the number of infiltrated cells observed. Up to a distance of 80 mm a few single cells and some small cell clusters surrounded by granular material were evident. Attachment of these bacterial cells to the vessel surface may have occurred. The first symptoms of wilting were visible after 3–4 d of vase life.

VASE LIFE

Water uptake

The water uptake during the first 24 h of the vase life was significantly lower when the cut flowers were placed in microbial suspensions than when they were placed in sterilized tap water (Table 2). It was also shown that the ornamental value of the cut flowers became critical when the uptake of vase water was decreased to <3 ml/d for *Gerbera* cv. 'Fleur' and <6 ml/d for *Rosa* cv. 'Sonia'.

The number and the size of micro-organisms added to the vase water

The greater the number or the size of micro-organisms initially suspended in vase water, the shorter was the vase life of the cut flowers (compare: *B. polymyxa* 5×10^7/ml vase water and *B. subtilis* 5×10^6/ml and *Ps. putida* and *F. oxysporum* 10^6/ml vase water; *K. marxianus* and *B. subtilis* 5×10^6/ml vase water) (see Table 2). A detailed study by Put & Jansen of this phenomenon is in progress to supplement these cytological observations.

The microflora of the vase water when sterilized tap water was used

After 24 h of vase life in sterilized tap water, the number of viable micro-organisms was <10/ml of vase water. During the course of the vase life, some micro-organisms (notwithstanding the aseptic handling of the flowers) remained viable and attached to the outer epidermis of the stem. These were washed off into the vase water and subsequently multiplied, to an extent determined by their growth factor requirements and the availability thereof in the vase water. After 7 d of vase life the numbers of these micro-organisms were: 10^3 (minimal) and 10^6 (maximal) cfu of bacteria, and $<10^2$ (minimal) and 10^4 (maximal) cfu of fungi/ml of vase water.

Discussion

The vase life of cut flowers was decreased by microbial cells in the vase water when they plugged the cut vessels so much that the normal water demand for flower development was inhibited (see Table 2). Fungal mycelium had the strongest vessel-plugging capacity compared with single microbial cells and spores (see Figs 5 and 8; 16 and 17 and Stigter 1981). Stigter & Broekhuysen (1986) observed in their model system that macromolecules (dextran-D 1×3 μm) suspended in vase water enter cut xylem vessels of 'Sonia' roses along with the transpiration stream of water and plug the stem when *ca* 10 mg of the dextran particles has been taken up by the stem. The harmful effects on the flower being more acute with higher, and more chronically with lower concentrations and size of macromolecular materials in the vase water.

Table 2. The influence of micro-organisms suspended in vase water on the water uptake of cut flowers (ml/day)

	Gerbera cv. 'Fleur'								*Rosa* cv. 'Sonia'							
Vase life (days)	1	2	3	4	5	6	7	15	1	2	3	4	5	6	7	8
Micro-organism added per ml vase water																
Control: sterilized tap water	10	5	5	5	5	5	5	*	16	18	20	22	20	16	15	*
Bacillus subtilis–207 *ca* 5×10^6	6	4	4	4	4	4	4*		7	8	6*	6*				
Bacillus polymyxa–218 *ca* 5×10^7	5	4	3	3*					6	4*						
Fusarium oxysporum–7 microconidia *ca* 10^6	7	4	4	3	3*				10	12*	8*					
Fusarium oxysporum–7 mycelium particles *ca* 3×10^6	6	4	3	3*					8	8*						
Kluyveromyces marxianus–120 *ca* 5×10^6	6	5	5	4	4	3*			10	11	8*					
Pseudomonas putida–48 *ca* 10^6	not tested								9	7, 5	6	6	6*			

* Average vase life (d).
The figures indicate the water uptake in ml/day.

The vase life of sound *Gerbera* and *Rosa* flowers is long enough (8 and 14 d respectively) for colonization by infiltrated micro-organisms, although it is probably too short for any significant phytotoxin production and activity as might occur in whole plants. Formation of microbial metabolic compounds, e.g. polysaccharides, microbial enzymes and toxic compounds, responsible for plugging, swelling and destruction of the vascular system as a result of vascular gelation and degradation, (Fujino *et al.* 1983; Harling & Taylor 1985; Put & Rombouts 1988) may have reduced the vase life of the cut flowers studied here (see Table 2) compared with their natural state. This merits further investigations.

Formation of toxic components by infiltrating potentially phytopathogenic *Fusarium* and *Bacillus* cells was slight or nil, and compared with the non-toxic micro-organisms tested, no serious or typical necrotic diseases of the flowers were observed during the vase life (Table 2, Figs 3–8, 14–17). Surprisingly, no morphological or anatomical deviations of the xylem vessel systems of *Rosa* and *Gerbera*, due to infiltrating micro-organisms were found here, in contrast to the observations of Zimmermann (1983) and Put & Rombouts (1988).

The cellular reactions of the plant xylem to the invading microbial cells suggest only general stress responses in accordance with the findings of Put & Jansen (1988). In their studies it was shown that relatively low numbers of bacteria added to the vase water may infiltrate into the xylem vessels, causing a relatively large decrease of the water conductivity of the Rosa stems, irrespective of the potential pathogenicity of the bacterial strain tested (Billing 1982).

In SEM preparations, *Bacillus* and *Pseudomonas* cells showed adherence to the vascular xylem walls of *Gerbera* and *Rosa* stems, but *Fusarium* microconidia and *Kluyveromyces* cells remained unattached, possibly due to the adherent capacity of bacterial capsules, their cell envelopes or adhesive pili (Rogers 1979).

Further electron microscopy is required to understand precisely how micro-organisms adhere to cut surfaces or to xylem walls (Fletcher 1979). Much more information is also needed to reveal the chemical and physical properties of the surfaces of both the bacterial, fungal and the xylem wall surfaces to which micro-organisms either do or do not adhere. Biochemical studies are essential to explain the role of any polymers involved in adhesion, and in this context the phenomenon of signal exchange in plant-microbe interactions should be considered (Halverson & Stacey 1986).

Infiltration of micro-organisms into a single xylem vessel might occur up to the maximum length of any cut vessel (Put & Van der Meijden 1988); the penetration of intervessel pit membrane barriers by infiltrating micro-organisms, however, might be mechanically inhibited, although some plant pathogenic moulds (*Fusarium*) have energy reserves sufficient for both spore germination and germ tube penetration into non-damaged host plants (Carlile 1970; Harling *et al.* 1984; Harling & Taylor 1985).

Further SEM preparations of rose and carnation stem segments made after 1 week of vase life in vase water containing the natural developing vase water microflora (*ca* 10^7 cfu/ml) showed coccoid and rod-shaped micro-organisms. They formed clusters, which were naked or surrounded by granular, amorphous mucoid material. The micro-organisms covered much of the cut surface and also infiltrated into the xylem vessels. Some small microbial cell clusters were localized, attached around the vessel pits (Van Doorn 1985).

The work of Dansereau & Vines (1975) showed that many microbial species (bacteria and fungi) were present in the vase water containing cut *Antirrhinum*, but only two bacterial species were isolated from the internal vascular xylem of their cut stems; they were identified as *Flavobacterium lutescens* and *Pseudomonas marginalis*, and because of their known antagonistic activity they may have prevented the infiltration and multiplication of the remaining micro-organisms present in the vase water.

Antagonistic micro-organisms in the soil, e.g. *Pseudomonas* spp., *Trichoderma* spp. and *Gliocladium* spp., can cause a delay and/or a decrease in the appearance or development of wilt symptoms in rooted host plants (Lynch & Ebben 1986).

Xylem-residing micro-organisms may also play a role in the vase life of cut flowers. Thus far, xylem-residing micro-organisms have been isolated only from xylem vessels of Florida citrus trees by Gardner *et al.* (1982). About 10^2–10^4 bacteria/g xylem vessel were found and and thirteen genera of bacteria were isolated. *Pseudomonas*, *Enterobacter*, *Bacillus* and *Cory-*

nebacterium were most frequent, and some of these resident bacteria were potentially phytopathogenic.

Scanning electron microscope observations are not sensitive enough to assess the visually low numbers of either xylem-residing micro-organisms or infiltrated micro-organisms far from the cut ends of the xylem vessels of cut flowers. The plate count method as described by Put & Van der Meijden (1988) should therefore be used as this gives fairly reproducible data even at low levels of infiltrated microbial cells. This method is not applicable, however, to cut flowers with hollow stems like *Gerbera* (Van Meeteren 1980), unless prior disinfection of the hollow stem area is possible.

From the SEM observations reported here some interesting additional information was obtained on the infiltration ability of pure cultures of micro-organisms into the xylem vessels of cut flowers, but much more multidisciplinary research is needed to reveal precisely which factors may be related (directly or indirectly) to the mechanism of xylem water-stress, and the adhesion and plugging of xylem vessels by microbial cells (and/or their metabolites), leading to decreased water uptake and consequent shorter vase life of cut flowers.

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Chapter 5

The effects of the vase life of cut *Rosa* cultivar 'Sonia' of bacteria added to the vase water.

Rosa cv. 'Sonia': stage 5 of flower opening (Berkholst, 1980)

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The Effects on the Vase Life of Cut *Rosa* Cultivar 'Sonia' of Bacteria Added to the Vase Water

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ABSTRACT

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A marked decrease in the vase life of cut *Rosa* cultivar 'Sonia' was observed when water-washed viable or heat-inactivated cells of either *Bacillus subtilis, Enterobacter agglomerans, Pseudomonas fluorescens* or *P. putida* were added to the vase water. The concentrations of viable or heat-killed bacterial cells influenced the vase life, but different bacterial strains exerted similar effects. High initial numbers of bacterial cells added to the vase water reduced the water conductivity, shortened the vase life and delayed the onset of flower-bud development. Similar numbers of viable cells added to the vase water ($< 10^7$- $\geqslant 0^5$ cells ml^{-1}) decreased the vase life of the roses more than did heat-inactivated cells. Water-conductivity measurement was a rapid, reproducible method of assessing the effects of bacteria after only 24 h of vase life. Vascular plugging caused by bacterial cells was not the sole cause of decreased water conductivity in the stem segments of the roses.

Keywords: *Bacillus subtilis; Enterobacter agglomerans; Pseudomonas fluorescens; Pseudomonas putida; Rosa* cultivar 'Sonia'; vase life.

Abreviations: API = Appareils et Procédés d'Identification, La Balme Les Grottes - 38390, Montalieu Vercieu (France); LSD = least significant difference; RH = relative humidity; SEM = scanning electron microscope.

INTRODUCTION

The dominant microflora of freshly cut flower stems of *Chrysanthemum* cultivar 'Spider', *Gerbera* cultivars 'Appelbloesem' and 'Fleur', and *Rosa* cultivar 'Sonia' have been found to show typical cultivar-related differences, which were also observed between the stem flora of the whole plant and when the cut stems

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were placed in vase water (H.M.C. Put, unpublished data, 1989). The bacterial genera *Bacillus* and *Enterobacter* were dominant on freshly harvested *Rosa* stems, but they lost their dominance in the vase water. *Pseudomonas* spp. multiplied more rapidly in the nutrient-poor vase water.

Bacterial plugging of xylem vessel elements has been claimed to be the major cause of rapid senescence of cut flowers (Aarts, 1957). However, in many such studies, microbial numbers have not been adequately assessed (Zagory and Reid, 1986). Bacterial strains used to study cut-flower senescence have rarely been identified (Gardner et al., 1982; Accati-Garibaldi, 1983). It has also been suggested that some (usually unidentified) microbial products excreted into vase water cause vascular plugging and cut-flower senescence (Gentile and Accati, 1981; Put, 1986; Zagory and Reid, 1986).

Experiments to elucidate the role of certain (phytopathogenic and non-phytopathogenic) bacterial species, associated with fresh *Rosa* stems during the course of the vase life of *Rosa* flowers, have been carried out. Known numbers of pure cultures of bacteria were added to vases of cut *Rosa* flowers to assess their effects on the vase life (cf. Durkin and Kuc, 1966; Burdett, 1970; Lineberger and Steponkus, 1976; Zimmermann, 1983).

The purpose of this study was to elucidate the role which viable and inactivated bacterial cells play in the phenomena leading to xylem vessel plugging, disturbance of the water relations and vase life of cut flowers.

MATERIALS AND METHODS

Plant material. – *Rosa hybrida* 'Sonia' was supplied by local greenhouse growers. The roses were harvested at flowering stage 1 (Berkholst, 1980) and wrapped in parchment paper, transported to the laboratory and stored at 4°C for 24–48 h prior to carrying out the experiments.

Bacterial strains. – Five bacterial strains were tested, viz. *Bacillus subtilis* Strains 207 and 214, *Enterobacter agglomerans* Strain 41; *Pseudomonas fluorescens* Strain 52, *P. putida* Strain 48. They were isolated from freshly harvested *Rosa* stems and from vase water containing *Rosa* flowers. Identification was carried out by conventional methods.

In addition, commercially available identification systems were used. Oxi/Ferm tube II (La Roche[1]) was used for the identification of oxidase-positive Gram-negative rods (*Pseudomonas*) and Enterotube II (La Roche) for oxidase-negative Gram-negative rods (Enterobacteriaceae). For *Bacillus* strains (Gram-positive aerobic sporeforming rods) Enterotube II (La Roche) and API 50 CH were applied in combination with tests for the hydrolysis of: casein, gelatin and lecithin. The formation of bacterial polysaccharides, dextran and

[1]F. Hoffmann-La Roche & Co. Ltd., Diagnostica, CH-4002 Basle, Switzerland.

levan, was detected in API sucrose agar code 5013. Stock cultures were maintained on nutrient agar and stored at 5 °C.

Vase water suspensions. – Washed bacterial cells, grown on either nutrient agar (Difco) or sucrose agar (API), were aseptically suspended in sterile tap water to give 10^8 cells ml^{-1} (measured turbidimetrically). Cell concentrations were also counted microscopically (Roser et al., 1987) and by plate counting on plate count agar (Difco) incubated aerobically at 28 °C for 48 h.

Each suspension was divided; one aliquot was stored at 5 °C (<20 h), the second was heat-treated at 85 °C for 10 min. Just prior to the vase life evaluation, spiral plates of the suspensions were made on plate count agar (Difco) and incubated aerobically at 28 °C for 48 h.

Vase life evaluation. – The *Rosa* stems were cut aseptically to a standard length of 45 cm. All except the 3 upper leaves were removed. Each rose was placed singly in a sterilized cylindrical vase calibrated to 100 ml. The vases contained sterile tap water (control) or a freshly prepared bacterial suspension diluted to give 10^8, 10^7, 10^6, 10^5 or 10^4 viable or heat-inactivated cells ml^{-1} of vase water. Each dilution was replicated 6 times. Protection against air contamination was obtained by sealing plastic "Parafilm M" on the top of each vase. The vases were maintained at $20°C \pm 1°C$, 60% RH $\pm 2\%$ and 10.25 W m^{-2} photoradiation with a 12-h photoperiod.

Water conductivity of cut flowers. – Water-conducting ability of cut-flower stems was assessed by an apparatus described by Put and van der Meijden (1988).

Triplicate samples of roses were taken from the vase after 24 h of vase life in water or in a bacterial suspension. Three consecutive stem sections, of 5 cm each, were cut off from the basal end upwards and inserted on to the manifold of the apparatus in an inverted position. The water flow through the segments was assessed after 30, 60 and 90 min of water flow. The average water conductivity in ml per 30 min was then calculated (Durkin, 1979). Conductivity was defined as the reciprocal value of the resistance to water flow through xylem vessels of flowers, expressed per transverse-sectional area of 5-cm length. The higher the water conductivity (ml per 30 min), the lower the water-flow resistance of the xylem vessels (Gilman and Steponkus, 1972; Chin and Sacalis, 1977; Put and van der Meijden, 1988).

Water uptake by cut flowers. – Water uptake by cut roses in sealed cylinders containing water or a bacterial suspension was measured at 24-h intervals by recording the water level in the calibrated cylindrical vases. The vase fluid levels were held between 50 and 100 ml by aseptic addition of the corresponding suspension.

Vase life of cut flowers. – Vase life evaluation of the cut flowers was assessed by visually observing: (1) the condition of the flower and leaves; (2) the flower bud development (Berkholst, 1980); (3) the ornamental value. The ornamental value is negative when the acceptability of the flower in the vase is visually decreased to <50%, compared with the 'normal' bud development of cut *Rosa* flowers placed in sterile tap water. Negative features in ornamental value are considered to be: wilting of leaves and petals; bent neck; desiccation; discoloration, chlorosis, necrosis.

Acid-fuchsin test to detect plugged vascular tissues. – When the ornamental value of the roses was decreased to <50%, the roses were removed and placed for 30 min in vases containing an acid-fuchsin solution of 0.5% w/v in 50% v/v ethanol. The epidermis and cortex of the stems were peeled off and plugging was revealed by the extent and height of the red coloration of the vessels.

Statistical evaluation. – Analyses of variance were applied to the measurements and observations of the water conductivity, vase life and flower opening, and mean values were compared. A probability level of $P<0.05$ was used.

RESULTS

Effect of the concentration of the bacterial cells added to vase water. – When viable or heat-inactivated bacterial cells of pure cultures of the 5 bacterial strains were added to the vase water, there was a marked reduction in water conductivity through the xylem (Table 1), vase life (Table 2) and flower bud development (Table 3), compared with cut roses placed in sterilized tap water. It was furthermore shown that the higher the number of bacteria initially added ml^{-1} vase water, the lower the water conductivity (ml per 30 min), the shorter the vase life (days) and the more inhibited the flower opening.

Effects of the addition of viable vs. heat-inactivated bacterial cells to the vase water. – Results with the 5 bacterial strains were mainly similar. When averaging the results of the 5 strains, the water conductivity through the xylem vessels (Fig. 1), the vase life (Fig. 2) and the flower bud development (Fig. 3) of cut roses was decreased to a greater extent by viable bacterial cells than by the addition to vase water of the same concentration of inactivated cells.

The greatest difference between the effects of viable vs. inactivated bacterial cells were found when the initially suspended bacterial cell numbers varied between $\geq 10^5$ and $<10^7$ ml^{-1} vase water.

The least differences between the effects of viable vs. inactivated bacterial cells on the water relations and vase life of cut roses were exerted when $>10^7$ or 10^4 viable or heat-inactivated cells, respectively, were added ml^{-1} vase water.

TABLE 1

Water conductivity (ml per 30 min) of stems of *Rosa*. 'Sonia' after 24 h in vase water with 10^8–10^4 viable (v) or heat-inactivated (i) bacterial cells (5 bacterial strains) added ml^{-1}. The values represent the average conductivity of the stem segments of 3 roses measured 3 times consecutively (after 30, 60 and 90 min)

Log n bacterial cells added ml^{-1} of vase water	Stem segment tested (cm)	Bacterial strain: *B. subtilis* Strain 207		*B. subtilis* Strain 214		*E. agglomerans* Strain 41		*P. fluorescens* Strain 52		*P. putida* Strain 48	
		v	i	v	i	v	i	v	i	v	i
8	0–5	0.45^{a}	1.00^{abc}	0^{a}	0^{a}	0.02^{a}	0^{a}	0.01^{a}	0.01^{a}	0.01^{a}	-
	5–10	0.48^{a}	1.25^{abc}	0.32^{ab}	0.21^{a}	0.35^{abc}	0.16^{a}	0.22^{a}	0.50^{ab}	0.36^{a}	-
	10–15	0.72^{a}	1.40^{abc}	0.48^{bc}	0.31^{ab}	0.36^{abc}	0.15^{a}	0.38^{a}	0.45^{a}	0.41^{a}	-
7	0–5	0.20^{a}	1.55^{bcd}	0^{a}	0^{a}	0.23^{a}	0.57^{ab}	0.57^{bc}	0.02^{a}	0.30^{ab}	-
	5–10	0.42^{a}	1.75^{bc}	0.11^{a}	0.18^{a}	0.24^{ab}	0.21^{a}	0.51^{ab}	0.27^{a}	0.39^{a}	-
	10–15	0.85^{ab}	2.30^{cde}	0.19^{a}	0.25^{ab}	0.24^{ab}	0.29^{ab}	0.31^{a}	0.31^{a}	0.31^{a}	-
6	0–5	0.61^{ab}	1.95^{cde}	0.36^{a}	0.09^{a}	0.64^{ab}	1.48^{bc}	0.27^{ab}	0.34^{ab}	0.31^{ab}	-
	5–10	0.83^{ab}	2.30^{c}	0.92^{ab}	0.56^{ab}	0.65^{abc}	1.17^{cd}	0.24^{a}	0.59^{abc}	0.41^{a}	-
	10–15	1.12^{ab}	2.60^{de}	1.20^{cd}	0.88^{abc}	0.94^{bcd}	1.08^{cd}	0.27^{a}	0.68^{a}	0.47^{a}	-
5	0–5	0.65^{ab}	2.46^{de}	0.74^{ab}	0.77^{ab}	0.91^{abc}	2.31^{d}	0.64^{bc}	0.52^{abc}	0.58^{b}	-
	5–10	1.60^{bc}	2.98^{d}	1.17^{b}	1.02^{ab}	1.04^{bcd}	2.10^{e}	1.11^{bcd}	0.73^{abc}	0.95^{ab}	-
	10–15	1.64^{bc}	2.65^{e}	1.59^{de}	1.02^{bcd}	1.11^{cd}	2.38^{e}	1.43^{b}	0.77^{a}	1.10^{b}	-
4	0–5	1.56^{bcd}	2.73^{e}	2.68^{c}	1.87^{bc}	1.98^{d}	1.84^{cd}	1.61^{d}	1.14^{cd}	1.37^{c}	-
	5–10	2.27^{dc}	2.20^{c}	3.31^{d}	2.27^{c}	1.93^{ef}	1.32^{de}	1.16^{cd}	1.42^{d}	1.29^{b}	-
	10–15	1.74^{bcd}	2.90^{e}	2.25^{e}	2.21^{e}	1.92^{e}	1.58^{d}	1.70^{b}	1.44^{b}	1.57^{b}	-
None	0–5	2.07^{de}		1.67^{bc}		1.72^{cd}		2.34^{e}		2.34^{d}	
	5–10	1.99^{bc}		2.21^{c}		1.20^{d}		2.08^{e}		2.08^{c}	
	10–15	2.71^{e}		1.69^{e}		1.66^{de}		2.32^{c}		2.23^{c}	
LSD ($P<0.05$)	0–5	1.01		1.16		0.99		0.54		0.54	
	5–10	1.20		0.92		0.82		0.63		0.63	
	10–15	0.90		0.80		0.77		0.50		0.50	

- = not tested.

Mean values per stem segment and per bacterial strain followed by different superscripts are significantly different, $P<0.05$.

TABLE 2

The influence on the vase life (days) of *Rosa* 'Sonia' of the numbers of viable (v) or heat-inactivated (i) bacterial cells (5 bacterial strains) added to the vase water

Log n bacterial cells added ml^{-1} of vase water	Bacterial strain									
	B. subtilis Strain 207		*B. subtilis* Strain 214		*E. agglomerans* Strain 41		*P. fluorescens* Strain 52		*P. putida* Strain 48	
	v	i	v	i	v	i	v	i	v	i
8	1.0^{a}	2.5^{b}	1.0^{a}	2.5^{ab}	1.5^{a}	1.5^{a}	1.0^{a}	0.5^{a}	1.5	-
7	2.5^{b}	4.5^{c}	2.0^{ab}	3.0^{abc}	1.5^{a}	1.5^{a}	2.0^{b}	1.0^{a}	3.5	-
6	3.0^{b}	5.5^{cd}	2.5^{ab}	5.0^{cd}	2.0^{a}	3.5^{b}	2.5^{b}	1.0^{a}	4.5	-
5	5.5^{cd}	6.5^{de}	3.5^{bc}	6.0^{de}	4.5^{bc}	5.5^{cd}	5.5^{c}	6.5^{d}	6.0	-
4	6.5^{de}	7.0^{e}	6.0^{de}	7.0^{de}	5.5^{cd}	6.0^{d}	7.5^{e}	7.5^{e}	7.5	-
None	7.0^{e}		7.5^{e}		7.5^{e}		7.5^{e}		8.0	
LSD	1.4		2.3		1.0		0.9		-	

- = Not tested.

Mean values per bacterial strain followed by different superscripts are significantly different, $P < 0.05$.

TABLE 3

The influence on the flower bud development of *Rosa* 'Sonia' of the numbers of viable (v) or heat-inactivated (i) bacterial cells (5 bacterial strains) added to the vase water

Log n bacterial cells added ml^{-1} of vase water	Bacterial strain									
	B. subtilis Strain 207		*B. subtilis* Strain 214		*E. agglomerans* Strain 41		*P. fluorescens* Strain 52		*P. putida* Strain 48	
	v	i	v	i	v	i	v	i	v	i
8	1.5^{a}	2.5^{ab}	2.0^{a}	2.0^{a}	1.5^{a}	2.0^{ab}	<0.5	0.5^{a}	2.0	-
7	2.0^{a}	3.5^{bc}	2.0^{a}	3.0^{ab}	3.5^{cd}	3.0^{bc}	1.0^{a}	1.0^{a}	3.0	-
6	2.0^{a}	5.0^{de}	2.5^{ab}	6.0^{c}	4.5^{de}	5.0^{ef}	1.5^{ab}	2.5^{b}	4.0	-
5	4.5^{cd}	6.0^{e}	3.5^{b}	6.5^{cd}	5.0^{ef}	6.0^{fg}	5.5^{c}	5.5^{c}	5.5	-
4	4.5^{cd}	7.5^{f}	7.0^{cd}	7.5^{d}	6.5^{g}	6.5^{g}	7.0^{d}	7.0^{d}	7.5	-
None	7.5^{f}		7.5^{d}		8.0^{h}		7.0^{d}		8.0	
LSD	1.3		1.0		1.1		1.1		-	

- = Not tested.

Mean values per bacterial strain, followed by different superscripts are significantly different, $P < 0.05$.

No effect on the vase life and flower bud development was exhibited when 10^4 inactivated cells ml^{-1} vase water were added, compared with the roses placed in sterilized tap water.

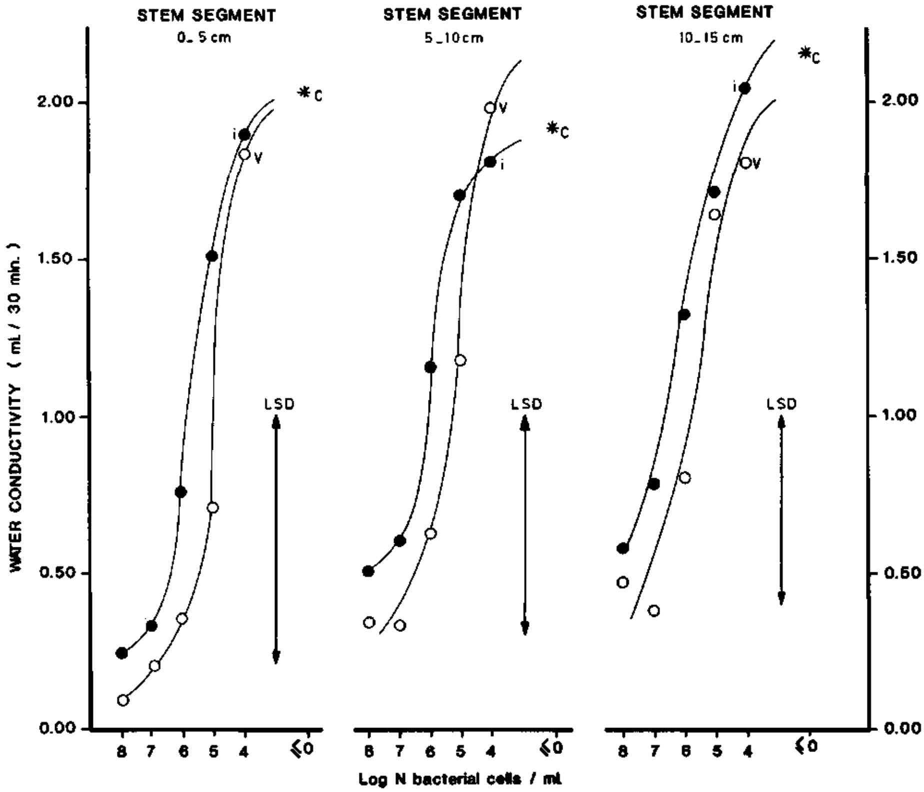

Fig. 1. The influence of the numbers of viable or heat-inactivated bacterial cells (ml^{-1}) added to the vase water, on the water conductivity of stem segments of *Rosa* 'Sonia' after 24 h of vase life. ○ = viable cells (v); ● = heat inactivated cells (i); * = control (c) value for sterilized tap water. The values shown are means of 3 triplicate tests of the 5 different bacterial strains.

Water conductivity of consecutive stem segments. – The lowest water conductivity was measured at the basal end of the *Rosa* stem when the highest initial numbers of bacteria were added to the vase water. The two consecutive tested segments of the corresponding rose stems showed a slightly increased water conductivity (Table 1 and Fig. 1).

Vase water uptake (ml per 24 h). – The mean values of the daily water uptake of the individual roses when the same concentration of either viable or heat-inactivated bacteria were added to the vase water showed that the higher the concentration of bacteria initially added ml^{-1} of vase water, the lower the water uptake (ml day^{-1}, Fig. 4). Lower also was the uptake from vase water with viable cells than from vase water with the same initial concentration of inactivated cells. Besides, it was found that after >2 days of vase life the water uptake decreased, and this decrease was more extensive when viable cells, rather

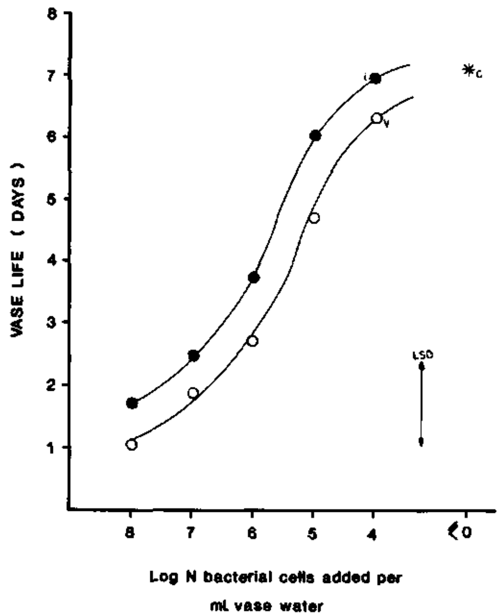

Fig. 2. The influence of the numbers of bacterial cells (ml^{-1}) added to the vase water, on the vase life of *Rosa* 'Sonia'. ○ = viable cells (v); ● = heat inactivated cells (i); * = control (c) value for sterilized tap water. The values shown are means of triplicate tests of the 5 different bacterial strains.

than an equal concentration of inactivated bacterial cells, were added to the vase water. The highest water uptake was measured when roses were placed in sterilized tap water.

Acid-fuchsin test to detect vascular plugging. – The higher the initial concentration of bacterial cells ml^{-1} of vase water, the lower the infiltration ability of the acid fuchsin solution into the xylem vessels of the roses. Initial concentrations of 10^8 bacteria ml^{-1} vase water showed entirely blocked vessels. Fuchsin-red infiltration was observed in a few vessels at 10^7 ml^{-1} viable cell concentrations. A few more vessels were coloured when the same number of inactivated bacterial cells were added. Initial concentrations of 10^6 viable ml^{-1} showed a nearly half-blocked vessel system. The non-blocked vessels coloured up to the flower head and the leaves. The vessel colouration ability of fuchsin red increased further at initial concentrations of 10^5 and 10^4 bacterial cells ml^{-1} vase water. Small differences remained visible between equal initial numbers of viable and inactivated cells. Of all treatments roses placed in sterilized tap water showed the highest colour intensity through the xylem system of stem, leaves and into the flower petals.

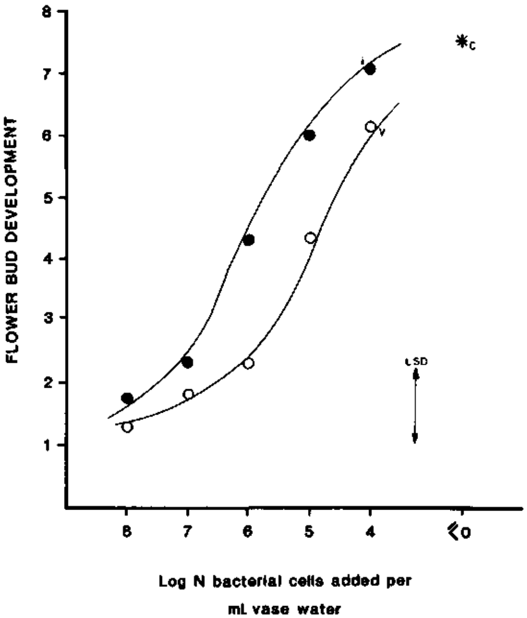

Fig. 3. The influence of the numbers of bacterial cells (ml^{-1}) added to the vase water, on the bud development of *Rosa* 'Sonia'. ○ = viable cells (v); ● = heat inactivated cells (i); * = control (c) value for sterilized tap water. The values shown are means of triplicate tests of the 5 different bacterial strains.

DISCUSSION

Viable or heat-inactivated water-washed cells of the 5 bacterial strains caused a marked decrease in the vase life of the roses.

The initial number of bacteria added affected the decrease of the vase life of the roses, but surprisingly the individual bacterial strains behaved very similarly (Table 2), notwithstanding their differences in: (1) cell shape; (2) cell motility; (3) slime production on agar media; (4) formation of a cellular capsule; (5) production of levan from sucrose; (6) ability to decompose pectin or cellulose; (7) the opportunistic pathogenicity of the different strains tested (Anderson, 1984; Halsall and Gibson, 1985; Put and Rombouts, 1989).

Water-washed suspensions of strains of many other bacterial species, isolated from flower stems, showed the same phenomena regarding the effects of the initial bacterial cell load added to the vase water on the vase life of cut roses. These observations (not detailed here) applied to strains of: *Bacillus cereus; B. cereus* var. *mycoides; B. circulans; B. licheniformis; B. polymyxa; Enterobacter cloaca; E. sakazaki; Pseudomonas aeruginosa; P. cepacia; P. maltophilia.*

Initial numbers of 10^8 viable or heat-inactivated bacterial cells ml^{-1} added

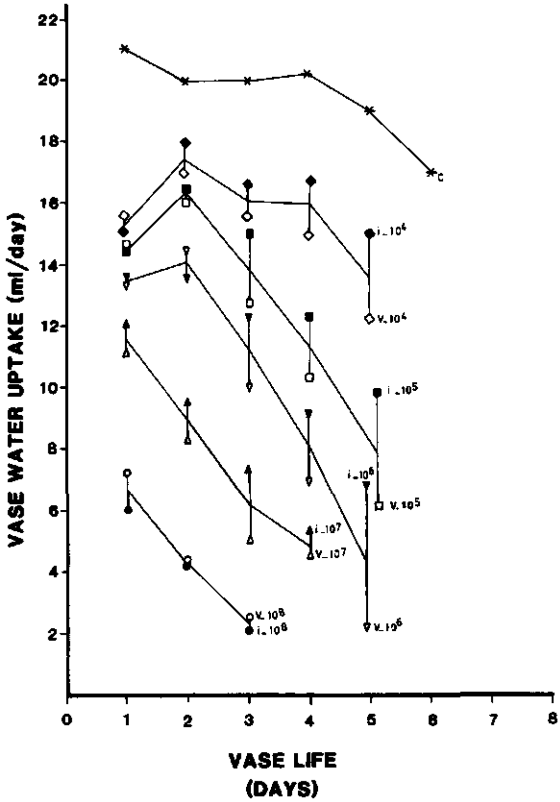

Fig. 4. The influence of the numbers of bacterial cells (ml^{-1}) added to the vase water, on the daily water uptake of *Rosa* 'Sonia'. The values shown are means of triplicate tests of the 5 different bacterial strains. * = control (c) values for sterilized tap water; ○ = viable cells (v) 10^8 ml^{-1}; ● = heat inactivated cells (i) 10^8 ml^{-1}; △ = viable cells (v) 10^7 ml^{-1}; ▲ = heat inactivated cells (i) 10^7 ml^{-1}; ▽ = viable cells (v) 10^6 ml^{-1}; ▼ = heat inactivated cells (i) 10^6 ml^{-1}; □ = viable cells (v) 10^5 ml^{-1}; ■ = heat-inactivated cells (i) 10^5 ml^{-1}; ◇ = viable cells (v) 10^4 ml^{-1}; ◆ = heat-inactivated cells (i) 10^4 ml^{-1}.

to vase water caused xylem element occlusions at the basal end of the stem; this was seen in SEM observations (Put and Clerkx, 1988). The water conductivity data and blockage of infiltration of acid fuchsin reported here confirm these observations (Table 1 and Fig. 1).

Initial numbers of 10^7 viable or heat-inactivated bacterial cells ml^{-1} of vase water, however, showed only a slightly increased xylem water conductivity and vase life of the roses compared with 10-fold higher initial bacterial numbers. SEM observations and the acid-fuchsin test indicated that the xylem vessels were partially blocked by bacterial cells. This suggests that in addition to the physical–mechanical blockage of the xylem vessels, there were some non-specifiable secondary reactions between the infiltrating bacteria and the vascular

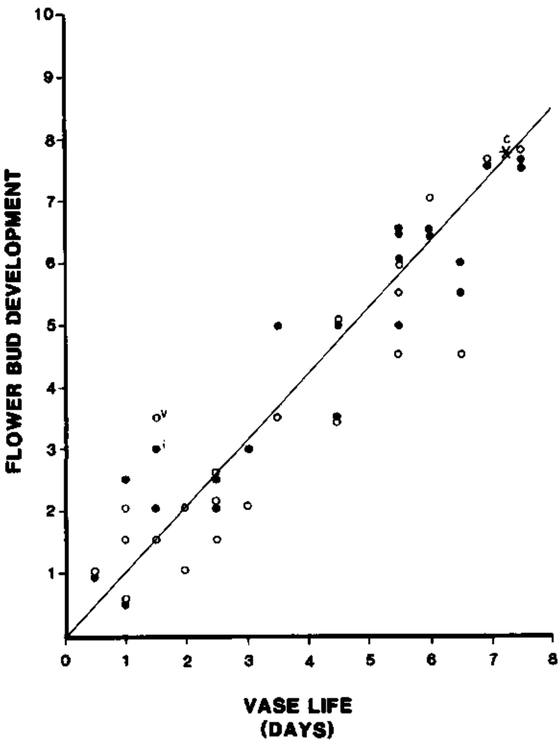

Fig. 5. The maximal vase life (days) plotted against the maximum flower bud development of *Rosa* 'Sonia' placed in water initially containing decimal dilutions of 10^8-10^4 ml^{-1} of cells of a bacterial strain. The results are a summation of the results using the 5 strains separately. ○ = viable cells (v); ● = heat-inactivated cells; * = control (c) value for sterilized tap water.

water-transport system. These might have contributed to the observed water stress, wilting and premature senescence of the roses.

At lower initial numbers ($\geq 10^5$–$< 10^7$ ml^{-1}) of bacteria added to the vase water, the differences in the effects of viable and heat-inactivated bacterial cells on the vase life of 'Sonia' roses became significant (Table 2 and Fig. 2). Thus, not only inert bacterial propagules, but also bacterial metabolic activities probably played a significant role in the course of the vase life of the roses (Gentile and Accati, 1981; Accati-Garibaldi, 1983; Zagory and Reid, 1986; Put and van der Meijden, 1988).

A reduced water conductivity of *Rosa* stems measured after 24 h of vase life can implicate a much later observable decrease of the ornamental value of the flowers.

To reveal whether a reduced water conductivity is caused by viable and/or non-viable bacteria, a microscopic counting method should be combined with a plate-count method.

It is not known why the infiltration of viable or heat-inactivated bacterial cells into the xylem vessels resulted in water stress measured as decreased water conductivity. The similarities between the reactions of different bacterial strains tested (Fig. 5) suggests that these reactions were merely non-specific stress responses (Konings and Veldkamp, 1980; Halverson and Stacey, 1986).

Initial numbers of 10^4 bacteria ml^{-1} added to the vase water only slightly influenced the water relations or decreased the vase life of *Rosa* flowers. For maximum longevity of *Rosa* flowers their handling and storage should ideally be under virtually aseptic conditions. This can be estimated within a short time after harvest by rapid methods such as stem conductivity measurements or direct microscopic counts of bacteria in the flower vase water (Roser et al., 1987) and in the xylem vessels (Put and van der Meijden, 1988).

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Chapter 6

The effects of microbial exopolysaccharides (EPS) in vase water on the water relations and the vase life of *Rosa* cv. 'Sonia'.

Rosa cv. 'Sonia': stage 6 of flower opening (Berkholst, 1980)

The effects of microbial exopolysaccharides (EPS) in vase water on the water relations and the vase life of *Rosa* cv. Sonia

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Pure cultures of five microbial species were used to test the formation of exopolysaccharides (EPS) when grown in agitated sucrose (5% w/v) containing liquid cultures. These test species were isolated from stems of freshly harvested cut flowers (*Chrysanthemum*, *Gerbera* and *Rosa*) or from the vase water of these flower cultivars. The partial conversion of sucrose into other saccharides was demonstrated by HPLC and colorimetric analysis. The final polymeric character of the newly formed saccharides was investigated. SEM preparations of xylem vessels of *Rosa* maintained in EPS-containing vase water showed blockage, disorganization and injury of the vessel structure. EPS were shown not to pass the xylem pit membranes. Recovery from the first symptoms of disturbed water flow (wilting) due to EPS was possible in young flowers by cutting off the blocked part of the stem (15–20 cm. The higher the microbial conversion rate of sucrose into polysaccharides, the more disturbed were the water relations of the roses placed in the EPS-containing fluid, as was demonstrated by the decrease of: (1) water conductivity of *Rosa* stem segments (ml/30 min); (2) water uptake (ml/d); (3) *Rosa* vase life (d); and (4) flower bud development. Bacterial EPS (presumably levans and dextrans) could be concentrated in the retentate by molecular filtration with a cut-off level of 10 000 Da. Filtrates did not cause *Rosa* xylem blockage and 'bent-neck' of the flower stems, but still may be toxic to roses. Two simple methods were also used for diagnostic investigations: (1) the beetroot tissue cube test to detect microbial products causing injury of the plant cell membranes, (2) the acid fuchsin test, to show the extent and location of *Rosa* xylem vessel occlusion.

In general, the vase life of cut flowers is enhanced by a sucrose addition (up to 0·135 mol/l) in the vase water (Aarts 1957; Chin & Sakalis 1977; Durkin 1979), although vase life of cut roses such as cv. *Sonia* is negatively correlated with bacterial growth and bacterial numbers in the vase water (Put 1986; Put & Jansen 1989).

Exogenously added sucrose contributes to cell metabolism as an energy source, increases the osmotic value, and enhances the ability of the tissue in which the sucrose accumulates to absorb water and maintain turgidity (Halevy & Mayak 1979; Paulin 1980; Paulin *et al.*, 1981; Paulin & Jamain 1982). These effects may be reversed, however, by enhancement of bacterial growth which may be sucrose-mediated.

Many of the microbial species isolated from cut flower stems and cut flower vase water can produce extracellular polysaccharides as capsules attached to the outer cell walls or as slime released to the environment, or both (Sutherland 1977).

An increasing resistance to water flow in vascular tissue is the major cause of water stress (wilting) in cut flowers (Aarts 1957). Purified bacterial polysaccharides induced wilting of

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sunflower and tomato stem cuttings (Corey & Starr 1957), and Hodgson *et al.* (1949) found that the effectiveness of artificially synthesized polymers correlated with the molecular size.

In alfalfa plants (*Medicago sativa* L.) interference by dextran of vascular conductance was directly correlated with the molecular weight (mol.wt < 250 000 rarely affected vascular flow). However, different sections of the vascular system showed different susceptibilities to plugging by dextrans of known mol. wt (Van Alfen & Allard-Turner 1979). Macromolecules probably disrupt vascular flow by accumulating on pit membranes (Van Alfen *et al.* 1983), so the frequency of pit membrane occurrence in the water flow pathway may thus be an important wilting determinant.

In most of the work reported by other authors, purified bacterial polysaccharides (see Corey & Starr 1957) or artificially synthesized polymers were applied to elucidate the mechanisms of phytopathogenicity. The effects of polysaccharides on ornamental cut flowers, however, have rarely been studied (Hodgson *et al.* 1949; Van Alfen & Turner 1975; Van Alfen & Allard-Turner 1979; Van Alfen *et al.* 1983; de Stigter & Broekhuysen 1986; Neumann 1987). In the present work, molecular filtrates and retentates of supernatant fluids of pure cultures of several micro-organisms grown in an agitated sucrose-containing medium were used as a vase medium for cut roses cv. Sonia. The micro-organisms tested originated from stems of cut flowers or from cut flower vase water.

The aim of our investigations was to reveal a possible correlation between vase life, water flow and the quantity of microbial polysaccharides in the vase water.

Materials and Methods

PLANT MATERIAL

Rosa hybrida cv. Sonia was supplied by local greenhouse growers. The roses were harvested at flowering stage 1 (Berkholst 1980) and wrapped in parchment paper, transported to the laboratory and stored at 4°C for 24–48 h before doing the experiments. In addition some comparative tests were done with *Chrysanthemum* cv. Daymark and *Gerbera* cv. Rebecca.

MICRO-ORGANISMS

Five microbial species were tested: *Bacillus subtilis* strains 207 & 214; *Pseudomonas fluorescens* strain 52; *Ps. putida* strain 48; *Botrytis cinerea* strain 196; *Fusarium oxysporum* strain 07. These organisms were isolated from stems of freshly harvested cut flowers of *Chrysanthemum*, *Gerbera* or *Rosa*, or from vase water containing these cut flowers.

VASE WATER PREPARATION

Shaken cultures

Bacteria: the growth of a 24–48 h old streak plate culture on Nutrient Agar (Difco) incubated at 28°C was suspended in 20 ml sterile tap water and vortex-mixed at maximum speed. After mixing, 2·5 ml of the suspension was added to each of a series of 500 ml screw-capped bottles containing 250 ml of sterilized 5% w/v sucrose solution in tap water. Five ml of a clear supernatant fluid of a sterilized mashed *Rosa* stem suspension (5% w/v) was added to each bottle.

Fungi: growth was harvested from 2–4 d old streak plate culture on Malt Extract Agar (Difco), incubated at 25°C. The harvesting and inoculation of the fungus cultures into the bottles was as described for bacteria.

All inoculated bottles were shaken at 60 rev/min and an amplitude of 20 cm during *ca* 3 weeks at 20 ± 1°C to obtain maximum polysaccharide formation.

Static cultures

The medium used in static cultures consisted of glucose 1–3% w/v, or sucrose 1% w/v, and a clear supernatant fluid of a sterilized mashed *Rosa* stem suspension (0·1% w/v) in tap water. The microbial inoculum was the same as for the shaken cultures. Incubation was at 20 ± 1°C for 2–3 d. The single pure cultures thus obtained were used as controls, simulating microbial vase water. Young, static cultures in media with a relatively low carbohydrate content will show little or no microbial exopolysaccharide formation. Pectolytic enzymes may or may not be formed. Fungus cultures were slightly agitated to prevent surface growth.

Harvest of microbial products

The grown pure cultures were centrifuged at 10°C and 10 000 **g** (Beckman J-21C). The supernatant fluid was decanted, and microbial cells and mycelium particles removed by pressure filtration through a 0·22 μm sterile disposable filter unit (Millipak-60, MPGL-06-Hz). The sterile filtrate was stored at *ca* 5°C.

Molecular filtration

A Pellicon recirculating cassette system for 'molecular sieving' was used with a nominal cut-off level of mol. wt 10 000 Da (Millipore, a PT packet 10K NMWL, retentate separator 10/PK and pump-motor 23 OV 50/60 HZ at 10 PSI). When the retentate could not be recovered aseptically, an additional filtration through a 0·22 μm Millipak sterile disposable filter unit was applied. Sterilized tap water was added to get equal volumes of filtrate and retentate. Storage of sterile filtrates and retentates was at 2–5°C up to a maximum of 60 h.

ANALYSIS OF CULTURE LIQUIDS

Assay for the presence of polysaccharide

The total concentration of saccharides (as anhydrohexose) in culture liquids was estimated by the phenol-sulphuric acid method (Dubois *et al.* 1956), wherein glycosidic linkages are split and the reducing groups formed react to give coloured compounds, which absorb at 488 nm (absorption E1). When the reaction mixture was incubated for 20 min at 80°C before cooling, the molar absorptions for glucose, fructose and anhydrohexose from the sucrose value are nearly equal. In a parallel test, existing reducing endgroups were first reduced to alcohol groups with $NaBH_4$ solution, followed by the phenol and sulphuric acid reaction. The resulting absorption, E2, is less than E1 and the average degree of polymerization, DP′, is given by, DP′ = E1/(E1 − E2) (Timell 1960). Because any remaining sucrose gives equal contributions to E1 and E2 (for pure sucrose E1 = E2) the measured absorptions have to be corrected for the contribution of sucrose, (S). Glucose and fructose, when present, contribute only to E1 thus lowering the measured DP′; therefore E1 is also corrected for this (GF). Therefore the average degree of polymerization of the mixture of saccharides, other than glucose, fructose or sucrose is given by:

$$DP' = \frac{E1 - (GF + S)}{E1 - (GF + E2)} \qquad (1)$$

Glucose, fructose and sucrose were determined in aliquots of culture liquids by a Waters Associates liquid chromatograph equipped with a 6000 A pump, U6K injector and R401 RI detector. The sugars were separated on a Lichrosorb-10-NH_2 column (Chrompack, Vlissingen, The Netherlands) with ethyl acetate–acetone–water 300 : 555 : 150 (v/v) at a flow rate of 1·3 ml/min. Column and detector temperature was 30°C.

Absorption value (E1 or E2) with the phenol-sulphuric acid method were converted to molar concentration (mol/l), with a calibration curve for sucrose and the appropriate dilution factor.

SCANNING ELECTRON MICROSCOPE OBSERVATION (SEM)

SEM preparations were made after 24 h of vase life, as described by Put & Clerkx (1988). The *Rosa* stem segments studied were from vase water containing added filtrate or retentate of culture fluids of: *B. subtilis*, *F. oxysporum* and *Ps. fluorescens*.

VASE LIFE EVALUATION

The vases contained 100 ml of sterile tap water, sucrose 1% w/v in sterile tap water (control), or decimal dilutions in sterile tap water (maximal 10^{-3}) of the retentate or the filtrate of one of the microbial vase water supernatant fluids.

The vase life of the roses was assessed as described by Put & van der Meijden (1988), and consisted of: (1) water conductivity measurements of stem segments (ml/30 min); (2) determinations of water uptake (ml/d); (3) visual observations of the flower bud development (Berkholst 1980); and (4) assessment of the ornamental value of the flowers.

ACID FUCHSIN TEST TO DETECT PLUGGED VASCULAR TISSUES

At the end of the vase life, when the ornamental value of the roses had decreased to $<50\%$, the roses were removed and placed for 30 min in vases containing an acid fuchsin solution of 0·5% w/v in 50% v/v ethanol. The epidermis and cortex of the stems were then peeled off, and plugging was revealed by the extent and height of the red coloration of the vessels (Parups & Molnar 1972).

BEETROOT TISSUE CUBE TEST

To demonstrate the ability of micro-organisms to form cell-membrane- or cell-wall-damaging products, raw beetroot was peeled and cut into cubes of *ca* 7 mm and immediately washed in running tap water until the washwater remained colourless; they were then dried with paper tissue and transferred singly into sterile screw-capped polystyrene 15 × 45 mm tubes. Five ml of the supernatant fluid, retentate or filtrate of a culture of the strain to be tested was added to each test tube, which was then shaken gently before static maintenance at room temperature. The colour intensity of the fluid (indicative of cell membrane damage) was examined after 30 min and 4 h at 20°C and compared visually with a control tube containing sterile tap water (see Plate 1).

PECTINASE TEST

The method described by Wieringa (1956) was used to demonstrate any possible formation of pectic enzymes in the different microbial cultures used.

STATISTICAL EVALUATION

Analyses of variance was applied to all the measurements and observations of the water uptake, water conductivity, vase life, and flower opening of the *Rosa* flowers.

Results

MICROBIAL EXOPOLYSACCHARIDE (EPS) FORMATION IN SHAKEN CULTURES

Sucrose conversion

The composition of the supernatant fluids and some retentates after molecular filtration of supernatant fluids of pure cultures of six strains is given in Table 1. The molar concentrations pertaining to culture supernatant fluids (SN) are in accord with the molar balance of sucrose conversion: $GF + S + P + SM = 0{\cdot}292$ mol/l where SM is metabolized sucrose.

It appears that the percentage conversions of sucrose into polysaccharides by *B. subtilis*, *Ps. fluorescens*, *Ps. putida* and *Bot. cinerea* are 51–61%, 14–41%, 31% and 22% respectively. Results for the supernatant fluid of *F. oxysporum* are not available, but the molar fractions of polysaccharides in the retentates R1 and R2 account for partial conversion of sucrose into polysaccharides.

It was also observed that shaken cultures inoculated with the same mother culture did not show significantly different *EPS* production. Replicate cultures, however, sometimes produced different quantities of *EPS* as is shown by *Ps. fluorescens* in Table 1; the toxicity of molecular filtrates (*B. subtilis; Ps. fluorescens* and *F. oxysporum*) was also different.

Average degree of polymerization

Relative standard deviations for E1, E2 and for the lower saccharides were 4% and 8% respectively. The absolute standard deviation in the numerator and denominator of equation (1) was calculated using a standard procedure. The denominator was considered to be near zero when its numeric value was less than the calculated absolute standard deviation. In that case only the lower limit for the DP′ value is given in Table 1.

High DP′ values (2–12) were also measured in supernatant fluids of shaken cultures of *B. licheniformis* and *B. polymyxa* while a moderate DP′-value of 1·5–2 was measured in culture retentates of *Enterobacter agglomerans* and *Kluyveromyces marxianus* (results not presented).

Molecular filtration

The proportion of polysaccharides, P/E1, with *B. subtilis*, strain 214, was 70% for both supernatant fluid and its retentate. The latter, however, had a significantly higher average degree of polymerization than the original supernatant fluid (Table 1). With *Ps. putida* the DP′ values were barely different, but the retentate had a higher proportion of P (50%) than

Plate 1. The beetroot tissue cube test, to assess cell-membrane damage due to supernatant fluids of microbial broth cultures: (1) control, in sterile tap water, –; (2) no cell-membrane damage, –; (3) weak cell membrane damage, w; (4) cell-membrane damage, +; (5) cell membrane damage +/+ +; (6) cell-membrane damage + +. Only a slightly increased colour-intensity occurred at 4 h compared with 30 min at 20°C.

Plate 2. Showing the ornamental value of *Rosa* cv. 'Sonia' after 2 d of vase life with various additives from a culture supernatant fluid of *F. oxysporum*-07 added to the vase water. F^{-1} = plus molecular filtrate diluted 10^{-1}; F^{-2} = plus molecular filtrate diluted 10^{-2}; R^{-1} = plus retentate diluted 10^{-1}; R^{-2} = plus retentate diluted 10^{-2}; W = in sterilized tap water.

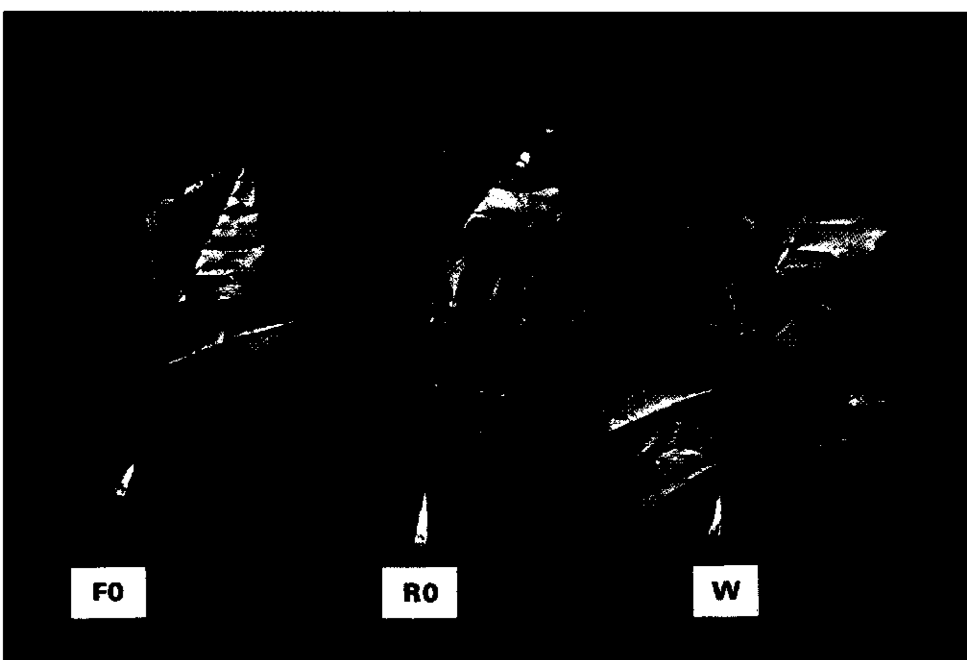

Plate 3. Showing the leaves of *Rosa* cv. 'Sonia' after 2 d of vase life, with various additives prepared from a culture supernatant fluid of *F. oxysporum*-07 added to the vase water. F0 = plus molecular filtrate; R0 = plus retentate; W = in sterilized tap water.

Table 1. Conversion of sucrose by pure cultures of micro-organisms. Results for saccharides, including denominator (*D*) of equation 1 (see text) are expressed as mol/l anhydrohexose

Micro-organism with strain no. and sample type		Glucose + Fructose GF	Sucrose S	Total saccharides: Before reduction E1	Total saccharides: After reduction E2	Polysaccharides (= Nominator of of equation 1) P	Denominator of equation 1 D	Average degree of polymerization DP′
Bacillus subtilis								
strain 207	SN	0·058	<0·001	0·237	0·052	0·179 (0·010)	0·127 (0·010)	1·4 (0·1)
strain 214	SN	0·018	0·047	0·214	0·175	0·149 (0·009)	0·021 (0·011)	7·1 (3·7)
	R*	0·071	<0·001	0·243	0·169	0·172 (0·011)	0·003 (0·013)	>12
Pseudomonas fluorescens								
	SN1*	0·015	0·058	0·194	0·173	0·121 (0·009)	0·006 (0·010)	>11
strain 52	SN2*	0·024	<0·001	0·103	0·050	0·079 (0·004)	0·029 (0·005)	2·7 (0·6)
	SN3*	0·127	<0·001	0·183	0·051	0·042 (0·013)	0·005 (0·013)	>2
Ps. putida								
strain 48	SN	0·137	0·009	0·237	0·110	0·091 (0·012)	−0·01 (0·013)	>6
	R	0·089	<0·001	0·177	0·098	0·088 (0·010)	−0·01 (0·011)	>7
Botrytis cinerea								
strain 196	SN	0·108	0·008	0·179	0·070	0·063 (0·010)	0·001 (0·010)	>5
Fusarium oxysporum								
strain 07	R1	0·082	0·056	0·214	0·126	0·076 (0·012)	0·006 (0·012)	>5
	R2	0·094	<0·001	0·198	0·100	0·104 (0·011)	0·004 (0·012)	>8

SN, culture supernatant fluid; R, retentate of supernatant fluid.
* Highly viscous.
P = E1 − GF − S, D = (E1 − GF − S) − (E2 − S) = E1 − GF − E2, DP′ = P/D (see text for further details).
Data between brackets are absolute standard deviations.

the supernatant (38%). The four retentates shown in Table 1 did not contain glucose, so GF here refers to fructose; the high proportions are inexplicable. Because no filtrates were analysed and no constant volume of retentate was obtained, a comparison of the saccharide concentrations of *SN* and *R* was not possible.

MICROBIAL EPS FORMATION IN STATIC CULTURES

No EPS was found, except in the supernatant fluid of a stationary culture of *Ps. fluorescens*-52 which contained a very low concentration of EPS.

WATER RELATIONS AND VASE LIFE OF 'SONIA' ROSES IN EPS-CONTAINING VASE WATER

The water conductivity values of the roses measured after 24 h of vase life as influenced by the composition of the vase water used, are set out in Figs 1a–5a. The water uptake (ml/d) from these vase waters is given in Figs 1b–5b. In addition the vase life and the flower opening shown by these roses (Berkholst 1980) are shown in Figs 1c–5c.

The higher the conversion of sucrose into polysaccharides, the greater was the disturbance of the water relations of the roses; this was measured as decreases in the water conductivity, the water uptake, and the vase life of the *Rosa* flowers.

The influence of the specific culture additives on the water relations and the vase life of *Rosa* flowers are as follows.

Bacillus subtilis *strains 207 & 214*

The *EPS* production and polymerization in shaken cultures of *B. subtilis* was so high that even retentate dilutions up to 10^{-2} (strain 214) decreased the vase life of *Rosa* as a consequence of xylem occlusions. Wilting, shrivelling, 'bent-neck', as well as chlorotic spots on the leaves were evident, but at the end of the vase life even necrotic spots. The 10^{-3} retentate dilution showed only minor visual deviations compared with the control roses (strain 214). See Fig. 1a–c.

The non-diluted *Bacillus* culture filtrate promoted flower wilting, shrivelling and at the end of the vase life leaf chlorosis and necrotic spots, and sometimes also 'bent-neck' were evident. The non-diluted retentate of the static cultures caused a decrease of the vase life of about 50%.

Diluted retentate, however, showed no visual differences compared with roses in sterilized tap water.

Pseudomonas fluorescens strain 52

The deviations and disturbance of the water relations and the vase life of *Rosa* flowers in vases supplemented with *Ps. fluorescens*-52 shaken culture retentate were (but to a somewhat lesser extent) similar to those induced by *B. subtilis* retentate. At the end of the vase life, flower shrivelling and 'bent-neck' were observed. The undiluted filtrate also caused some flower wilting. After two days of vase life the non-diluted retentate of the *Ps. fluorescens*-52 static culture promoted an acute 'toxic' reaction towards the flowers, shown by a fall in the vase fluid uptake, and 'bent-neck' and shrivelling of the flowers, although the EPS content of the static culture was very low, indeed. The static culture filtrate caused only a slightly decreased vase life. See Fig. 2a–c.

Pseudomonas putida strain 48

A heavy disturbance of the water relations (wilting and 'bent-neck') of *Rosa* flowers was caused by shaken culture retentates of *Ps. putida*, even at 10^{-1} dilution, although to a lesser extent than caused by the non-diluted retentate. Non-diluted filtrates of these shaken cultures caused a slightly decreased vase life (Figs 3a–c), as did also the supernatant fluids of static cultures of this strain.

Fusarium oxysporum strain 07

Vase fluids with additions from shaken cultures of *F. oxysporum*-07 caused the most significant effects on the vase life of the *Rosa* flowers compared with the other microbial strains tested (see Plates 2 and 3 facing p. 371). Besides xylem vessel occlusions and the consequences thereof on the water relations of the roses, the flower bud development was accelerated significantly, compared with control roses (Fig. 4a–c). This phenomenon might have been initiated by ethylene production by the *Fusarium* strain or elicited thereby (Albersheim *et al.* 1977). It is not yet clear whether low molecular weight compounds collected in the filtrate might have played a certain role, not only in the disturbance of the water uptake of the *Rosa* flowers, but also in the accelerated senescence. The end of the vase life, when retentate or filtrate was added to the vase fluid, was marked by flower wilting, 'bent-neck' and leaf chlorosis alongside the veins. In addition, leaf necrosis and dehydration of the leaves was observed preceding the 'bent-neck' phenomenon. Only a slightly reduced ornamental life was shown when roses were subjected to supernatants of a young static culture of *F. oxysporum*-07.

Botrytis cinerea strain 196

Roses in vase fluids with a concentrated molecular retentate supplement from shaken cultures of *Bot. cinerea*-196 showed xylem vessel occlusions, disturbance of the water relations and a decreased vase life, even when retentate dilutions up to 10^{-2} were added. To a significantly lesser extent, the culture filtrate caused decreased water conductivity, water uptake and vase life (Fig. 5a–c). At the end of the vase life, in both types of vase fluids the roses showed flower wilting and shrivelling and chlorotic as well as necrotic spots on the leaves. Supernatant fluids of young static cultures, however, only slightly reduced the vase life of 'Sonia' roses.

PECTINASE PRODUCTION IN MICROBIAL CULTURES

No pectinase activity was observed in shaken cultures (with 5% w/v sucrose) of the six microbial strains tested, nor in the young static control cultures (with 1% w/v sucrose) of these microbial strains. In older glucose broth cultures which were inoculated with washed cells obtained from a pectin-containing growth medium, pectinase activity was positive (*B. subtilis, Ps fluorescens, F. oxysporum, Bot. cinerea*) or variable (*Ps putida*) (Put & Rombouts 1989). Thus microbial pectinase could not have played a role in the vase life of the roses.

BEETROOT TISSUE CUBE TEST

The tissue-permeability damaging reaction of supernatant fluids of the shaken cultures tested against cubes of beetroot tissue was variable (*Ps. putida*) or strongly positive (remaining

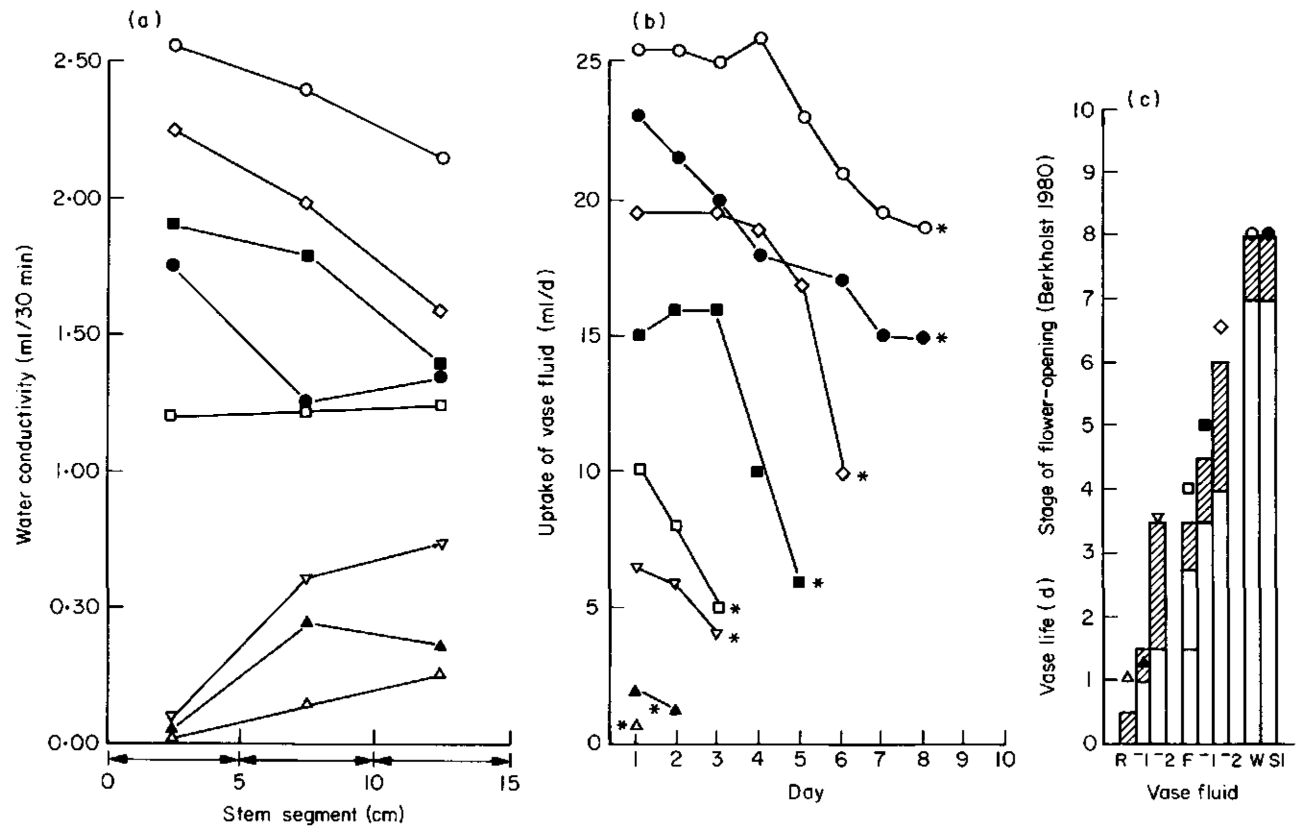

Fig. 1. Water relations and vase life of *Rosa* in vase water containing added filtrate and retentate of culture fluids of *Bacillus subtilis*-214. (a) Showing the conductivity (ml/30 min) of three consecutive *Rosa* stem segments of 5 cm each after 24 h of vase life. Additives were prepared from the supernatant fluid of a shaken culture of *Bacillus subtilis*-214), separated by a molecular separator at 10 000 Da. Dilutions of culture retentates (R) and filtrates (F) were prepared in sterilized tap water. △, plus retentate (R); ▲, plus retentate 1 : 10; ▽, plus retentate 1 : 100; □, plus filtrate (F); ■, plus filtrate 1 : 10; ◇, plus filtrate 1 : 100; ○, control, in sterilized tap water (W); ●, control, in sucrose 1% w/v in sterilized tap water (S1). (b) Showing the uptake of vase fluids (ml/d). Symbols used are as in (a). *, end point of the vase life of *Rosa* flowers. (c) Illustrating the vase life of *Rosa* flowers (d). The shaded part of the vase life bar indicates the number of days during which the ornamental value of the *Rosa* flowers was mediocre. The stage of the flower opening at the end of the vase life is indicated by the location of the symbols used in (a) for the different vase-water conditions.

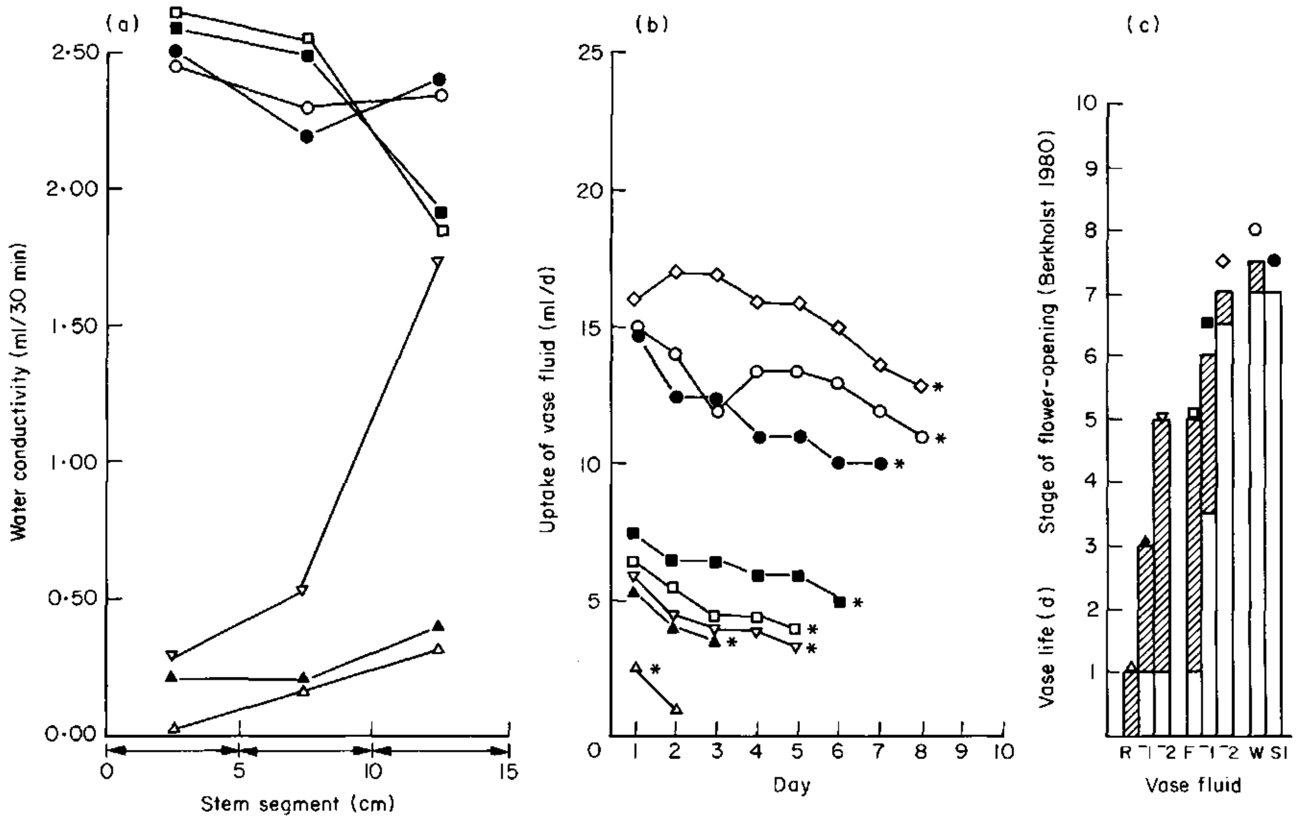

Fig. 2. Water relations and vase life of *Rosa* in vase water containing added filtrate and retentate of culture fluids of *Pseudomonas fluorescens*-52. (a) Conductivity; (b) uptake of vase fluids; (c) vase life of *Rosa* flowers. For details see Fig. 1.

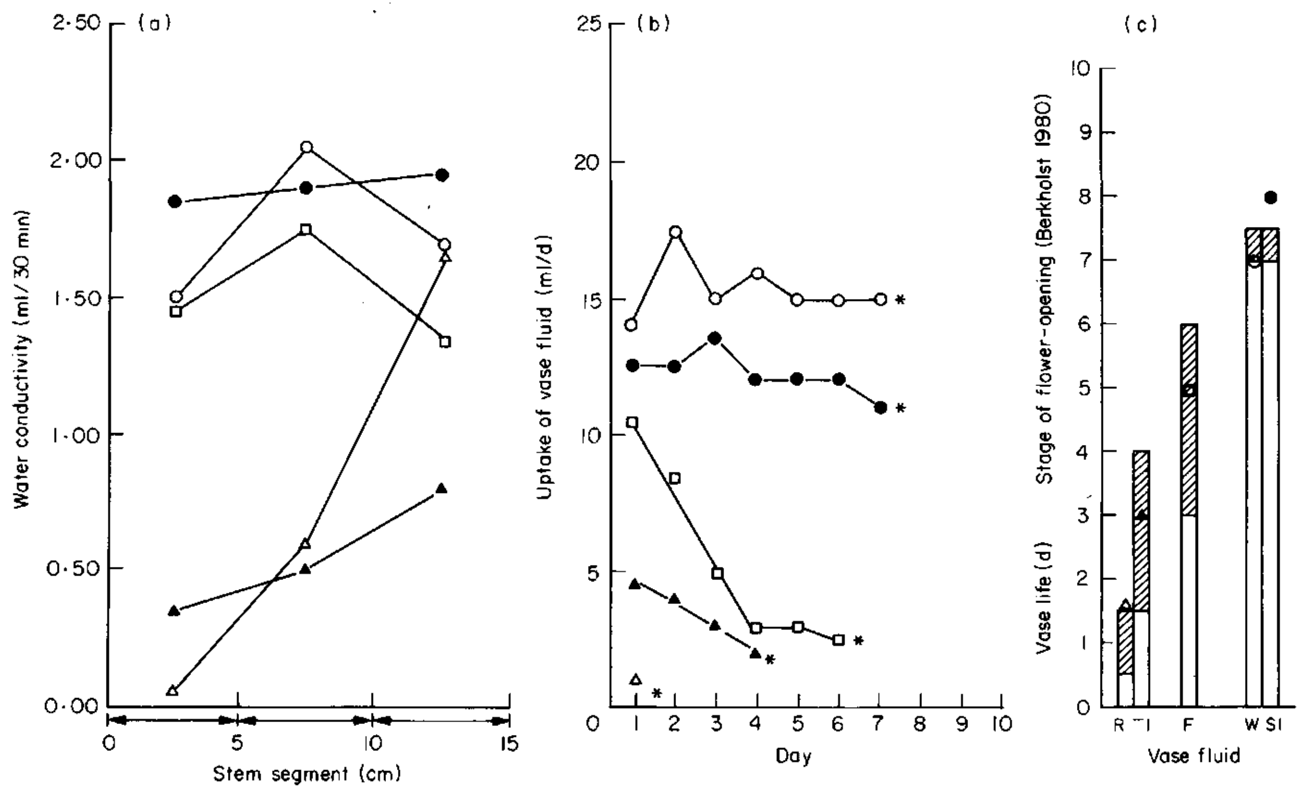

Fig. 3. Water relations and vase life of *Rosa* in vase water containing added filtrate and retentate of culture fluids of *Pseudomonas putida*-48. (a) Conductivity; (b) uptake of vase fluids; (c) vases life of *Rosa* flowers. For details see Fig. 1.

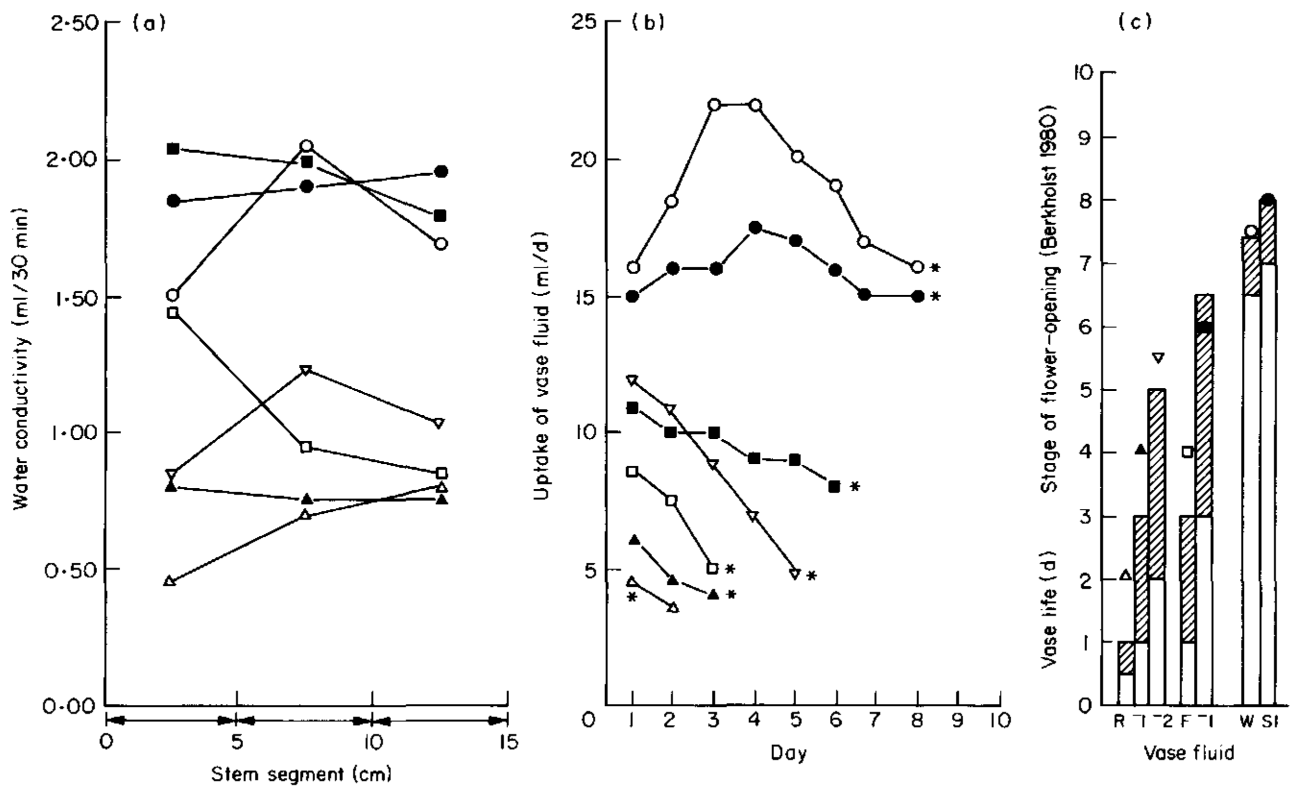

Fig. 4. Water relations and vase life of *Rosa* in vase water containing added filtrate and retentate of culture fluids of *Botrytis cinerea*-196. (a) Conductivity; (b) uptake of vase fluids; (c) vase life of *Rosa* flowers. For details see Fig. 1.

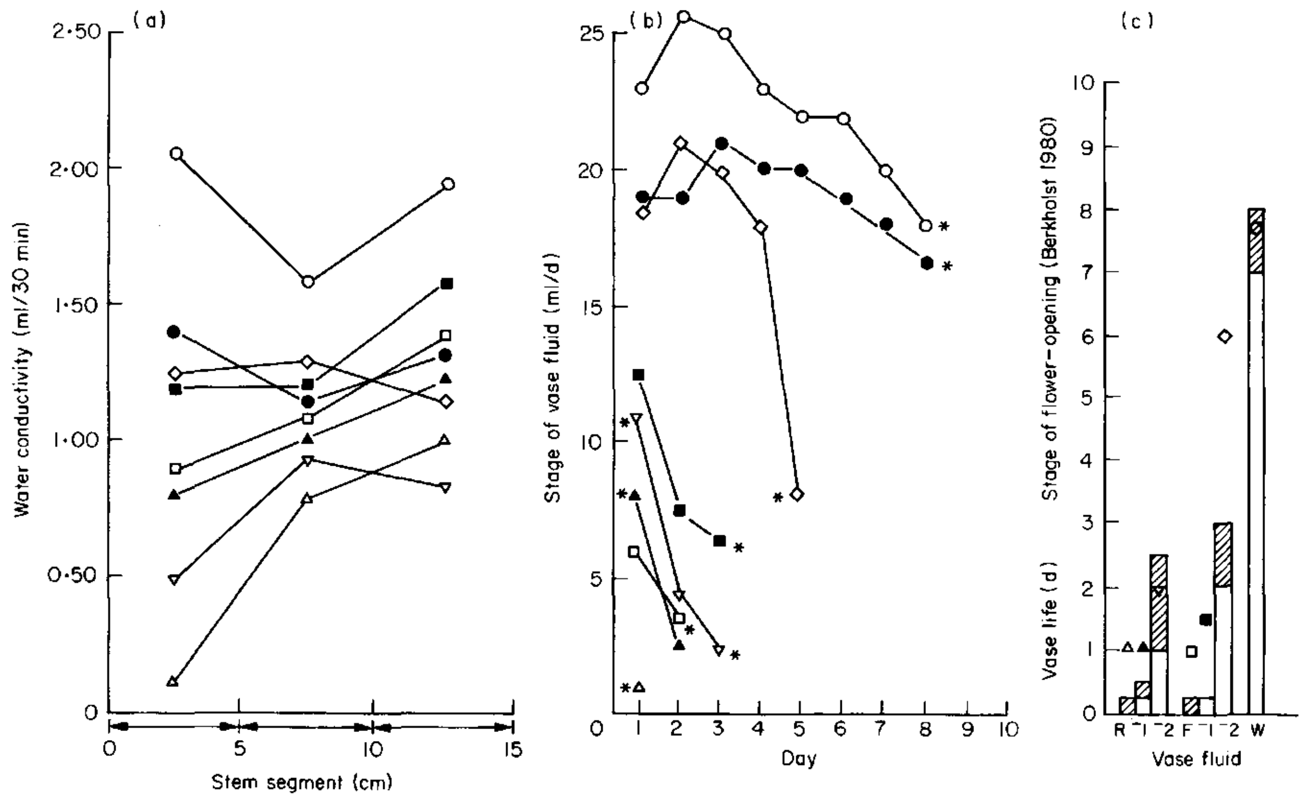

Fig. 5. Water relations and vase life of *Rosa* in vase water containing added filtrate and retentate of culture fluids of *Fusarium oxysporum*-07. (a) Conductivity; (b) uptake of vase fluids; (c) vase life of *Rosa* flowers. For details see Fig. 1.

strains). After molecular filtration of these cultures the retentate was shown to give a somewhat stronger beetroot-damaging reaction. The filtrates, however, were beetroot negative (*Ps. putida*), weakly positive (*Bot. cinerea*), positive (*B. subtilis*) or strongly positive (*Ps. fluorescens* and *F. oxysporum*) in their ability to damage beetroot tissue. Static culture supernatant fluids almost always showed no effects on beetroot tissue permeability.

ACID FUCHSIN TEST

EPS-containing culture retentate

The greater the disturbance of the water relations of the roses, the greater was the reduction in the vase life of the roses, and the ability of the fuchsin red to infiltrate into the *Rosa* xylem vessels was also markedly reduced, to values mainly from 1–2 mm up to 2–3 cm upwards into the vessels. Roses placed in diluted retentates were equally blocked (or to a somewhat lesser extent compared with the non-diluted retentate) although a few vessels remained open, as observed by their red coloration. Further dilutions showed a decreased vessel blockage: the dye penetrated *ca* 12–25 cm up the stem.

Culture filtrate

When culture filtrates were present in the vase water a minor (with *F. oxysporum*) or no (with the remaining strains) vessel blockage was observed, even when the filtrate was shown to be toxic to roses.

CONTROL

Roses placed in sterilized tap water, or sucrose 1% w/v or filter-sterilized mashed *Rosa* stem suspension (0·1% w/v) showed no vessel blockage and a fuchsin red coloration of xylem vessels due to fuchsin uptake was observed even in the flower petals and the leaves.

SCANNING ELECTRON MICROSCOPE OBSERVATIONS

When *F. oxysporum*-07 retentate was present in the vase water, at one cm upwards from the cut surface of the rose stems, the SEM images of the cross sections showed occlusion and blocking of a part of the xylem vessels by amorphous material. Diluted retentates also showed, although to a lesser extent, similar aberrations, compared with cross-section images of the control roses placed in sterilized tap water. In addition it was found that the *F. oxysporum* retentate injured and partly deformed (Figs 6 and 7) the vessel structure of the *Rosa* stems. In non-diluted filtrates of *B. subtilis* or *Ps. fluorescens*, the rose stem vessels showed no or only slight morphological deviation. ***Fusarium oxysporum*** filtrate, however, caused heavy disorganization of the vascular structure, even when present at lower concentrations.

Discussion

THE ROLE OF SUCROSE IN ROSA VASE WATER

In commercial practice, 2–10 d may elapse between the harvest of the flowers and the commencement of their ornamental vase life. During the pre-ornamental phase, plant sucrose is used by the cut flower as a source for respiratory energy, because complete flower development is energy-requiring. Sucrose interaction with plant hormones, i.e. ethylene, abscisic acid and cytokinins, moderates the programmed flowering process, and sugars added to vase water contribute towards the osmotic adjustment of the developing flowers (Halevy & Mayak 1979) as well as to cell metabolism (Paulin *et al.* 1981 and Paulin & Jamain 1982). The general protective effect of sugar on membrane integrity also maintains mitochondrial structure and function (Halevy & Mayak 1981). An exogenous supplement of sucrose (or glucose) during the post-harvest life of cut flowers such as *Rosa* is thus advantageous to obtain mature flowers. Furthermore, sucrose plays an important role in the uptake, transport and storage of carbohydrates in leaves and petals of cut *Rosa* flowers (summarized diagrammatically in Fig. 8). Externally applied sugars are first accumulated in rose leaves and only then translocated from the leaves to the flowers (Paulin 1980). Normally relative high concentrations of chemical solutions are used for pulsing cut flowers, intermediate for bud-opening, and low concentrations for holding solutions (Halevy & Mayak 1981). The supplied sugar may also reduce naturally

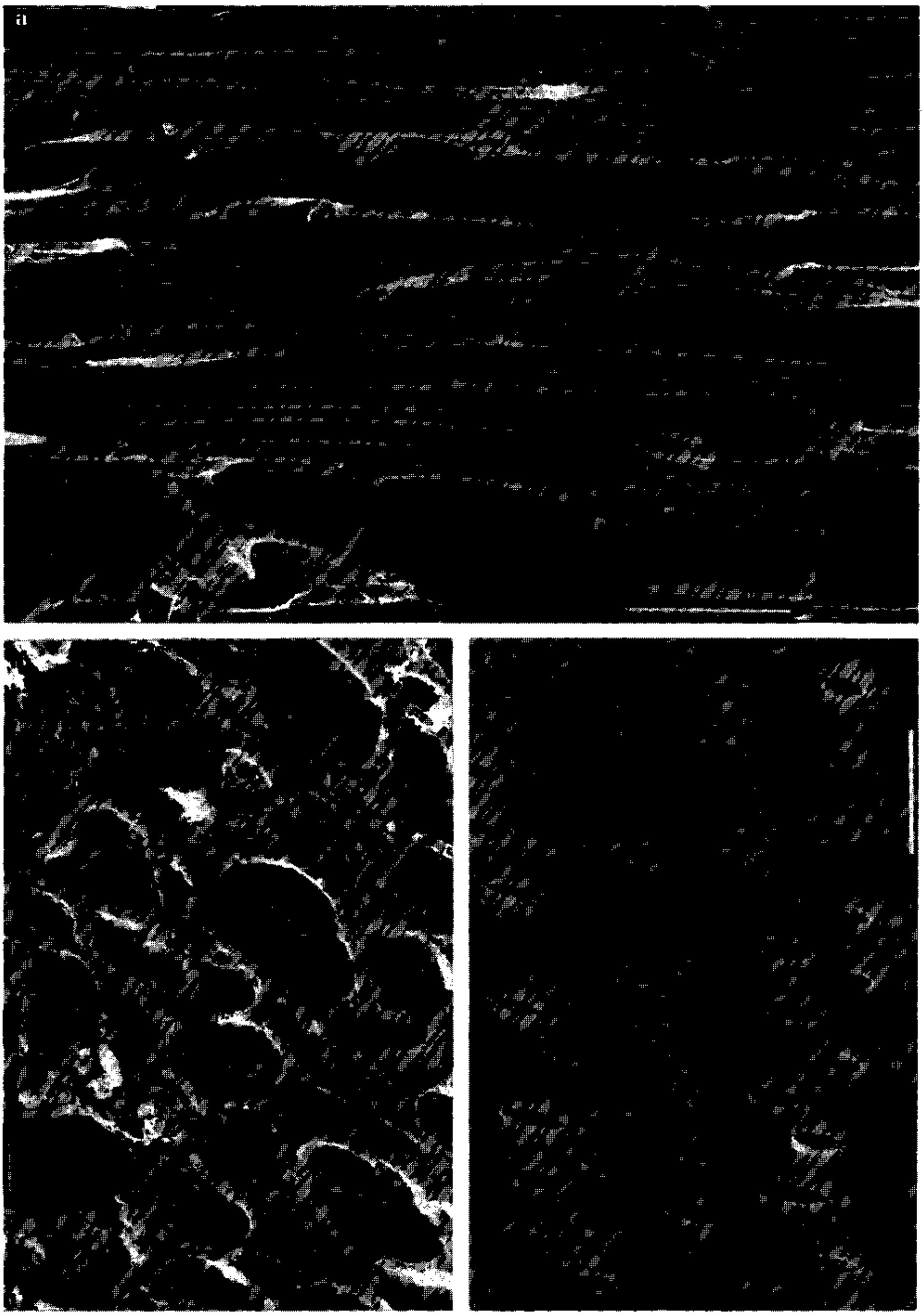

Fig. 6. *Rosa* cv. 'Sonia', held in sterilized tap water 1 d. (a) Longitudinal section from near the cutting point. Bar represent 100 μm. (b) Transverse section from near (ca. 1 cm) the cut surface. Bar represents 10 μm. (c) Detail showing pit openings in a xylem vessel. Bar represents 10 μm. (SEM microphotographs, TFDL, Wageningen, The Netherlands.

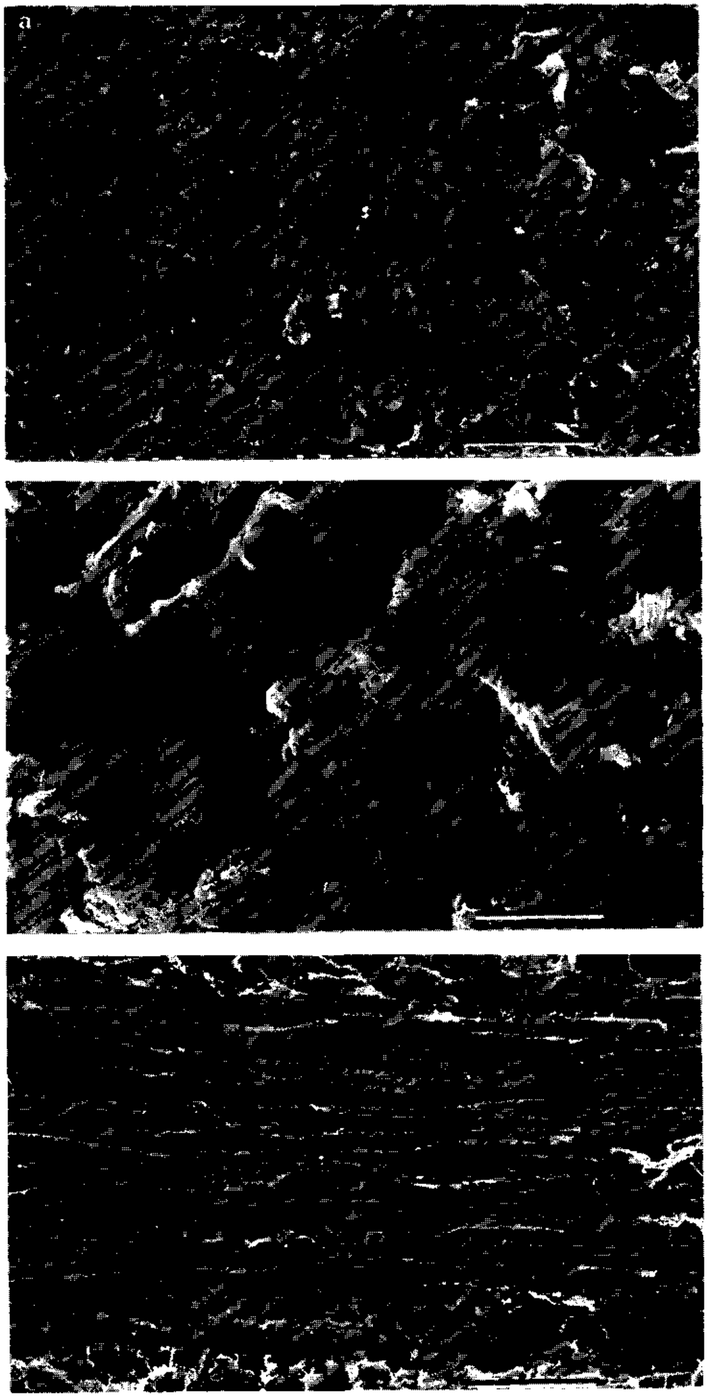

Fig. 7. *Rosa* cv. 'Sonia' after 1 d in vase fluid containing some of the filter retentate of a culture supernatant fluid *F. oxysporum*-07, diluted 10^{-1}. (a) Transverse section *ca* 10 mm from the cut surface of the stem. Bar represents 100 μm. (b) Detail of (a). Bar represents 10 μm. (c) Longitudinal section of the stem, near the point of cutting. Bar represents 100 μm (SEM microphotographs, TDL, Wageningen, The Netherlands).

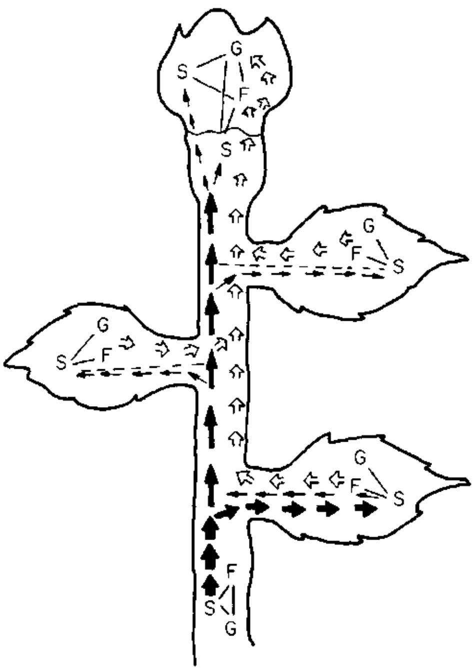

Fig. 8. Diagram representing (semiquantitatively by means of arrow thickness) carbohydrate transport in the floral branch of 'Carina'-rose supplied with a solution of glucose or sucrose (G = glucose; F = fructose; S = sucrose), after Paulin *et al.* (1981). When the floral branch of a rose was supplied with a solution of 0·16 mol/l glucose or 0·08 mol/l sucrose the level of C(arbon) rose at the same rate in both cases. Glucose was immediately transformed into sucrose in the stem axis. Sucrose migrated to the leaves and flower and was equally distributed between the ovary and the petals. In the flower, sucrose is immediately hydrolysed, in the leaves its hydrolysis occurs more slowly. A strong isomerase activity occurred in the petals.

occurring starch hydrolysis and lipid degradation in cut *Rosa* stems held in water (Molnar & Parups 1977, Paulin 1980). The first post-harvest addition of sucrose is mainly given in the greenhouse directly after cutting when the roses are placed in basins with circulating water, but other metabolic sugars like glucose and fructose are similarly effective (Halevy & Mayak 1981, Paulin *et al.* 1981, Paulin & Jamain 1982). In these basins the first contact of the cut flower with microbial EPS or EPS-producing micro-organisms may also occur.

Further handling of the *Rosa* flowers (at the point of sale, during transport, storage and at the retail market), involves further opportunities for the flowers to contact microbial EPS and EPS-producing micro-organisms. $Al_2(SO_4)_3$, 50–100 μg/ml, has been used in many preservative formulation for pulsing and holding solutions for cut roses. It contributes to a lowering of the pH, but does not generally contribute to a lowering of the microbial activity in the vase fluid to below an acceptable level. Ag-thiosulphate (STS) is a more effective germicide. It acts as inhibitor of ethylene production of many cut flower cultivar, but is not successful for pulsing of roses (Halevy & Mayak 1979; Veen 1986, 1987).

In our experiments the roses were tested under strictly hygienic conditions within 1–2 d after the harvest. Therefore, the carbohydrate reserve of the cut flower may have been optimal and not dependent on exogenous sucrose supplementation for flowering.

POLYSACCHARIDE FORMATION

Among the different kinds of bacterial exopolysaccharides constituting the EPS of capsules or slime are $\beta(2 \rightarrow 6)$-linked poly-D-fructans (levans). In bacterial cultures they may be synthesized from the fructose moiety of sucrose, present in the medium. Reports of levan-forming *Bacillus* and *Pseudomonas* species are mentioned by Sutherland (1977, 1979) and by Powell (1979). Most of the organisms used in our experiments are known commonly to form exopolysaccharides (possibly levans) (Wilkinson 1958, 1977; Gorin & Spencer 1968; Govan *et al.* 1979; Powell 1979; Anderson 1984; Marquës *et al.* 1986; Neumann 1987).

The culture liquids of our test organisms contained a high proportion of saccharides (other than glucose, fructose or sucrose) with an average degree of polymerization above 2. Although nothing is known about the distribution of monomers, oligomers and polymers in a saccharide solution with DP′ = 2, (as an example), two distribution limits are possible in such a solution: (1) The whole fraction consists of dimers (other than sucrose); (2) the anhydrohexose units are equally distributed between monomers and high polymers. Moreover, some culture liquids with a DP′ above 2 were highly viscous, and may be interpreted as indicating exopolysaccharide synthesis from sucrose.

In our experiments the DP value as well as the quantity of EPS produced, was found to depend on the microbial species, the microbial strain and the physiological state of the culture used. The quantity of EPS in most shaken cultures was so high that even retentate dilutions of 10^{-2} used as vase fluid caused blockage of *Rosa* xylem vessels, as is shown in Figs 1–5, Plate 2.

THE INFLUENCE OF MICROBIAL EPS ON THE WATER RELATIONS OF ROSES

The greater the microbial conversion of sucrose into polysaccharides, the more disturbed were the water relations of the roses placed in the EPS-containing fluids. EPS taken up with the sap stream by cut *Rosa* flowers, does block the open xylem vessels at the base. The smaller the DP′ value or the lower the EPS concentration (Table 1), the higher did the EPS infiltrate upwards into the open xylem vessels. The microbial EPS produced could not have passed the capillary holes in the pit membranes (0·02–0·10 μm diameter) nor would it cause mechanical rupture of the membranes (Van Alfen & Allard-Turner 1979; Halevy & Mayak 1981; Neumann 1987). The highest point which could have been reached by infiltration of EPS up the stems was shown to be the longest open vessel (*ca* 25 cm according to de Stigter & Broekhuysen 1986).

It is not clear, however, if microbial EPS infiltrated into partially blocked *Rosa* xylem vessels; it might have blocked the pit membranes in these vessels and thus also inhibited the sap stream (Zimmermann 1983).

In addition, it was observed that EPS-containing culture retentates of *Bacillus*-214 and *Pseudomonas*-48 & 52 disturbed to a considerable extent the vase life of *Chrysanthemum* cv. 'Daymark' and *Gerbera* cv. 'Rebecca' (results not reported).

LOCALISATION OF XYLEM OCCLUSIONS

With the acid fuchsin test, xylem occlusions were easily rendered visible to the naked eye. This, however, does not give any information about the nature and chemical structure of the occluding material.

Cytochemical staining methods such as the use of aqueous ruthenium red (0·02% w/v) involve microscopic examination (Peresse 1974; Rasmussen & Carpenter 1974; Roth 1977). Moreover, cytochemical studies show that not only microbial EPS but also other plant-derived carbohydrates such as pectins material may be coloured by ruthenium red (Parups & Molnar 1972).

EFFECTS OF THE INFILTRATION OF MICROBIAL COMPOUNDS ON XYLEM CELL WALLS

It is noteworthy that cytotoxic polysaccharides may be released by partial hydrolysis of plant cell walls by enzymes. Thus, in addition to diverting xylem flow, xylem-borne polymers might directly induce senescence of leaf cells (Neumann 1987). The apparent absence of visible xylem blockage by plant polysaccharides reported by Van Alphen & Turner (1975) and Neumann (1987) suggests that complex hormonal regulation may affect polysaccharide concentrations in specific sections of the xylem system. To reveal the occurrence of this phenomenon, further research is needed (Halverson & Stacey 1986).

THE EFFECTS UPON ROSA VASE LIFE OF LOW MOLECULAR WEIGHT MICROBIAL COMPOUNDS IN VASE FLUIDS

Elicitation of a significantly decreased vase life (accelerated bud opening) of Sonia roses was caused by the uptake of culture filtrates of *B. subtilis*-207; *Bot. cinerea*-196 and *F. oxysporum*-07. Although no physical plugging of xylem vessels was found, the water conductivity decreased significantly. By cutting-off *ca* 15–25 cm of the stem, no reversal of visual symptoms occurred if the roses were replaced in sterilized tap water. Hence eventual low molecular weight toxic microbial compounds in the vase fluid may have been flushed up in the sap stream through the pit membranes.

CONSEQUENCES OF MICROBIAL COMPOUNDS IN VASE FLUIDS FOR THE POSTHARVEST HANDLING OF CUT ROSES

Many of the micro-organisms coming into contact with cut flowers and their vase water are able to excrete high molecular weight compounds (exopolysaccharides) and also low molecular weight toxins (or toxoids) which can adversely affect the longevity of the flowers (Accati 1980; Accati-Garibaldi 1983). Thus contact of cut flowers with micro-organisms should be prevented, and conditions should be such that microbial growth is minimal.

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Chapter 7

The influence of purified microbial pectic enzymes on the xylem anatomy, water uptake and vase life of *Rosa* cultivar 'Sonia'.

Rosa cv. 'Sonia': stage 7 of flower opening (Berkholst, 1980)

The Influence of Purified Microbial Pectic Enzymes on the Xylem Anatomy, Water Uptake and Vase Life of *Rosa* cultivar 'Sonia'

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ABSTRACT

Put, H.M.C. and Rombouts, F.M., 1989. The influence of purified microbial pectic enzymes on the xylem anatomy, water uptake and vase life of *Rosa* cultivar 'Sonia'. *Scientia Hortic.*, 38: 147–160.

This paper describes macroscopic and microscopic symptoms following the addition of purified pectic enzymes (pectate lyase of *Pseudomonas fluorescens* and polygalacturonase of *Kluyveromyces fragilis*) to the vase water containing cut flowers of *Rosa* cultivar 'Sonia'. These symptoms were: decreased water uptake; slightly decreased water conductivity after 24 h and 48 h of vase life; decreased flower-bud development; a deep red colouration of desiccated flower petal edges; chlorotic spots on the leaves, which became necrotic in the center of the spots. Scanning electron microscope (SEM) observations of the stem xylem vessel system after 40 h vase life showed: (1) degradation of wall structures causing disorder and loose particles in the vessels; (2) injury of the vessel pit structure; (3) released spiral vessels. This promoted desiccation of the flower petals and leaves, but not vessel blockage or wilting. The structural abnormalities apparently were not the reason for the reduced water uptake caused by plugging of the xylem vessels, as was shown by water conductivity experiments after 24 and 48 h of vase life.

Keywords: cut flowers; pectic enzymes; roses; vase life; xylem vessel structure degradation.

Abbreviations: M = molar (mol l^{-1}); mol.wt. = molecular weight; RH = relative humidity; SEM = scanning electron microscope; U = units; W = watt.

INTRODUCTION

Microbial contamination and microbial products have been implicated as causes of reduced vase life of cut flowers (Aarts, 1957; Mastalerz, 1977; Accati et al., 1980). However, vascular plugging also may have been caused by cutting

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injury, enzymatic activity or endogenous ethylene formation (Abelis, 1973; Fujino et al., 1983; Vandermolen et al., 1983). In studying the influence of the microflora on the stems of cut flowers on the vase life of *Rosa* 'Sonia', *Gerbera* 'Fleur' and *Chrysanthemum* 'Spider' (Put, 1986), it was observed that not only microbial occlusions, but also microbial products (e.g. enzymes), may decrease the vase life of cut flowers, which must receive a minimal amount of water (and carbohydrates) to remain alive and turgid (Conrado et al., 1980).

A major factor contributing to the rapid senescence of cut *Rosa* flowers appears to be the blockage of water-conducting vessels of the xylem, which accounts for the lack of turgor in the flower pedicel, causing "bent neck" and wilting of petals (Parups and Molnar, 1972; Halevy and Mayak, 1981). Histochemical studies of xylem blockage in cut roses furthermore showed that the occluding material stained positively with ruthenium red, indicating pectinaceous material and other high mol.wt. polysaccharides (Lineberger and Steponkus, 1976; van Alfen and Allard-Turner, 1979; Vandermolen et al., 1983; Zimmermann, 1983; Neumann, 1987).

The major cell-wall constituent of herbaceous plants which influences the water-holding capacity of the intact plant is pectin (Domsch and Gams, 1969). A number of pectolytic enzymes are responsible for the process of pectin degradation (Codner, 1971), and soil is a large reservoir of pectolytic microorganisms; $>10^6$ pectolytic microorganisms g^{-1} soil can be counted. The phyllosphere of healthy plants also harbours a wide variety of pectolytic bacteria with a preponderance of *Pseudomonas, Bacillus* and *Flavobacterium* and the rhizosphere also contains abundant aerobic pectolytic microorganisms (Rombouts and Pilnik, 1980).

It is not surprising that many plant pathogens secrete pectin-degrading enzymes because the cell wall is a barrier which must be breached by most microbial pathogens (Domsch et al., 1980). It is possible that these pectin-degrading enzymes may activate host enzymes that release phytoalexins, thus acting as an endogenous elicitor (Halverson and Stacey, 1986). Phytoalexin accumulation can also occur in the absence of exogenous materials when the tissue is wounded or when some of the cells are killed, e.g. by cutting or pruning.

To prove that any enzyme produced by a specific microorganism really causes the disease of the plant or decreases the vase life of cut flowers, and that this is not due solely to chance contaminant(s), it was thought to be desirable to test the influence of single purified enzymes. Moreover, by the addition of purified pectic enzymes to vase water, the side effects of microbial products other than pectic enzymes can be avoided and therefore it is possible to study specifically the effect of added pectic enzymes on the vase life of cut flowers. The results of a series of experiments with purified microbial pectic enzymes added to the vase water containing cut *Rosa* flowers are given in this paper.

MATERIALS AND METHODS

Plant material. – *Rosa hybrida* 'Sonia' flowers were supplied by local greenhouse growers. The roses were harvested between 15 February and 15 March 1986 at flowering Stage 1–2 (Berkholst, 1980), wrapped in parchment paper, transported to the laboratory, stored at 4°C for 24–48 h and the experiments then carried out.

Pectic enzymes. – The enzymes tested were: pectate lyase from *Pseudomonas fluorescens* and polygalacturonase from *Kluyveromyces fragilis* (cf. Rombouts and Pilnik, 1980). These enzymes were produced and purified according to Rombouts et al. (1978) and Phaff (1966), respectively. The enzyme preparations contained: 50 U ml^{-1} of pectate lyase and 20 U ml^{-1} of polygalacturonase, measured by the methods of Rombouts et al. (1978) and Phaff (1966), respectively. The pectate lyase was diluted in 1/80 M sterile Tris–HCl buffer at pH 7.5. The polygalacturonase was diluted in 1/80 M sterile citrate buffer at pH 4.5. Heat sterilized non-chlorinated Ca^{2+}-containing tap water was used for preparation of the buffered dilutions.

Vase water. – Dilutions containing: 0 (control), 1, 0.5, 0.1, 0.05 U of buffered pectate lyase ml^{-1} of sterile vase water and 1, 0.5, 0.1, 0.05 U of buffered polygalacturonase ml^{-1} of sterile vase water were prepared under aseptic conditions. Freshly-harvested 'Sonia' roses were cut off to a stem length of 45 cm and put singly into cylindrical vases calibrated up to 100 ml, containing 40 ml of sterile vase water. Each of the dilutions were at least tested in triplicate. The vases were held at 20 ± 1°C, 60 ± 2% RH (relative humidity) and 10.25 W m^{-2} photoradiation at flower height during a 12-h photoperiod each 24 h. Protection against air contamination was obtained by sealing a plastic film on the top of each vase (Parafilm "M", American Can Cy.).

Vase life. – Every 24 h observations were made on the water uptake (ml), the flowering stage (Fig. 1; Berkholst, 1980) and the ornamental value of the roses.

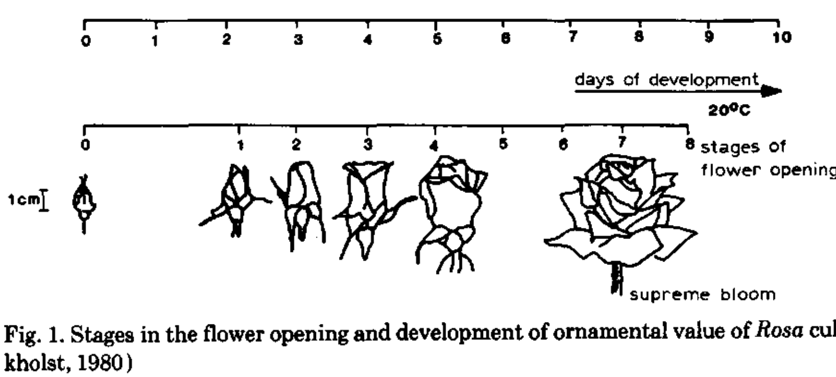

Fig. 1. Stages in the flower opening and development of ornamental value of *Rosa* cultivars (Berkholst, 1980)

When the ornamental acceptability of the flower in the vase was visually decreased to <50% of the "normal" flower-bud development in sterile vase water, the ornamental value was negative. Negative features in ornamental value are considered to be: wilting, drying, curling and discolouration of leaves and petals; bent neck; chlorotic and necrotic spots on the leaves; retardation of flower-bud development. After 24 and 48 h the water conductivity of the stems was measured according to the method of Chin and Sacalis (1977). The apparatus used consisted of an overhead 10-l bottle filled with sterile glass-distilled water, connected by medical plastic tubing to a glass-manifold of 2×12 pieces (Fig. 2). A Mariotte tube was inserted through a rubber stopper into the 10-l bottle to establish constant water pressure. The distance or height of the water column measured from the base of the Mariotte tube to the upper end of the stem segment was 60 cm. A piece of soft plastic tubing was attached to each T-piece for connection to a single stem segment.

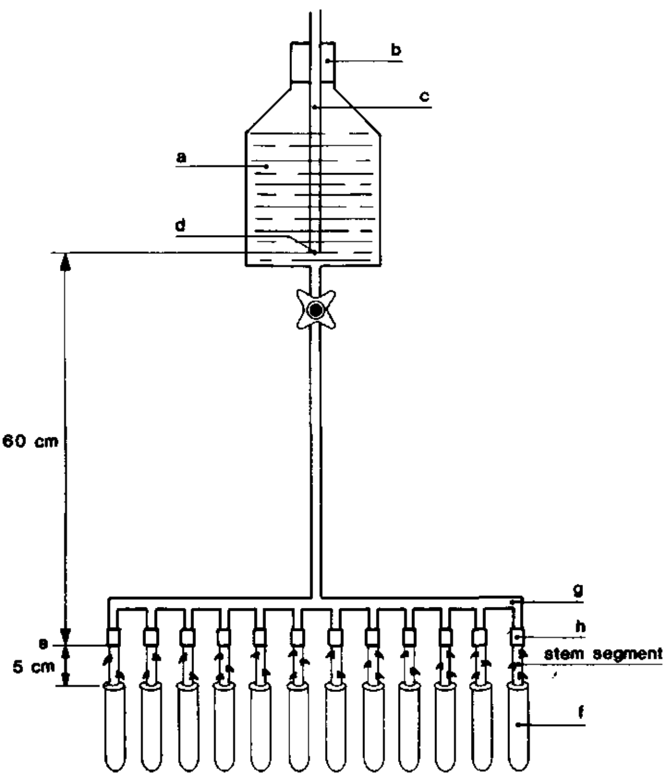

Fig. 2. Apparatus designed to measure xylem conductivity (Chin and Sacalis, 1977). (a) Bottle containing 10 l sterile glass-distilled water; (b) rubber stopper; (c) Mariotte tube; (d) base of the Mariotte tube; (e) upper end of the (upside down) inserted stem segment of 5 cm length; (f) plastic tube of known weight to collect water flowing through the stem segment; (g) glass manifold with 12 connectors (h) of soft medical plastic tubing.

TABLE 1

The influence of the addition of purified polygalacturonase in buffered vase water on (1) stage of flower development and (2) ornamental value of cut flowers of 'Sonia'; + = normal; ± = acceptable; - = unacceptable (see Fig. 1)

	Days of vase life										
	0	1	2	3	4	5	6	7	8	9	
Control: citrate buffer											
1/80 M pH 4.5	(1)	1	2	3	5	6	6	7	7	8	8
	(2)	+	+	+	+	+	+	+	+	±	-
Plus polygalacturonase											
1 U ml^{-1}	(1)	1	2	3	4	4	4				
	(2)	+	+	±	±	-	-				
0.5 U ml^{-1}	(1)	1	2	3	4	4	4				
	(2)	+	+	±	±	±	-				
0.1 U ml^{-1}	(1)	1	2	3	4	4	4	5	5		
	(2)	+	+	+	+	+	±	±	-		

Values are means of 6 replications and are rounded off. Significant differences of flower development and vase life are associated with addition of polygalacturonase: 1 U, 0.5 U and −0.1 U ml^{-1} vase water ($P<0.05$).

Triplicate samples of roses were taken from the vase water after 24 and 48 h vase life. Immediately thereafter 3 consecutive stem sections of 5 cm each were cut off from the basal end upwards and inserted onto the manifold in an upside down position. The water solution was allowed to flow through the segments (i.e. the natural direction) for 60 min to purge air in the lines and obtain an equilibrated water flow. Thereupon the water was collected in plastic tubes of known weight. The water flow through the segments was assessed by weighing the tubes after 30, 60 and 90 min of water flow. The average water conductivity 30 min^{-1} was then calculated (Durkin, 1979). Conductivity was defined as the reciprocal value to the resistance to flow, expressed per transverse-sectional area^{-1} of 5-cm stem length (Burdett, 1970; Gilman and Steponkus, 1972; Put and van der Meijden, 1988).

Microscopic and macroscopic observations. – After 40 h vase life SEM preparations of transverse and longitudinal sections of the stem and xylem vessels were made by the Technical and Physical Engineering Research Service, Mansholtlaan 12, Wageningen, The Netherlands (Dr. Ir. A. Boekestein and Anke Clerkx).

Statistical treatment of data. – Separation of mean values was done by analysis of variance (ANOVA) followed by a test for least-significant differences (LSD) at a probability level of $P<0.05$. A computer programme of the Lawes Agri-

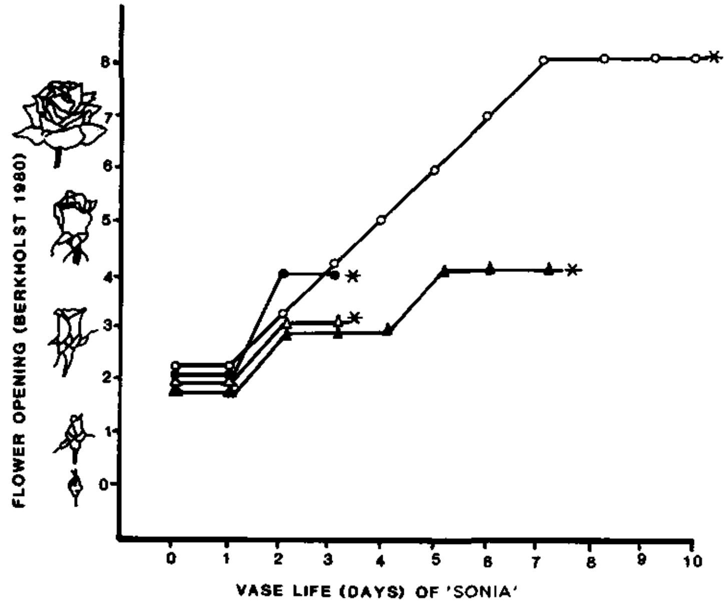

Fig. 3. The influence of the addition of purified pectate lyase to buffered vase water on the flower opening of cut roses. Vase water: o, 1/80 M Tris buffer at pH 7.5; •, plus pectate lyase 1 U ml^{-1}; ▵, plus pectate lyase 0.5 U ml^{-1}; ▴, plus pectate lyase 0.1 U ml^{-1}; *, end of the vase life.

cultural Trust (Rothamsted Experimental Station, Herts, England) was used, carried out by the Statistical Department of the Sprenger Institute, Wageningen, The Netherlands.

RESULTS

Vase life observations. – The vase life of the control roses was 9 ± 1 days. After the addition of 1 and 0.5 U ml^{-1} of buffered (pH 7.5) pectase lyase or 1.0 or 0.5 U ml^{-1} of buffered (pH 4.5) polygalacturonase to the vase water, the vase life was decreased to 3 ± 1 days, while 0.1 U ml^{-1} of either pectic enzyme added separately to the vase water resulted in a decreased vase life of 6 ± 1 days (Table 1, Fig. 3). However, no effect was shown when 0.05 U ml^{-1} of either of the pectic enzymes was added to the vase water.

Both pectic enzymes showed similar effects on the vase life of the roses, so detailed data concerning only pectate lyase are presented here. During the first 36 h of the vase life no effects of the enzyme treatment were visible on the flowering stage of the roses, which developed from Stage 1+ to Stage 4. Also the water-uptake level (Fig. 4) and the water-conductivity values (Table 2) were not significantly less compared with the control vases. However, after ≥ 2 days of vase life the uptake of water decreased compared with the control; at the same time the outer petals of the flowers showed signs of inadequate water

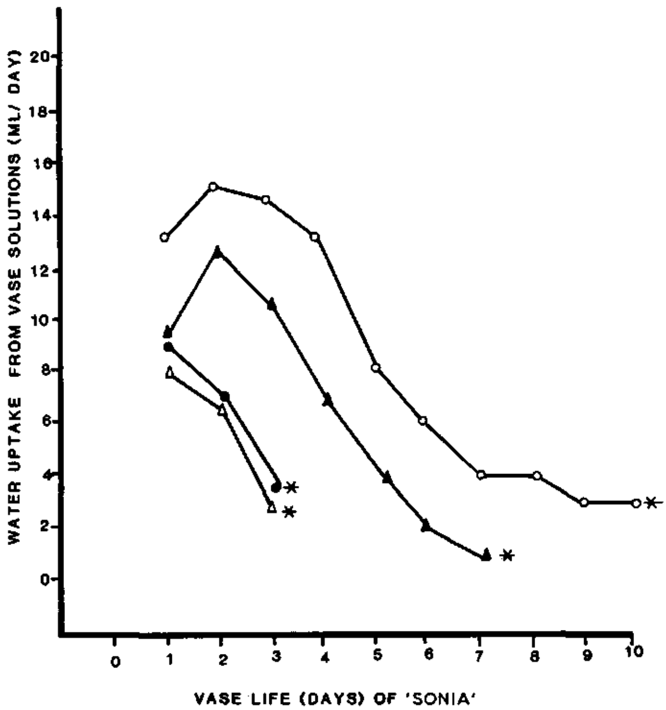

Fig. 4. The influence of the addition of purified pectate lyase to buffered vase water on the uptake of water by cut roses. Vase water: ○, 1/80 M Tris buffer at pH 7.5; ●, plus pectate lyase 1 U ml^{-1}; △, plus pectate lyase 0.5 U ml^{-1}; ▲, plus pectate lyase 0.1 U ml^{-1}; *, end of the vase life.

uptake by the curling, drying and dark-red colouring of the petal edges. In addition, the leaves showed water-deficiency symptoms, at first by chlorotic spots, which became larger and a few days later became partly necrotic (Fig. 5).

Scanning electron microscope observations. – Transverse and longitudinal sections of control roses showed "clear" xylem vessels and normal pit structures (Figs. 6 and 7), but numerous loose vessel fragments (Fig. 8), loose spiral xylem vessels (Fig. 9) and injured pits (Fig. 10) were seen in the xylem vessels of enzyme-treated cut roses after 40 h of vase life.

DISCUSSION

When purified pectolytic enzymes, viz. pectate lyase of *Pseudomonas fluorescens* and polygalacturonase of *Kluyveromyces fragilis*, were added to buffered vase water of cut 'Sonia' roses, their water relations were disturbed. The cause thereof may have been due to enzymatic degradation of the pectin-linked structures of the xylem vessels as observed in SEM preparations. Neither wilting nor "bent necks" of rose stems were evident, yet desiccation of petals and leaves and inhibition of flower-bud development occurred.

TABLE 2

Water conductivity (ml 30 min^{-1}) of stems of 'Sonia' after 24 and 48 h of vase life in buffered vase water with 1 U of pectic enzymes added ml^{-1}

Vase water		Vase life (h)	Distance from the base of the stem of the stem segment tested (cm)		
Enzyme added	Buffer 1/80 M (pH)		0–5	5–10	10–15
Control: no pectic enzyme added	7.5	24	1.85	2.20	3.00
		48	2.25	3.45	3.30
Plus pectate lyase: 1 U ml^{-1}	7.5	24	2.25	1.60	2.30
		48	0.90	1.65	1.50
Control: no pectic enzyme added	4.5	24	3.60	2.45	2.70
		48	2.35	2.90	2.75
Plus polygalacturonase: 1 U ml^{-1}	4.5	24	3.25	2.30	2.60
		48	2.90	3.45	2.50
Control: sterilized tap water. No pectic enzyme added	7.2	24	2.80	3.90	3.11
		48	2.50	3.10	3.35

Values are means of 9 replications. A significant effect on the water conductivity is observed after addition of pectate lyase and 48 h of vase life ($P < 0.05$).

It is conspicuous that a pronounced ability to decompose pectin is in almost all pectic enzyme-producing microorganisms linked with a high variability between different strains of the same microbial species (Rombouts and Pilnik, 1980). Polygalacturonase production by certain microbial strains (bacteria and fungi) may be remarkably stimulated by maintaining the microorganism in a pectin-containing culture medium (Nyeste and Hollò, 1962; Verhoeff and Warren, 1972); other factors may also affect pectin enzyme production or activity. *Bacillus circulans, B. polymyxa, B. pumilis, B. sphericus, B. subtilis,* and *Pseudomonas fluorescens* isolated by us from flower stems produced only slight amounts of endopectate lyase when grown in/on plain yeast extract media (Difco). In our experiments, pectic-enzyme production of these strains could greatly be stimulated by addition of pectin or pectate to the growth medium. Nevertheless, only cell-free culture liquids obtained from the post-stationary growth phase of these *Bacillus* strains showed a markedly increased endopectate lyase activity. Pectic enzyme-producing fungi isolated from flower stems, on the contrary, maintained their enzyme activity, even after transfer into plain malt extract agar (Difco).

The symptoms caused by the addition of purified pectic enzymes to the vase water containing cut flowers of 'Sonia' differed from those observed when water-washed microorganisms or microbial products, e.g. polysaccharides, and phy-

Fig. 5. The influence of purified pectate lyase added to buffered vase water on the ornamental value of cut roses after 3 days of vase life. Pect 1E = plus pectate lyase 1 U ml^{-1} in Tris buffer pH 7.5; pH 7.5 = 1/80 M Tris buffer.

totoxins, were suspended or mixed with vase water (van Alfen and Allard-Turner, 1979; Neumann, 1987; Put and van der Meijden, 1988). Washed microbial cells of *Bacillus, Enterobacter, Kluyveromyces* and *Pseudomonas* spp. added to vase water were shown to infiltrate into the cut xylem vessels of roses; this resulted in blockage of the water-transport system, decreased water uptake, decreased water conductivity and "bent-neck" flower stems within 24–48 h vase life (Put and van der Meijden, 1988; Put and Jansen, 1988). Microbial polysaccharides (mol.wt. > 10 000) in the vase water caused a sudden arrest of the flower development of the roses, "bent-neck" and total blockage of the water transport system within 24 h (H.M.C. Put and W. Klop, personal observations, 1988). However, it is not yet clear whether the interaction of the microbial pectic enzymes and the plant cell-wall carbohydrates of the cut roses induced the release of plant cell-wall oligosaccharins, resulting in the elicitation of: (1) host defence responses as shown in many intact plant; (2) abnormal growth; (3) morphogenetic changes induced by disturbed growth hormone-regulating mechanisms (Albersheim and Valent, 1978; Halverson and Stacey,

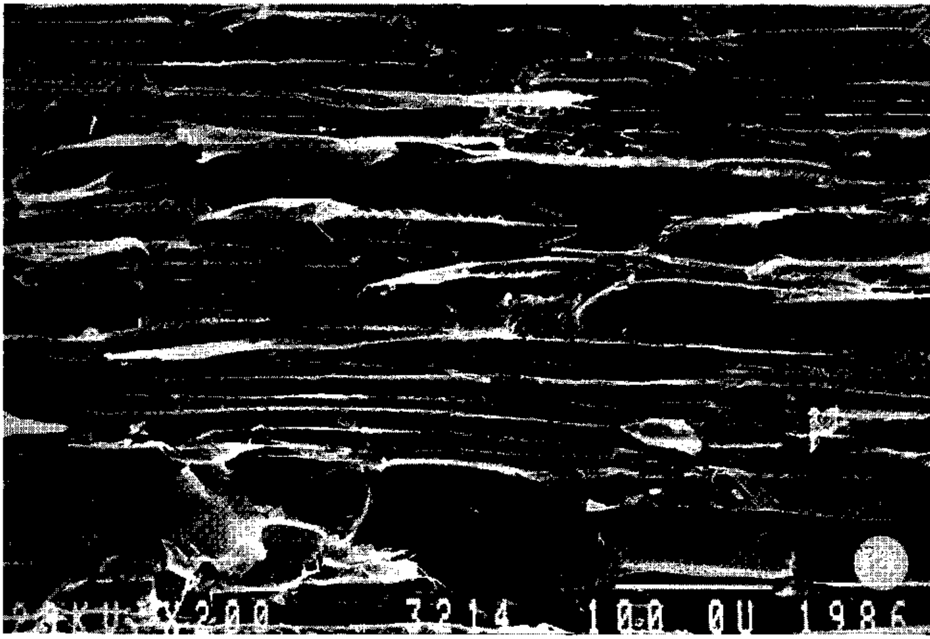

Fig. 6. Longitudinal section of xylem vessels of 'Sonia' near the base of the stem. Cut flowers were held in sterile vase water for 40 h and the vessel structure was normal. Bar mark represents 100 μm.

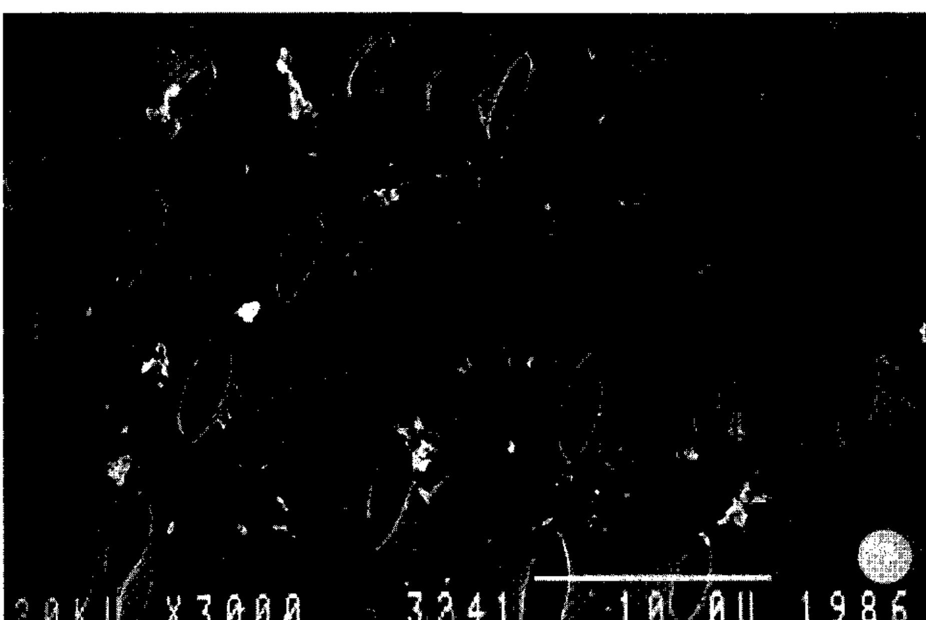

Fig. 7. Longitudinal section made at 5 cm from the base of the stem of 'Sonia' cut flowers held in sterile vase water for 40 h. A normal xylem vessel pit structure is shown. Bar mark represents 10 μm.

Fig. 8. Longitudinal section made at 2–10 mm distance from the base of the stem of 'Sonia' cut flowers held in a buffered sterile pectate lyase solution of 1 U ml^{-1} for 40 h. The vessel structure is seen to be degradated. Bar mark represents 100 μm.

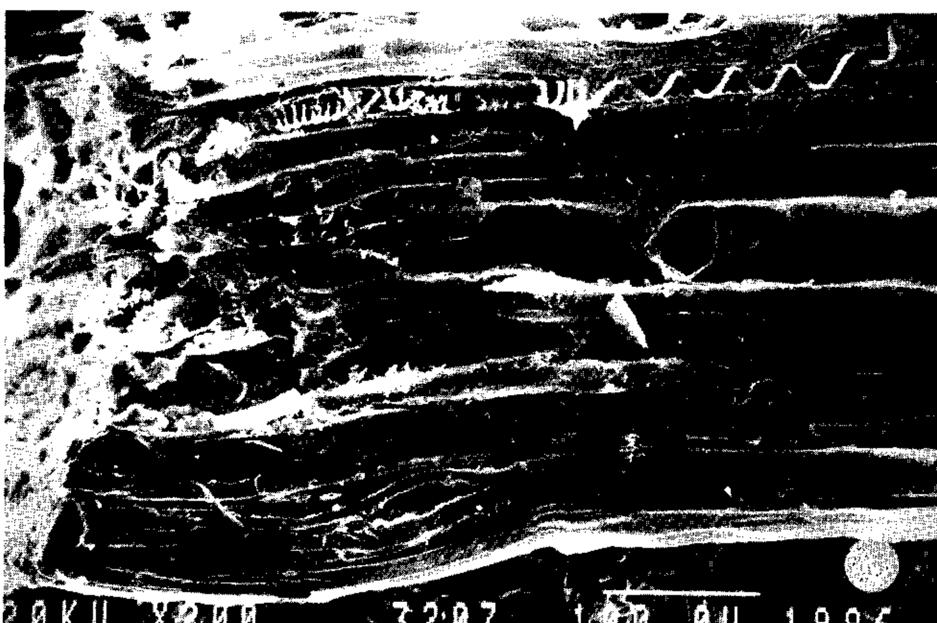

Fig. 9. Longitudinal section made at 2–10 mm distance from the base of the stem of 'Sonia' cut flowers held in a buffered sterile pectate lyase solution of 1 U ml^{-1} for 40 h. Loose spiral fragments of vessels are evident. Bar mark represents 100 μm.

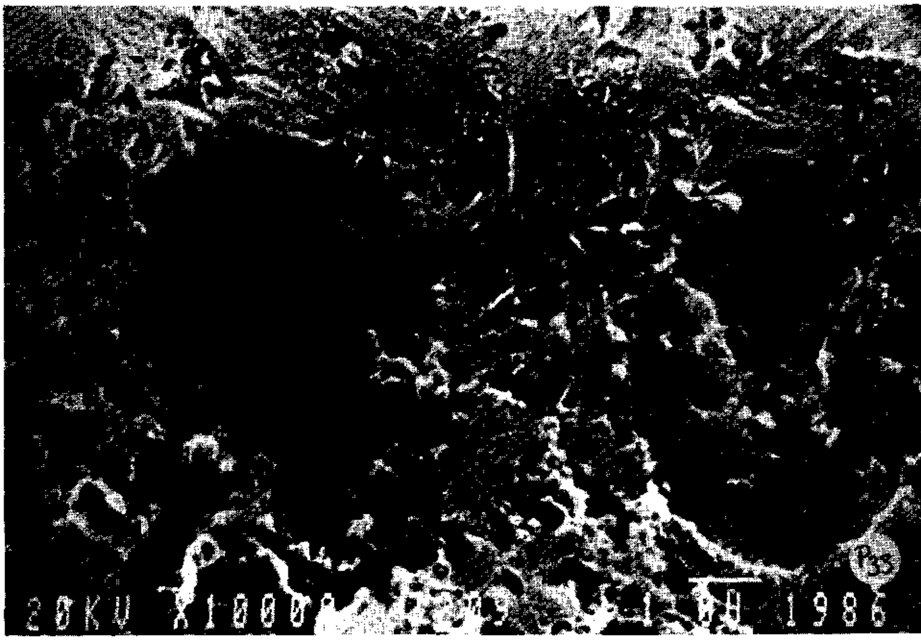

Fig. 10. Longitudinal section made at 10 mm distance from the base of the stem of 'Sonia' cut flowers held in a buffered sterile pectate lyase solution of 1 U ml^{-1} for 40 h. The xylem pit structure is damaged and many loose vessel fragments and mucoid materials are shown. Bar mark represents 1 μm.

1986; Zagory and Reid, 1986). The many ways in which microorganisms, through their pectic enzymes, may interfere with plant xylem cells, are very complicated, and their mechanisms are far from clear (Rombouts and Pilnik, 1980; Billing, 1987).

Further experiments are needed to reveal whether other purified microbial pectic enzymes cause similar symptoms during the vase life of *Rosa* and other cut flowers.

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Chapter 8

The influence of A12 (SO4) 3 added to the vase fluid on the infiltration ability of *Bacillus subtilis* cells into xylem vessels of cut *Rosa* cv. 'Sonia': a scanning electron microscope study.

The heart of a fully blooming Rosa (Photograph: Judith Tromp)

Chapter 8

The influence of $Al_2(SO_4)_3$ added to the vase fluid on the infiltration ability of *Bacillus subtilis* cells into xylem vessels of cut *Rosa* cv. 'Sonia': a scanning electron microscope study.

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ABSTRACT

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Aluminium sulphate, $Al_2(SO_4)_3.18H_2O$, 500 $\mu g\ ml^{-1}$ added to deionized water (1.5 mM Al^{3+}) results in a slightly opalescent solution with a pH of approx. 4, due to hydrolysis. The insoluble $Al(OH)_3$ forms a glassy colloidal solution at pH 4. On standing at room temperature, the $Al(OH)_3$ flocculates to small white flocky deposit. The quantity of precipitated $Al(OH)_3$ is increased by increasing of the pH from pH 4 - 5.5. Al^{3+} ions react easily with some inorganic and organic anionic substances e.g. bacterial cell wall peptides, substances of damaged stem cells and stem xylem cell wall compounds of cut *Rosa* flowers immersed into vase fluids containing Al_-^{3+} and *Bacillus subtilis* cells.

The Al-compounds formed, aggregate and flocculate. The *Bacillus*/ aluminium compounds contribute to an increase of flocky deposits on the cut surface and open xylem vessels of the roses, preventing the infiltration of the *Bacillus* cells which is demonstrated by cryo-SEM observations and X-ray microanalyses of pieces of the stem tissue.

The aluminium sulphate effect in *Rosa* vase fluid consists of three factors: (1) an almost biostatic (or very weak biocidal) activity towards *Bacillus subtilis* cells; (2) preventing of *Bacillus subtilis* cells to infiltrate (and plug) into open xylem vessels; (3) slightly decreasing of the daily water uptake as well as the *Rosa* bud development, extending the longevity (ornamental value) of the flowers.

Cryo-SEM observations showed that the aluminium sulphate and the *Bacillus subtilis* cells added to the vase fluids did not affect the xylem ultra structural morphology of the roses within 2 days of vase life.

Short title: Cryo-SEM observations of *Rosa* flower xylem vessels

Keywords: aluminium sulphate, *Bacillus subtilis*; infiltration ability; Cryo-SEM; *Rosa* cv. 'Sonia'; xylem vessels.

INTRODUCTION

In supplying cut flowers with sugar as source for their energy, it is necessary to add simultaneously a germicide to the vase solutions in order to inhibit microbial growth (Aarts, 1957 and Paulin, 1971). For this purpose 8-hydroxyquinoline-citrate (HQC) as well as aluminium sulphate have found widespread use in post-harvest handling of cut flowers (Halevy & Mayak, 1981). Sometimes these chemicals are used together with calcium nitrate (Goszczynska *et al.*, 1989).

Intended as vase-solution disinfectants, both $Al_2(SO_4)_3$ and HQC are also reported to have various important physiological effects on the cut flowers. They lower the pH of the vase solution and suppress the growth of non-acidophilic bacteria (Aarts, 1957). In addition the Al^{3+} ions affect the stomatal status by diminishing stomate opening and subsequently decreasing water evaporation (Schnabl & Ziegler, 1975; Schnabl, 1976). De Stigter (1981) however, observed that the ornamental value and longevity of Al^{3+}-treated roses were only slightly better compared to the non-treated controls, although their water loss was reduced by stomatal closure. Similar observations were reported by Halevy & Mayak (1981). These authors found that the effect of Al^{3+} on cut roses was attributed to the lowering of the rose petal pH and stabilizing the anthocyanins, while also acidifying the vase water, and thus reducing bacterial growth and improving water uptake. A certain reduction of the number of viable bacteria infiltrated into the lower part of the xylem vessels of roses, treated with aluminium sulphate, was shown by Van Doorn et al. (1990) when the number of cfu (colony forming units) were assessed at the 7th day of the vase life.

Therefore, our study was aimed at the effects of Al^{3+} and *Bacillus subtilis* cells added to vase water of roses with respect to: (1) the xylem morphology of the roses; (2) the infiltration ability of Al^{3+} with the sap stream; (3) the infiltration of bacterial cells into the xylem system; (4) the multiplication ability of bacterial cells added to the vase fluid and (5) the water uptake of the flowers.

For this purpose a cryo preparation method of stem specimens for scanning electron microscope observations has been used, as well as X-ray microanalysis of stem pieces in order to investigate the infiltration of bacterial cells and Al^{3+} into xylem vessels.

MATERIALS AND METHODS

- Plant material

Rosa hybrida cv. 'Sonia' were supplied by a local greenhouse grower. The roses were harvested at flower stage 1-2 (Berkholst, 1980). The experiments were started the same day, within 4 h after harvest.

- Micro-organisms

Bacillus subtilis, was inoculated on Nutrient agar (Oxoid), incubated for 48 h at 32°C, washed off into sterile deionized water, mixed and diluted in sterile deionized water to a cell concentration of ca. $5x10^8$ per ml.

Cell concentrations were assessed microscopically, and by plate counting on Plate Count agar (Oxoid). The suspensions were stored at -20°C.

- Vase life evaluation

Flowers were cut with a stem length of 45 cm and a stem edge of 45°, using an anatomic razor blade and aseptical handling. The stems were wiped off with paper tissue soaked in ethanol 98% (v/v) to minimize contamination, and placed singly into sterile cylindrical vases containing the respective vase fluids to be tested: (1) sterile deionized water; (2) sterile deionized water to which was added $5x10^6$ or $5x10^7$ *Bacillus subtilis* cells per ml; (3) sterile deionized water to which was added 500 μg $Al_2(SO_4)_3.18H_2O$ (Merck) per ml; (4) sterile deionized water to which was added 500 μg $Al_2(SO_4)_3.18H_2O$ per ml as well as *Bacillus subtilis* cells $5x10^6$ or $5x10^7$ per ml.

The vase fluid was adjusted to pH 5 and compared with the effects at pH 4 (normal pH after addition of Aluminiumsulphate). Each modification was measured in triplicate.

The vases were incubated at 20°C, 60% r.h. (relative humidity) and 10.25 W/m^2 photoradiation at flower height during a 12 h light/dark photoperiod each 24 h. Every 24 h of the vase life, observations were made of: the water uptake (ml), the flowering stage and the ornamental value (Berkholst, 1980) up to a vase life of 4 days. After 0, 2 & 4 d of vase life the microbiological state of well mixed samples of the vase fluids was assessed by counting of cfu (colony forming units) on Plate Count agar (Oxoid), as well as by direct microscopic counts of Gram-stained smears of the vase fluid samples by a modified Breed smear technique.

Scanning electron microscope preparations were made after 48 h of vase life (see below).

- Cryo-SEM observations and X-ray microanalysis

Pieces of rose stem of 1 cm were taken at the first cm, and at 5, 10 and 20 cm from the cutting point respectively. The pieces were mounted on brass stubs and subsequently frozen in nitrogen slush. Specimens were transferred to the preparation chamber of a Hexland - Oxford CT 1000/CP2000 system and freeze-fractured. The specimens were etched for ca. 30 min at 190 K (temperature of anticontaminator was 90 K). Finally the specimens were sputtered with gold and examined in a Philips SEM 535 at ca. 110 K at 15 kV accelerating voltage (Boekestein & Henstra, 1990). For X-ray microanalysis the EDAX 9900 system was used. Analysis conditions: 100 live sec. counting time, 15 kV accelerating voltage, spot 500 nm. For X-ray microanalysis specimens were coated with carbon. Each stem piece was measured five times, at different points (Boekestein & Henstra, 1990).

RESULTS AND DISCUSSIONS

- Precipitation of Al-compounds

$Al_2(SO_4)_3$ added to deionized water (1.5 mM Al^{3+}) results in a slightly opalescent solution with a pH of about 4 due to hydrolysis and formation of colloidal $Al(OH)_3$. In a sterile buffered solution of pH 5-5.5 at 20°C, insoluble colloidal $Al(OH)_3$ is flocculated to a white loose deposit. The quantity of precipitated $Al(OH)_3$ is raised with increasing pH.

During and shortly after the addition of the non-buffered suspension of *Bacillus subtilis* cells to the aluminium sulphate solution, the pH increased from 4 to 4.5 and a precipitate of loose flocky material was formed. After buffering of the aluminium sulphate/*Bacillus* suspension at pH 5-5.5, the quantity of the flocky material increased further, probably by the formation of more insoluble $Al(OH)_3$.

Al^{3+} and SO_4^{2-} ions can easily penetrate the capillary holes of the intervessel xylem pit walls, and thus infiltrate together with the sapstream upwards into the vessels as shown by X-ray micro-analysis. Obviously the flocky Al-containing material, is not able to pass these intervessel capillary holes. These Al-compounds are therefore only found on the cut surface and in the open xylem vessels.

Concerning the nature of the flocky material, it should be taken into account that Al^{3+} ions may form insoluble compounds with negative inorganic or organic ions, e.g. (1) phosphates; leaking out of disrupted plant cells; (2) peptides (peptidoglycan) of the *Bacillus* cell wall (Rogers, 1970; Dawes & Sutherland, 1976); (3) organic anionic vascular material. The compounds formed may not be able to pass the intervessel capillary pit holes, or to enter with the sap stream into the vascular system of the cut roses.

- The influence of $Al_2(SO_4)_3$ on the water uptake and vase life

Aseptically handled roses placed in Al^{3+} containing vase fluid showed a slightly slower bud development according to the Berkholst criteria (Berkholst, 1980) and slightly lower daily water uptake compared with non-treated control roses (see Table 1).

Our observations confirm the results of previous observations of De Stigter (1981), who studied the effects of aluminium sulphate on the water balance of cut 'Sonia' roses; he reported that the water balance of cut roses was improved by the addition of Al-sulphate + glucose to the vase fluid. The Al-treated roses gave only slightly better outgrowth and flowering than the (tap) water controls. De Stigter (1981) concluded that: (1) glucose had a physiological effect; (2) glucose + antimicrobial agents (8-HQS or aluminium sulphate) interacted with some physiological processes of the cut roses, such that the effects were different; (3) the results differed from the Al^{3+} effects reported by Schnabl & Ziegler (1975) and Schnabl (1976); (4) the mechanism(s) related to the interaction of antimicrobials and the water balance of the roses were unknown. The antimicrobial activity of both agents, $Al_2(SO_4)_3$ and 8-HQS, however, was not tested and pH of the vase fluids was not measured.

- Anti-microbial activity of $Al_2(SO_4)_3$

Our observations indicate that the Al-compound added to vase water was of less importance as a germicide than was the accompanying lowering of the pH of the vase fluid. Only acidophilic and acidotolerant micro-organisms are able to survive or multiply in the Al-containing vase fluid at or below pH 4 (Kushner, 1971).

In our observations a weak biocidal activity was shown towards *B. subtilis* cells at $\leq$ pH 4, but no effect or only a slight biostatic activity was found at pH $\geq$ 5 (Table 1).

It was furthermore found that in the vases treated with the Al-compound, the *Bacillus* cells did not stay suspended homogeneously. In the supernatant ca. 10^4 cfu ml^{-1} of *Bacillus* cells were enumerated after 1-2 d of vase life, while in the deposit ca. 10^7 cfu ml^{-1} were recorded. In the well mixed sample, however, ca. 10^6 cfu ml^{-1} were counted (Table 1).

These observations might suggest that the multiplication ability of the viable *Bacillus* cells was decreased by interaction of the cells (cell wall) with $Al(OH)_3$ resulting in co-precipitation of these cells on the cut surface of the roses, and gravitational deposition of cell aggregates at the bottom of the vase.

- Cryo-SEM observations of cut *Rosa* stems after 48 h of vase life

Results summarized in Table 2 merit further comments:

.. Sterilized vase water

Cryo-SEM micrograph objects may suffer less from preparation artefacts and showed more relevant structural details compared with those retained in SEM objects fixed with glutaraldehyde (Fraser & Gilmour, 1986 and Put & Clerkx, 1988). Cryo-SEM images of the vascular xylem cell wall showed good detailed structures (Plates 1-4).

.. $Al_2(SO_4)_3$ in sterilized vase water

A thin white granular layer was formed on the cut surface partly covering the smaller (≤ 1 μm) xylem vessels, as shown in Plate 5.

In the adjoining open vessels, at increasing distance from the cutting point, decreasing amounts of Al-flocks were observed.

.. *B. subtilis* cells added to sterilized vase water

Cryo-SEM images confirmed previously reported observations (Clerkx *et al.*, 1989), that the cut surface of *Rosa* stems can act as a coarse-threaded filter. Only a certain fraction of the *Bacillus* cells were infiltrated by the sap stream higher up into the open xylem vessels (Plates 7 & 8). The remaining fraction was attached to the cut surface (Plate 6) or adhered on the lower part of the vessels, subsequently partly blocking the water uptake (Plate 9).

.. *B. subtilis* and $Al_2(SO_4)_3$ in sterilized vase water

Cryo-SEM images of the cut surface and of stem specimens 5 cm up into the *Rosa* stem (Plates 10-14) show that the *Bacillus subtilis* cells are surrounded by an Al-complex, thickening the cells, causing them to adhere to the cut surface. The vascular xylem system was partially blocked and thus affecting the water uptake compared with *B. subtilis* in sterilized water. SEM observations also show that a much smaller fraction of the Al-complexed *B. subtilis* cells was able to enter with the sapstream higher up into the open vessels, compared with non-Al-treated *Bacillus* cells.

It is not yet known, however, whether other microbial genera and species e.g. Gram-negative bacteria, yeasts, fungal mycelium and spores might react similarly as a consequence of the addition of Al-sulphate to the vase water of roses.

- X-ray microanalysis

No aluminium was detected on the cut cross section of roses placed in deionized water (Fig. 1).

Aluminium was abundantly present on the cut cross section (Fig. 2) and, to a much lesser extent, in the first cm of the xylem vessels of the stem of a rose immersed in an aluminium sulphate containing vase fluid of pH 5 (Fig. 3). Yet, only in one out of five points measured at 5 cm from the cutting point, a small aluminium peak was observed, while at 20 cm from the cutting point, no aluminium was detectable.

These observations reveal that most of the $Al(OH)_3$ flocks formed at pH 5 and precipitated in the vase fluid, were not taken up by the sap stream of the rose, but remained as a filtered layer attached to the cut surface.

Rose stems immersed in an aluminium sulphate containing vase fluid of pH 4, on the contrary, showed in the xylem vessels, even higher aluminium peaks as far as 10 cm from the cutting point, compared with these detected on 5 different points of the cut cross section (Fig. 4). At pH 4, the $Al(OH)_3$ is a glassy colloid, slightly flocculated by the formation of very small particles which easily entered the xylem system of roses. They may also be able to pass the capillary holes in the pit membranes. It should be noted, however, that we have localized and analyzed only limited Al-concentrations; lower concentrations especially of the Al_3^+ may be transported higher up into the flower stem.

Other elements like P and K, are frequently found in biological tissues, but their concentrations may vary. S peaks can be attributed to the $Al_2(SO_4)_3$ added to the vase fluid.

- The role of flower 'preservatives'

Chemical compounds added to vase fluids as "flower preservatives" are - mainly chosen and evaluated for a specific property, although they may show further effects as a consequence of interaction. E.g. (1) Co^{2+} improves the water balance of roses but is also fungicidal and is an ethylene (C_2H_4) binder (Reddy, 1988), (2) Ag^+ is an ethylene binder but has also germicidal properties (Veen, 1987), (3) Al^{3+} at pH ≤ 4 is biocidal and decreases the infiltration of microbial cells into the xylem system as shown in our investigations, but also slightly improves the water balance of cut 'Sonia' roses, as also observed by De Stigter (1980 & 1981), (4) however, at pH 5 - 5.5 colloidal $Al(OH)_3$ may mainly act as a physical filter layer on the stem cross section, blocking bacterial infiltration and also slightly decreasing the water uptake. A typical phenomenon unfolded by SEM, X-ray and microbiological examinations is presented in this paper. These further effects of antimicrobial agents added to the vase water of roses, need further elucidation, and should be taken into account in the developnment of the methodology used for their evaluation.

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Table I The influence on the bacterial activity and the ornamental value of the roses of $Al_2(SO_4)_3.18H_2O$ (500 $\mu g\ ml^{-1}$) and *B. subtilis* cells ($5x10^6$-$5x10^7$ ml^{-1}) added to sterilized deionized vase water of *Rosa* cv. Sonia

Vase fluid contents	pH	Vase life (days)	Bacterial count ml^{-1} vase water		Water uptake ml/d	Stage of bud development (*)
			cfu	Microscopic		
Control-sterile deionized vase water	5.5	0	< 50	-	0	1-2
		2	< 50	-	50	5
		4	< 50	-	40	7^-
"	5.5	0	< 50	-	0	1-2
		2	< 50	-	45	5
		4	< 50	-	35	6-7
2) + $Al_2(SO_4)_3$	5.5	0	< 50	-	0	1-2
		2	< 50	-	35	3-4
		4	< 50	-	30	6-7
"	4	0	< 50	-	0	1-2
		2	< 50	-	35	4-5
		4	< 50	-	30	6-7
3) + *B. subtilis* $5x10^6\ ml^{-1}$	5.5	0	$5x10^6$	ca.10^6	0	1-2
		2	10^7	ca.10^7	45	3
		4	$5x\geq10^7$	$5x10^7$-10^8	20	4-5
+ *B. subtilis* $5x10^7\ ml^{-1}$	4	0	$5x10^7$	ca.10^7	0	1-2
		2	$\geq10^7$	ca.10^7	45	3
		4	$\geq5x10^7$	ca. $5x10^7$	20	6^-
4) + $Al_2(SO_4)_3$ + *B. subtilis* $5x10^6\ ml^{-1}$	5	0	$5x10^6$	ca.10^6	0	1-2
		2	$5x10^6$	ca.10^6	35	5
		4	ca.10^7	ca.10^7	25	6-7
+ $Al_2(SO_4)_3$ + *B. subtilis* $5x10^7\ ml^{-1}$	4	0	$5X10^7$	ca.10^7	0	1-2
		2	ca.10^6	ca.10^6	30	4
		4	ca.10^5	ca.10^6	25	6-7

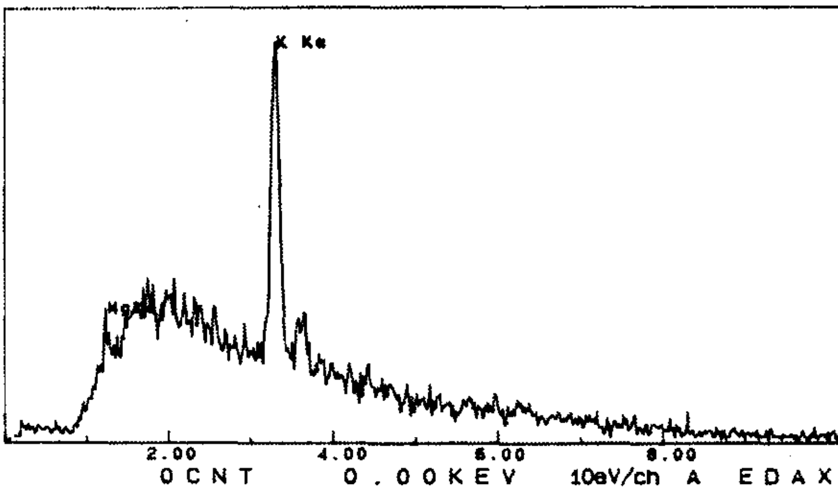
cfu colony forming units

* Berkholst, 1980

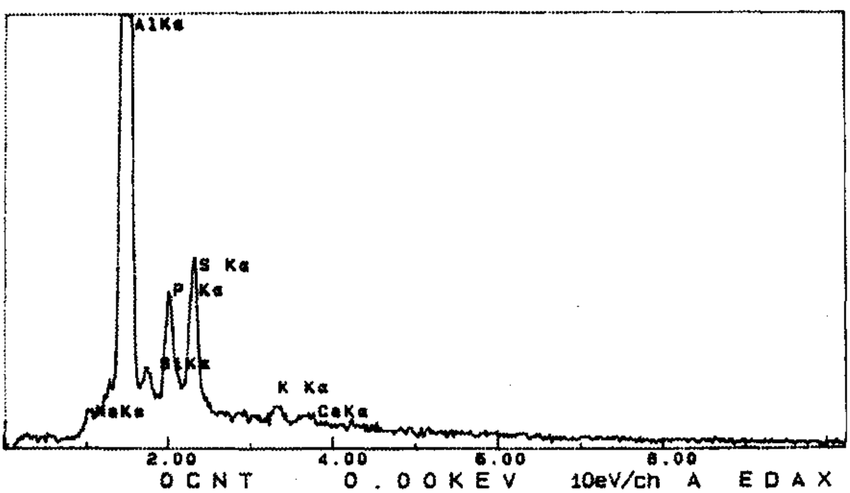
\- = no bacterial cells observed

Table II Cryo-SEM observations of xylem vessels of stems of Rose flowers after 48 h of vase life in vase fluids containing: sterilized deionized water (1) to which was added: (2) $Al_2(SO_4)_3$; (3) *B. subtilis* cells; (4) $Al_2(SO_4)_3$ and *B. subitilis* cells

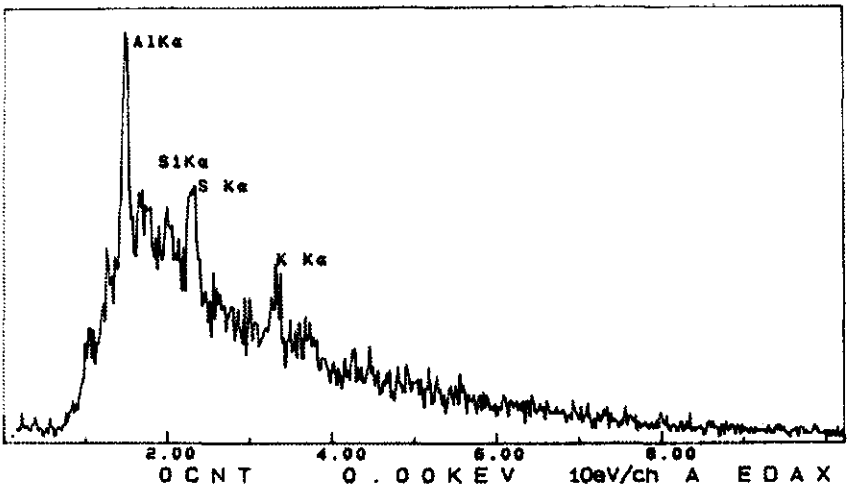
Vase fluid Code	pH	Sampling point: Cutting point	Plate no.	50 mm from the cutting point	Plate no.
(1)	5.5	normal; diam. of vessels from < 0.5-50 μm	1,2,3	normal defined images of longitudinal section of the xylem	4
(2)	4	Al-compound visible, both macroscopicaly and on cryo-SEM images, as a thin white granular layer on the cut surface and as thin white flocks in the vessels.	5	A few white flocks in the vessel, normal vessel ultrastructure.	-
	5.5	Thick white flocky layer on the cut surface.	-	Thin white flocks Normal vessel ultra structure.	-
(3)	5.5	On the cut surface a few *Bacillus* cells, in the adjacent vesses non, a few or plugged with *Bacillus* cells.	6	Some vessels plugged with *Bacillus* cells. Normal vessel ultra-structure.	7,8
	4	Images as at pH 5.5	9	Images as at pH 5.5	-
(4)	5	On cut surfaces *Bacillus* cells obscured by Al-flocks; in adjacent vessels a decreasing number of *Bacillus* and Al-flocks.	10,11	A few white flocks of Al-compound. No bacterial cells observed. Normal vessel ultra-structure.	-
	4	At ca. 0.1 mm from the cutting point many *Bacillus* cells amd Al-flocks are shown. At 1-1.5 mm only a few *Bacillus* and thin Al-flocks observed	12,13, 14	A few white flocks of Al-compound and sporadic some *Bacillus* cells. Normal vessel ultra-structure.	-



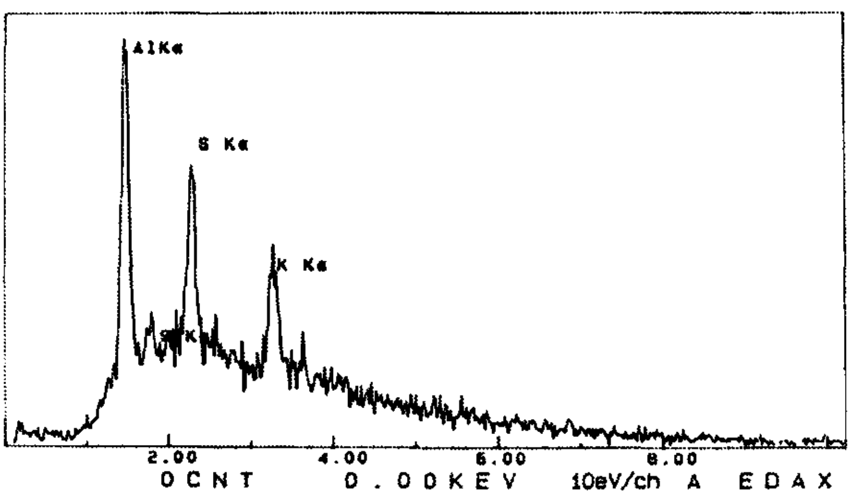
<u>Fig. 1.</u> Energy-dispersive X-ray spectrum of a xylem vessel in the first cm of the stem of a *Rosa* flower held in sterilized water of pH 5.5.



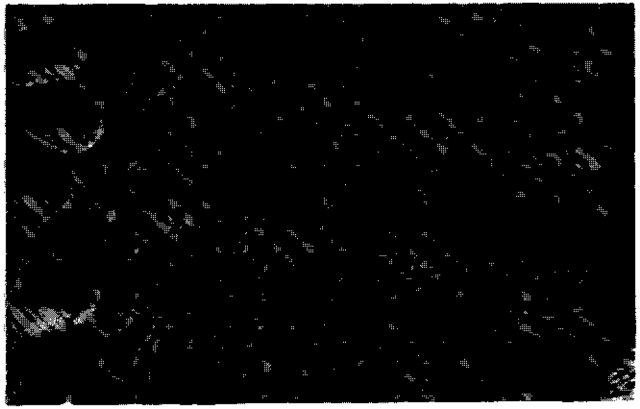
<u>Fig. 2.</u> Energy-dispersive X-ray spectrum of a xylem vessel close to the cut surface of a *Rosa* stem held in flocculated $Al_2(SO_4)_3$ solution of pH 5.5.



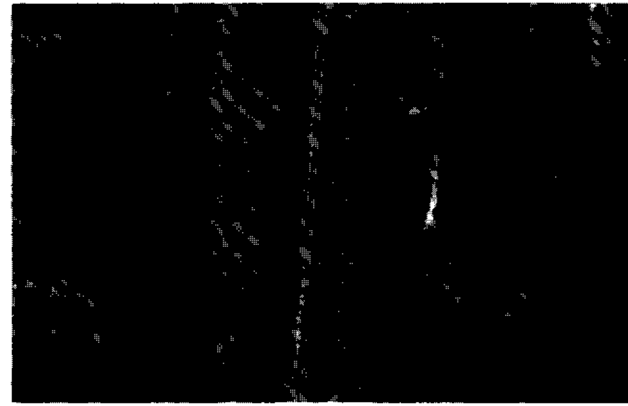
<u>Fig. 3.</u> Energy-dispersive X-ray spectrum of a xylem vessel in the first cm of the stem of a *Rosa* flower held in flocculated $Al_2(SO_4)_3$ solution of pH 5.5.



<u>Fig. 4.</u> Energy-dispersive X-ray spectrum of a xylem vessel at approx. 10 cm from the cutting point of a *Rosa* flower held in a clear $Al_2(SO_4)_3$ solution of pH 4.

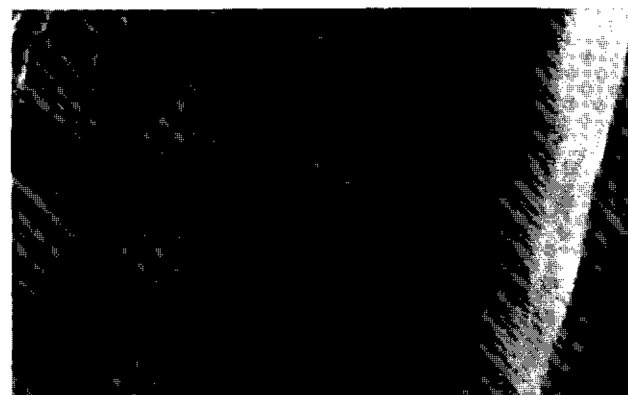


<u>Plate 1.</u> Transverse secton of the cut surface of a *Rosa* flower held in sterilized water of pH 5.5. Bar represents 0.1 mm.



<u>Plate 2.</u> Longitudinal section of xylem vessels near the cutting point of a *Rosa* flower held in sterilized water of pH 5.5. Bar represents 0.1 mm.

<u>Plate 3.</u> Longitudinal section of a xylem vessel near the cutting point of a *Rosa* flower held in sterilized water of pH 5.5 showing the vessel wall and pit structure. Bar represents 10 μm.



<u>Plate 4.</u> Longitudinal section of a xylem vessel at approx. 50 mm from the cutting point of a *Rosa* flower held in sterilized water of pH 5.5 showing the clear pit structure in the xylem vessel wall. Bar represents 10 μm.

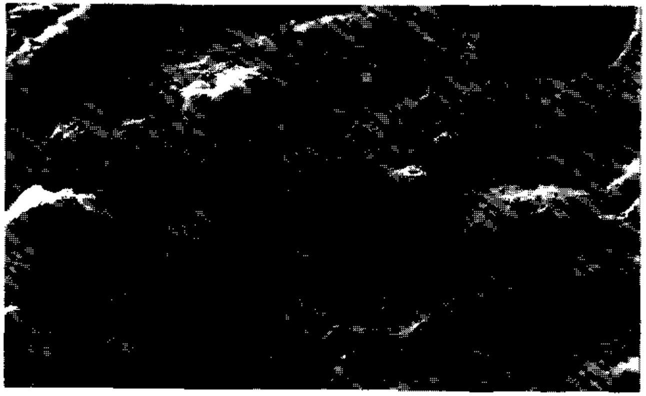


Plate 5. Transverse section of the cut surface of a *Rosa* flower held in an $Al_2(SO_4)_3$ solution of pH 4 showing a thin granular layer on the cut surface. Bar represents 10 μm.

Plate 6. Transverse section of the cut surface of a *Rosa* flower held in a *Bacillus subtilis* cell suspension of pH 5.5 showing *Bacillus* cells adhered onto the xylem wall cross section (compare with Plate 10). Bar represents 10 μm.

Plate 7. Longitudinal section of a xylem vessel approx. 50 mm from the cutting point of a *Rosa* flower held in a *Bacillus subtilis* cell suspension of pH 5.5 showing some infiltrated *Bacillus* cells and a normal vessel structure. Bar represents 10 μm.

Plate 8. Longitudinal section of a xylem vessel approx. 50 mm from the cutting point of a *Rosa* flower held in a *Bacillus subtilis* cell suspension of pH 5.5 showing a mass of infiltrated *Bacillus* cells, blocking that vessel. Bar represents 10 μm.

Plate 9. Longitudinal section of xylem vessels near the cutting point of a *Rosa* flower held in a *Bacillus subtilis* suspension of pH 4 showing or non, or a mass of infiltrated *Bacillus* cells, blocking that vessel. Bar represents 10 μm.

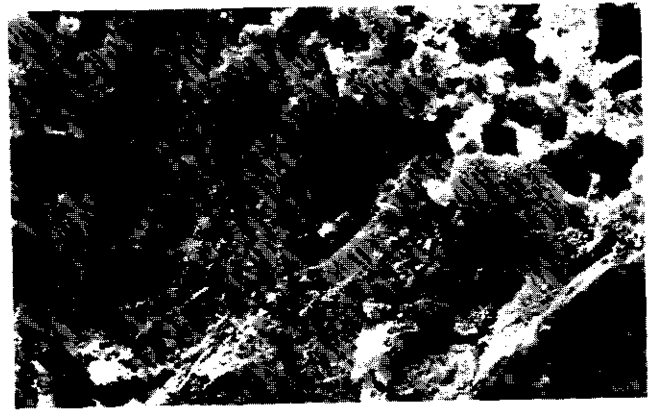


Plate 10. Transverse section of the cut surface of a *Rosa* flower held in an $Al_2(SO_4)_3$ solution of pH 5, to which *Bacillus subtilis* cells were added, showing *Bacillus* cells surrounded by a thin granular layer which also covered the cut surface. Bar represents 10 μm.

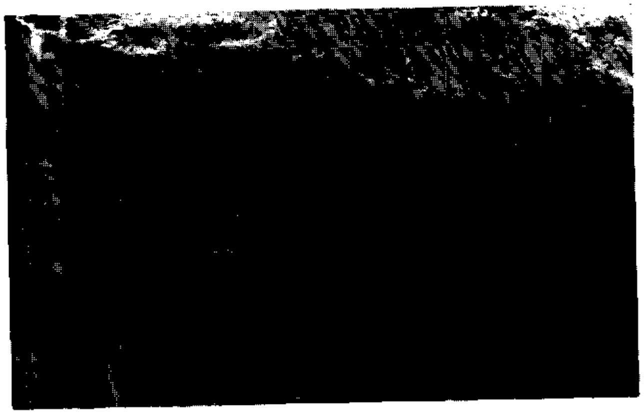


Plate 11. Longitudinal section of xylem vessels near to the cut surface of a *Rosa* flower held in an $Al_2(SO_4)_3$ solution of pH 5, to which *Bacillus subtilis* cells were added. Note the white granular layer on the cut surface and in some of the adjacent xylem vessels. Bar represents 0.1 mm.

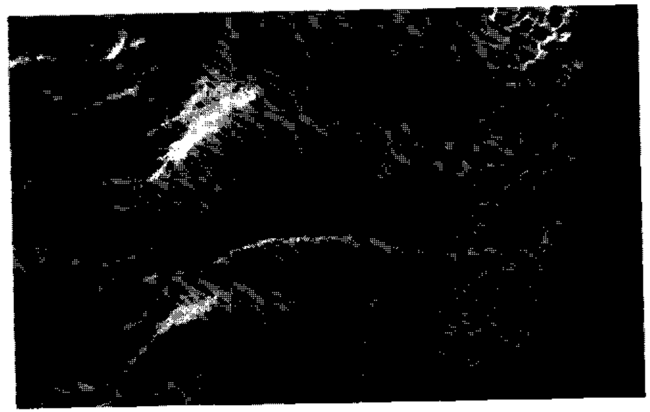


Plate 12. Transverse section of the cut surface of a *Rosa* flower held in an $Al_2(SO_4)_3$ solution of pH 4 to which *Bacillus subtilis* cells were added, showing a white granular layer covering a major part of the cut surface. Bar represents 1 mm.

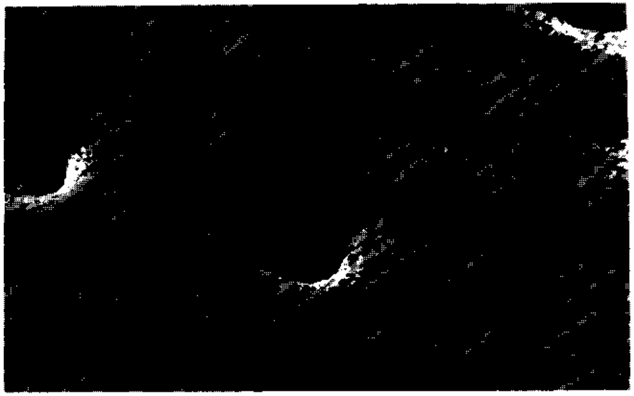


Plate 13. Transverse section of the cut surface of a *Rosa* flower held in an $Al_2(SO_4)_3$ solution of pH 4 to which *Bacillus subtilis* cells were added, showing the cut surface and the entrance of the widest xylem vessels covered with a thin granular layer, which also covered or masked the *Bacillus* cells. Bar represents 0.1 mm.

Plate 14. Longitidunal section of xylem vessels approx. 0.1 mm from the cutting point of a *Rosa* flower held in an $Al_2(SO_4)_3$ soluton of pH 4 to which *Bacillus subtilis* cells were added, showing in the upper vessels (see arrow), many infiltrated *Bacillus* cells and amorphous flocks of Al-compounds, and in the lower vessels no *Bacillus* cells or flocks. Bar represents 10 μm.

Chapter 9

Discussion

Rosa *(Judith Tromp)*

Chapter 9

DISCUSSION

In paleontology,it has been exerted that the life of wild roses may be at least seventy million years old. Breeding of roses, originates probably in China, and is already practiced for about 5000 (Shepherd, 1954). In Chinese cultural history, the rose is considered to be of great value. Most of the European *Rosa* cultivars descend from a number of about eight Asian cultivars.

The rose, as we know this flower today, is the 'Greta Garbo' among the ornamental flower cultivars. Its beauty is fascinating, its behaviour is capricious. For the vase life of roses is marked by an inconstancy which is difficult to become acquainted with, and which is still more difficult to get control on.

Postharvest microbiology of ornamental flowers and flower vase fluids, particularly of roses, appears to be a field which is hardly studied, in spite of the fact that the vase life of flowers can be dramatically influenced by micro-organisms (Aarts, 1957; Zieslin, 1989).

The purpose of the research presented in this thesis is: to reveal factors of the vase life of roses, in which micro-organisms play a dominant role.

The initial number of micro-organisms on cut flower stems

The activity of micro-organisms can be the main cause of rapid senescence of cut flowers, as already postulated by Aarts (1957). However, little is known concerning the initial microflora on stems of freshly harvested cut flowers. A series of investigations into the microbial load on stems of freshly picked *Chrysanthemum*, *Gerbera*, and *Rosa* cultivars showed that the numbers of viable micro-organisms enumerated as cfu (colony forming units) on the stems of *Rosa* flowers are very low, and much lower than those on *Chrysanthemum* and *Gerbera* stems, probably reflecting their distance from the soil when harvested, but possibly also due to the glossyness of the *Rosa* stem epidermis. Moreover, the numerous tiny side leaves of the *Chrysanthemum* stem and the hairy epidermis of the *Gerbera* probably facilitate the adherence of epiphytic and soil micro-organisms onto the stems (Chapter 2).

Identification of cut flower stem microflora

A wide variety of microbial species, bacteria as well as filamentous fungi and yeasts are isolated from stems of freshly harvested *Rosa*, *Gerbera* and *Chrysanthemum* flowers. These microbial species are identified as normal soil saprophytes or as epiphytic constituents of the phyllosphere, and some fungi are known as phyhtopathogenic: *Botrytis cinerea* and *Fusarium solani*. Yeasts as well as filamentous fungi are usually isolated in lower numbers than bacteria (Chapter 2).

In contrast the vase water of cut flowers has a more diversified flora. Microbial species not originating from soil or the flower phyllosphere must therefore have been introduced into the ecosystem of the flower stem and the flower vase fluid after harvest, e.g. by microbial contamination of the harvest materials, the water and the containers used.

Multiplication of cut flower stem microflora

The cut flower stem, the vase fluid and the flower stem immersed in the vase or container, represent an ecosystem in which certain microbial species show an optimal ability for multiplication. Although most of the microbial genera present on the cut stems were also present in the vase water, competition and antagonistic activities between the different microbial genera and species, as well as ecological conditions in the vase fluid, poor in nutrients, leads to the observed initial dominance of *Pseudomonas* species, not requiring organic growth factors (Chapter 2). In Chapter 2 it is also shown that the initial predominance of *Pseudomonas* species is followed by the predominance of more growth factors requiring *Enterobacter* and *Bacillus* species and from then on also fungal growth was stimulated as well as growth of other bacteria e.g. more demanding *Pseudomonas* species.

The evolution of the microflora of vase water during the vase life of flowers has so far been studied only fragmentarily (De Stigter & Broekhuysen, 1986; Woltering, 1987). However, to elucidate the influence of microbial factors on the vase life of cut flowers, it is indispensable to have knowledge of types, numbers and multiplication ability of the microflora - bacteria as well as fungi - during the whole course of the vase life.

The choice of the rose

The usually very low initial microbial contamination of hygienically picked greenhouse roses, makes this flower cultivar very appropriate for investigations into the influence of microbiological factors on its vase life. With careful handling of the flowers: (1) activities of the initial microbial flora can be minimized, (2) the addition of biocides to the vase fluid can be avoided and therewith their physiological activities, (3) suppletion of a nutritive sugar is not necessary for a normal bud development.

Added sugar influences the multiplication of micro-organisms and also the formation of certain metabolic products which can interfere - physiologically and microbiologically - with the factors to be studied (Chapter 1.6).

The methodology

The methods used in the consecutively executed investigations were standardized in order to make possible: (1) to check the reproducibility of the results obtained, (2) to apply statistical evaluation of the various measurements, visual observations and data, (3) to compare data and observations of the different test series, (4) to be able to attribute certain observations to specific phenomena of cut flower vase life, and events leading to a premature senescence.

Standardization of the plant material was realized by picking of the roses at the same stage of bud development (Berkholst, 1980). The cut flowers were stored for max. 48 h at 4°C before use. The flowers were cut to a stem length of 45 cm, and all except the 3 upper leaves were removed before the roses were placed singly into cylindrical vases containing 100 ml of the vase fluid to be tested.

All microbiological factors i.e. initial numbers of microbial cells, microbial metabolites (high mol. wt and low mol. wt compounds, pectic enzymes) were tested in triplicates and in series of at least three decimal dilutions. All the (vase) materials were sterilized. Pure cultures were used, and microbial products such as enzymes were thoroughly purified before use. Vase fluids were prepared from sterilized deaerated tap water.

Incubation of the vases was at controlled environmental conditions. Observations were made in 24 h intervals. Scanning electron microscope (SEM) studies, macro colour photographs, and conductivity measurements were made after 24 and 48 h of vase life.

A rose is a rose, is a rose, is a rose. In other words, roses although picked at the same time, in the same stage, at the same greenhouse are individual biological entities. Thus, it should be taken into account that from roses used after 24 h and 48 h of vase life for SEM preparations or conductivity measurements, only speculations can be made about how the course of their vase life might have been in case these flowers were held in their vases.

Vessel plugging

Bacterial plugging of xylem vessel elements has been claimed to be the major cause of rapid senescence of cut flowers (Aarts, 1957). This study confirms these claims with observations described in Chapters 3, 4 and 5.

Vessel plugging by micro-organisms causes water stress. However, water stress does not always need the consequence of microbial vessel plugging, although micro-organisms - viable as well as inactivated ones - do play an important role in the phenomena related to microbial vessel plugging and cut flower water stress.

In Chapter 5, it is shown that viable as well as heat inactivated water-washed bacterial cells prepared from pure cultures of different strains and species of *Bacillus*, *Enterobacter*, and *Pseudomonas*, added in decimal dilutions to the vase fluid, caused a marked decrease in the vase life of roses. The initial number of bacteria added affected the decrease of the vase life, but the individual bacterial strains behaved very similarly, notwithstanding the differences in their individual properties such as: cell shape, cell motility, slime production, capsular formation, levan production, enzyme activity and - opportunistic - pathogenicity.

Initial number of 10^8 viable or heat inactivated bacterial cells ml^{-1} added to the vase fluid of roses caused xylem element occlusions merely at the basal end of the stem, as shown by SEM observations, water conductivity data and the fuchsin red test (Chapters 4 and 5). At lower initial bacterial numbers added to the vase fluid, the differences in the effects on the vase life of the roses of viable and inactivated cells became significant, although the influence of inactivated bacterial cells on the vase life of the roses remained an important factor. The increasing differences of the effects of viable versus inactivated bacterial cells initially added in decimal dilutions to the vase fluids of the roses, can be related to the multiplication capacity of the viable bacterial species, and to specific metabolic compounds formed during growth, e.g. pectic enzymes, low mol. wt toxic compounds and high mol. wt vascular plugging compounds.

To reveal whether a reduced water conductivity is caused by viable and/or by inert microbial propagules, or by microbial metabolites, a microscopic counting method should be combined with plate count methods, to assess cfu (colony forming units) of bacteria and fungi in the vase fluid. In addition the beet root tissue cube test, to assess cell-membrane damage, such as caused by the activity of pectic enzymes, as well as quantitative chemical analysis of microbial metabolic compounds (EPS) should be applied (Chapters 6 and 7). Finally, by the acid fuchsin test, xylem plugging can be localized as shown in Chapters 5 and 6. With exception of the quantitative chemical analysis, these methods are easy to introduce in routine examinations.

The role of biotic elicitors

Microbial propagules, microbial metabolic compounds and flower vessel cell contents or fragments, may act as biotic elicitors. These elicitors may act alone or in combination (Halverson & Stacey, 1986; Chapter 1.5). These elicitors, taken up by the sap stream, may initiate a still unknown series of physiological reactions, which may disturb the water relations and the vase life of the roses (Hahn et al., 1989).

As a result of physical damage, cutting of the flower stem, the plant's own elicitors may be formed at the side of the cutting wound. These elicitors are: plant hormones, cell wall fragments and enzymes, which may reinforce the physiological reactions elicited by micro-organisms. Low numbers of bacterial propagules alone (10^4-10^6 ml^{-1}) added to the vase fluid, could not by themselves have caused the extent of water conductivity loss measured after 24 h and 48 h of vase life. These observations indicate that some still unknown physiological reactions of the water transport system, elicited by micro-organisms, may also play a role (Chapter 5).

In this thesis no direct experiments were caried out to show if any of these mechanisms, concerning biotic elicitors, were involved. However, it is tempting to ascribe some of the unexplained phenomena to such mechanisms. There is clearly a need for further research at this point (Hahn et al., 1989a).

SEM (scanning electron microscope) observations

The microbiological techniques and additional analytical methods used in Chapters 3-8, as well as the results obtained with SEM observations allow to conclude that: the cut surface of a flower, such as a rose, can act as a coarse threaded filter (Figure 1). Only a small fraction of microbial cells and high mol. wt compounds enters from the vase water into the vascular system with the transpiration stream. Most of the micro-organisms and high mol. wt EPS (exopolysaccharides) remains attached to the submerged cut surface, obstructing xylem vessels and blocking water uptake (Chapters 4 and 6).

This phenomenon was quantified by investigations done with *Pseudomonas putida* cell strain 48 (Chapter 3). Although the number of bacteria that infiltrated into open xylem vessels increased with time and increasing numbers of *Pseudomonas* cells added to the vase fluid, and decreased with increasing distance between cutting point and sampling point, the total load of infiltrated *Pseudomonas* cells was much lower than calculated by the volume of vase fluid taken up, as shown in Figures 4, 5 and 6 of Chapter 2. This finding suggests that the bacteria themselves, when deposited at the cut surface of the flower stem, may act as a filter preventing other bacteria from entering the vessels.

Further obstruction of xylem vessels at the cut surface depends on: (1) the number of vessels and their diameter. *Gerbera* cv. 'Fleur' vessels are wider (20-100 μm) than the xylem vessels of 'Sonia' roses (2.5-35 μm), (2) the number and the size of microbial cells added to the vase fluid. *Pseudomonas* cells are 0.5 x 1.5 μm and *Kluyveromyces* cells measure approx. 3 x 10 μm, (3) the capacity of fungal conidia e.g. *Fusarium oxysporum* to agglutinate and, of certain bacterial species e.g. *Bacillus polymyxa*, *Bacillus subtilis* as well as *Pseudomonas putida*, to form cell clusters by adhesion of capsular material onto the cut surface, (4) the capacity of filamentous fungi to form mycelium, covering the cut surface as another filter-layer through which only small mycelium particles and microbial cells could enter the vascular system with the sap stream (Chapter 4, Clerkx, Boekestein & Put, 1990), (5) the ability of micro-organisms to form adhesive high mol. wt compounds e.g. EPS (exopolysaccharides) as already mentioned above, and the amounts thereof (Chapter 6).

Light microscope and scanning electron microscope observations are essential methods to reveal the role of micro-organisms in water stress and vessel plugging of cut flowers during their vase life, as shown in Chapters 3, 4, 6, 7 and 8. Besides, it can be concluded that cryoSEM images are more defined than SEM images obtained after chemical fixation by means of glutaraldehyde. CryoSEM images of the vascular xylem cell wall show a finer, more detailed structure than SEM images of chemically fixed specimen (Chapter 8, Figures 1-4; Chapter 4, Figures 11-13; Fraser & Gilmour, 1988).

The role of pectic enzymes

Purified pectolytic enzymes, such as: pectate lyase of *Pseudomonas fluorescens* and polygalacturonase of *Kluyveromyces fragilis*, added to a buffered vase fluid, disturb the vase life of 'Sonia' roses. However, pectic enzymes in vase fluids need a certain lapse of time to show their typical effects on vessel morphology, water relations and vase life of *Rosa* flowers. The symptoms are very typical in as much these enzymes cause neither wilting, nor bent neck, but desiccation of petals and leaves, and inhibition of flower bud development (Chapter 7).

SEM figures show that pectic enzymes cause degradation of the pectin-linked structure of the *Rosa* xylem vessels. This may result in breakage of the capillarity of the pit membranes, causing water stress and also increased vulnerability for xylem cavitation and embolism (Sperry & Tyree, 1988; Dixon & Peterson, 1989).

It is obvious that pectic enzymes added to the vase fluid of *Rosa* flowers in low concentrations may cause strong effects on the vase life of roses. Besides the effects on xylem vessel morphology and water relations of roses, it is possible that in breakdown of xylem cell wall components, higher oligogalacturonic acid compounds are formed. These so-called 'oligosaccharins' are known to have a physiological effect on the plant. Oligosaccharins may act as endogenous biotic elicitors or signal molecules eliciting host defence responses, or abnormal growth (Chapter 1.5; Albersheim & Valent, 1978; Halverson & Stacy, 1986; Rickauer, 1989). As with the phytohormones, it remains to be determined how certain 'oligosaccharins' control growth and development of plants (Darvill et al., 1989). It is not known whether oligosaccharins affect the vase life of roses, nor whether the effects of pectic enzymes observed in Chapter 7, were mediated by oligosaccharins. An intriguing field for further investigations (Albersheim & Darvill, 1985; Cervone et al., 1989).

Aluminium sulphate in vase water

Aluminium sulphate shows hydrolysis in vase water, lowering the pH and forming an aluminium hydroxyde colloid, which reacts easily with bacterial cells, materials of wounded stem cells, and xylem vessel walls of the roses submerged in the vase fluid.

Bacterium-aluminium complexes develop a flocky deposit which adheres onto the cut surface of roses, obstructing infiltration of bacterial cells into the open xylem vessels. This phenomenon is clearly demonstrated by cryoSEM observations, photomicrographs, and X-ray microanalysis of stem specimens (Figure 2).

The aluminium sulphate effect on *Rosa* flowers in vase fluid containing bacterial cells, is caused by three factors: (1) a biostatic activity towards bacterial cells, (2) obstructing of bacteria to infiltrate into, and plug open xylem vessels, (3) maintaining of the daily water uptake and therewith the *Rosa* bud development, extending the longevity of the flowers (Chapter 8).

Biocides in flower vasewater

It is not surprising that the negative effects which micro-organisms in vase fluids may have on the vase life of ornamental flowers, have led to the addition of biocides to flower vase fluids. An application which is practiced lucratively. However, little is known concerning the initial microbial load of vase fluids, and of the microbial species to be inactivated. Also little is known concerning the sensitivity of the diverse microbial species, which can multiply in vase fluids, towards biocides normally added, as well as effects on the physiology of the cut flower, exerted by the biocides (Aarts, 1957).

In this context, the developing of a model system to test the biocidal and biostatic capacity of certain compounds is necessary. Typical differences in sensitivity towards biocidal compounds of bacteria, fungi, and the cut flower itself, should be taken into account in developing a widely applicable test model. Methods as described and normalized for biocides to be used in food hygiene practice, can be a basic example (Council of Europe, Strasbourg, 1987).

The use of biocides is only one element in a more general approach to maintain the quality of cut flowers. As in food technology this more general approach could be laid down in codes of hygienic practice. Good hygienic practices, from the greenhouse handling to the beginning of the vase life at the buyer's home, should provide the basis for high quality flowers with an extended vase life. Stat rosa pristina nomine (Eco, 1983).

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SUMMARY

The papers compiled in this thesis comprise a series of successively executed investigations into the role of micro-organisms in xylem plugging, and disturbance of the water relations and the vase life of cut flowers. For this purpose *Rosa hybrida* cultivar 'Sonia' (the hybrid tea-rose *Rosa* cultivar 'Sweet Promise') was selected.

Chapter 1 comprises an introduction into the economic importance of cut flowers and the capability of cut flowers for post harvest life. In addition, a brief review is given of literature on physiological and microbiological factors which can influence the vase life of cut flowers. Gaps in our knowledge of the mechanisms leading to vascular blockage of cut flowers are discussed. An outline is given of: (1) the selection of a greenhouse *Rosa* cultivar as plant material to study, (2) the experimental methods applied, (3) the results of preliminary investigations, and (4) the microbiological factors investigated.

Chapter 2 shows that stems of freshly cut flowers contain a wide variety and low numbers of microbial species. The initial microbial load on stems of *Rosa* flowers was found to be much lower than those on *Chrysanthemum* and *Gerbera* stems. Their distribution on the flower stem is not homogeneous.

The stem flora, predominantly *Enterobacter*, *Bacillus* and fungal species, lost its dominance in the vase fluid. The vase water showed an initial predominance of *Pseudomonas* species which do not require organic growth factors. In the course of the vase life *Enterobacter* and *Bacillus* species became dominant. These bacterial genera require organic nutrients and special growth factors for their multiplication.

Fungal growth was shown at a later stage, mainly in vase fluids of *Chrysanthemum* and *Gerbera* flowers, which have a much longer vase life and a higher initial number of fungi on their stems than *Rosa* flower stems.

Chapters 3, 4 and 5 demonstrate that the extent of infiltration of viable microbial cells into the xylem vessel system of *Rosa* and *Gerbera* cut flowers depends upon the number of microbial cells per ml initially added to the vase fluid, the shape and the size of the individual microbial cells and the width of the xylem vessels.

Scanning Electron Microscopic (SEM) observations as well as the assessment of the number of bacteria infiltrated into *Rosa* xylem vessels showed in addition that: (1) the number of bacteria which infiltrated into xylem vessels increased with time and, (2) this number increased with increasing numbers of bacteria initially added to the vase fluid, (3) this number decreased with increasing distance between cutting point and sampling point, (4) only a minor part of the bacterial and fungal cells suspended in the vase fluid was able to infiltrate into the xylem vessels of the flowers, a major part of the microbial cells remained attached to the cut surface, (5) even low numbers of infiltrated microbial cells caused a significantly decreased conductivity of stem segments, (6) a similar water conductivity decreasing phenomenon of stem segments was observed when low numbers of heat-inactivated microbial cells or low concentrations of microbial EPS (exopolysaccharides) were added to the vase fluid, as shown in Chapters 5 and 6.

Chapter 7 does show that purified microbial pectic enzymes added to the vase fluid of *Rosa* flowers cause a rapidly decreasing uptake of vase water, promoting desiccation of the flower petals and leaves. This phenomenon may have been due to enzymatic degradation of the pectin-linked structures of the xylem vessels as observed in SEM preparations. The SEM figures show: (1) degradation of the xylem vessel wall structure, (2) loose particles in the vessels, (3) released spiral vessels, and (4) injury of vessel pits.

The consequences for the ornamental value of the roses of vessel plugging and flower desiccation were easy to assess by means of macroscopic observations. Since pectic enzymes in extremely low concentrations exert a dramatic effect on cut flowers, it is likely that some of the products of the enzymatic activity elicit specific responses in the flowers, further affecting their vase life.

Chapter 8 covers the results of an electron microscopic study, using cryoSEM techniques and X-ray microanalysis to demonstrate the effects of aluminium sulphate added to the vase fluid of *Rosa* flowers on the ability of bacteria e.g. *Bacillus subtilis* cells to infiltrate into the xylem vessels of the *Rosa* flowers. The biocidal activity of aluminium sulphate added to the vase fluid was negligible. Aluminium sulphate probably acts as a flocculating agent, in which bacterial cells are embedded. Indeed, Al^{3+} ions react easily with inorganic and organic anionic substances e.g. *Bacillus subtilis* cell wall peptides, substances of wounded stem cells and stem cell wall compounds of cut *Rosa* flowers. The *Bacillus*/aluminium complexes contribute to the formation of flocky deposits which adhere onto the cut surface and the adjacent open xylem vessel wall of the roses. The flocculated material may form a filter bed at the cut surface of the flower stem, hardly decreasing the water uptake, but strongly obstructing the embedded *Bacillus* cells to enter the vascular system of the roses, as clearly shown by cryoSEM figures.

SAMENVATTING

Dit proefschrift is samengesteld uit een inleidend hoofdstuk en zeven publicaties (hoofdstuk 2-8). Deze publicaties zijn de neerslag van een reeks onderzoekingen naar de rol van micro-organismen in de verstoring van het vaasleven van gesneden bloemen. De hybride *Rosa* cultivar 'Sonia' is het geselecteerd uitgangsmateriaal voor deze onderzoekingen.

Hoofdstuk 1 bevat een overzicht van het economisch belang van snijbloemen in relatie tot het vermogen van snijbloemen tot ontplooiing en toename van de sierwaarde tijdens het vaasleven.

Vervolgens is een beknopt overzicht gegeven van relevante literatuur betrekking hebbend op fysiologische en microbiologische factoren die het vaasleven van snijbloemen kunnen beïnvloeden. Witte vlekken in onze kennis van mechanismen die kunnen leiden tot vatverstopping en verkorting van het vaasleven van gesneden bloemen, worden besproken. Enkele kanttekeningen zijn gemaakt bij: (1) de selectie van een kasroos cultivar 'Sonia' als uitgangsmateriaal voor de reeks onderzoekingen, (2) de gebruikte analyse-methoden, (3) de resultaten van oriënterend onderzoek, (4) de bestudeerde microbiologische factoren.

Hoofdstuk 2. Op de stengels van vers geoogste snijbloemen zijn geringe aantallen geteld van verschillende soorten micro-organismen. De initiële microbiologische besmetting van rozenstengels bleek veel geringer dan die van *Chrysanthemum* en *Gerbera* stengels. De soorten en aantallen zijn niet homogeen verdeeld over de bloemstengels.

Dominerende stengel micro-organismen - *Enterobacter*, *Bacillus* en schimmelsoorten - verliezen hun dominantie in het vaaswater waarin aanvankelijk *Pseudomonas* soorten domineren. Deze *Pseudomonas* soorten hebben geen organische groeifactoren nodig. In een later stadium van het vaasleven worden *Enterobacter* en *Bacillus* soorten dominant. Deze bacteriën hebben meer, en hogere concentraties nutrienten nodig voor vermenigvuldiging.

Het ecologisch milieu van plantestengels in vaaswater kan competitie tussen de diverse microbengenera en species hebben bevorderd. Lekkage van groeistoffen uit de aangesneden bloemstengels kan tevens hebben bijgedragen tot verschuiving van de dominante microflora. In een nog later stadium van het vaasleven is ook vermenigvuldiging van fungi waargenomen, vooral in vaaswater van *Chrysanthemum* en *Gerbera*. Beide cultivars hebben een langer vaasleven en een hogere initiële besmetting met fungi dan *Rosa* cultivars.

Hoofdstuk 3, 4 en 5 laten zien dat de mate van infiltratie van levende micro-organismen in de xyleemvaten van gesneden *Rosa* en *Gerbera* stengels afhangt van de aantallen cellen van micro-organismen die aanvankelijk aan het vaaswater zijn toegevoegd. Ook de vorm en de grootte van de individuele cellen spelen een rol, zowel als de wijdte en de spreiding van de doorsneden van de xyleemvaten.

Raster electronenmicroscopische waarnemingen (SEM) zowel als tellingen van bacteriën die zijn geïnfiltreerd in de xyleemvaten van *Rosa* stengels, laten zien: (1) de aantallen in de xyleemvaten geïnfiltreerde bacteriecellen nemen toe met de toename van de contacttijd van stengel en vaaswater, (2) de aantallen geïnfiltreerde bacteriecellen nemen toe afhankelijk van de aantallen bacteriën die aanvankelijk aan het vaaswater zijn toegevoegd, (3) de aantallen geïnfiltreerde bacteriecellen nemen af bij de toename van de afstand tussen het stengel-snijvlak en stengel-monsterpunt, (4) een klein deel van de in het vaaswater gesuspendeerde micro-organismen worden met de vochtstroom opgenomen in het xyleemsysteem van de roos; een groot deel van de vaaswater micro-organismen zal zich hechten aan het stengelsnijvlak, (5) reeds door geringe aantallen geïnfiltreerde micro-organismen kan de geleidbaarheid van het xyleem-watertransport in belangrijke mate afnemen, (6) overeenkomstige verschijnselen van afname van de watergeleidbaarheid van *Rosa* stengelsegmenten zijn gemeten na inoculatie van het vaaswater met geringe aantallen geïnactiveerde microben-cellen, en bij lage concentraties microbiële exopolysacchariden, zoals is aangetoond in de hoofdstukken 5 en 6.

Beide hoofdstukken (5 en 6) laten zien dat geïnactiveerde micro-organismen en verbindingen met een hoog moleculair gewicht zoals exopolysacchariden (EPS), zich in vaaswater gedragen als inerte deeltjes die met de sapstroom worden opgenomen door de plant. Deze inerte deeltjes verstoppen in zekere mate de watertransportbaan, verstoren de waterhuishouding en verkorten het vaasleven van de roos.

Micro-organismen, noch de geteste hoog moleculaire EPS-verbindingen zijn in staat de pitmembranen van de xyleemvaten te passeren via de capillaire openingen met een doorsnede van ca. 50 nm (De Stigter & Broekhuysen, 1986) en veroorzaken een verstopping van deze capillairen.

Het vatverstoppend vermogen van microben en microbiëel EPS is aangetoond door: (1) de fuchsine-rood test, (2) de watergeleidbaarheid test van stengeldelen, (3) electronenmicroscopische waarnemingen.

Met de sapstroom worden ook laag-moleculaire microbiële metabolieten opgenomen door de plant. Deze metabolieten kunnen de pitmembranen passeren en binnendringen in hoger gelegen plant-, blad-, en bloemdelen. Deze metabolieten kunnen toxisch zijn voor de gesneden bloem en de sierwaarde doen afnemen, het vaasleven verstorend, zoals in hoofdstuk 6 is aangetoond.

Hoofdstuk 7 toont aan dat gezuiverde pectolytische enzymen: pectaat lyase van *Pseudomonas fluorescens* en polygalacturonase van *Kluyveromyces fragilis*, toegevoegd aan het vaaswater van rozen, de vochtopname vrij plotseling vrij snel kan doen verminderen. Hierdoor wordt bevorderd dat blad- en bloembladen verdrogen. De oorzaak van deze verschijnselen kan mogelijk worden toegeschreven aan enzymatische afbraak van pectine-verbindingen die stevigheid verlenen aan de xyleem-vatwand-structuur. SEM-preparaten tonen aan: (1) aantasting van de structuur van xyleemvaten, (2) aantasting van pitstructuren, (3) losse deeltjes in de xyleemvaten, (4) losse spiraalvaten.

Het onderscheid tussen vatverstopping en bloemverdroging en de gevolgen ervan voor de sierwaarde van gesneden rozen kan macroscopisch worden vastgesteld.

Extreem lage concentraties pectolytische enzymen in vaaswater veroorzaken reeds een dramatisch effect op de sierwaarde van de gesneden bloemen. Deze verschijnselen wijzen op de mogelijkheid dat bepaalde producten van plantecelwand-pectolyse (oligosaccharinen) een prikkel (elicitor) kunnen zijn tot het optreden van specifieke reacties die het vaasleven van rozen verstoren.

In hoofdstuk 8 is een cryo-SEM techniek toegepast, gecombineerd met radio-micro-analyses. Met deze methoden zijn de effecten bestudeerd van aluminiumsulfaat en *Bacillus subtilis* cellen in vaaswater op het vermogen van *Bacillus subtilis* cellen tot infiltratie en verstopping van xyleemvaten van gesneden rozen.

Bij pH $\geq$ 4, werkt het aluminiumsulfaat als een uitvlokkingsagens, dat bacteriecellen omhult. De geflocculeerde deeltjes vormen een filterbed op het snijvlak van de bloem. Hierdoor neemt de wateropname in geringe mate af, terwijl de infiltratie van de omhulde bacteriecellen in belangrijke mate wordt verhinderd en daarmee tevens de mate van vatverstopping, verstoring van de waterbalans en verkorting van het vaasleven.

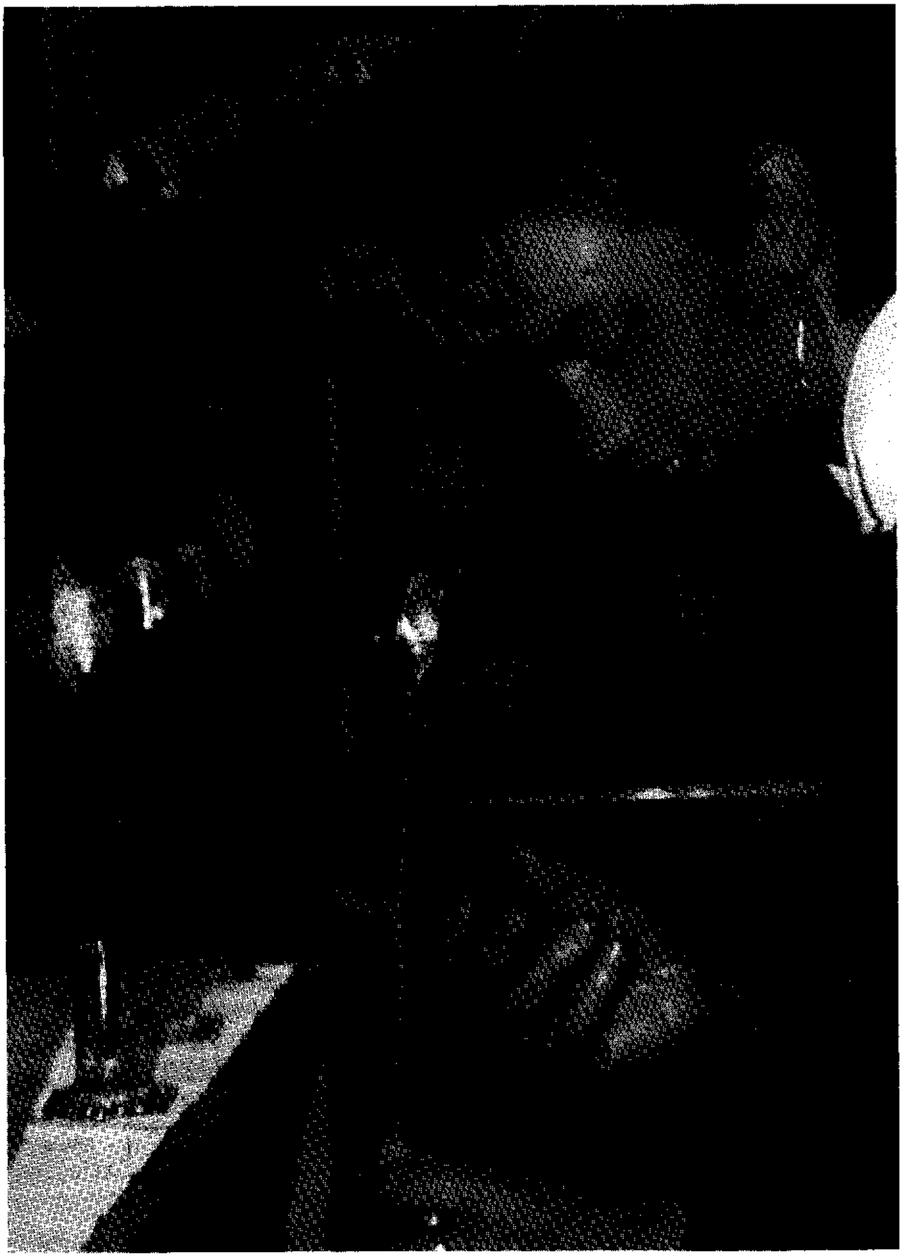

Fig. 1. Lieke Jansen observing Rosa flower bud development and ornamental value.

Fig. 2. Ton Van der Meijden measuring water conductivity of Rosa cv. 'Sonia' stem segments.

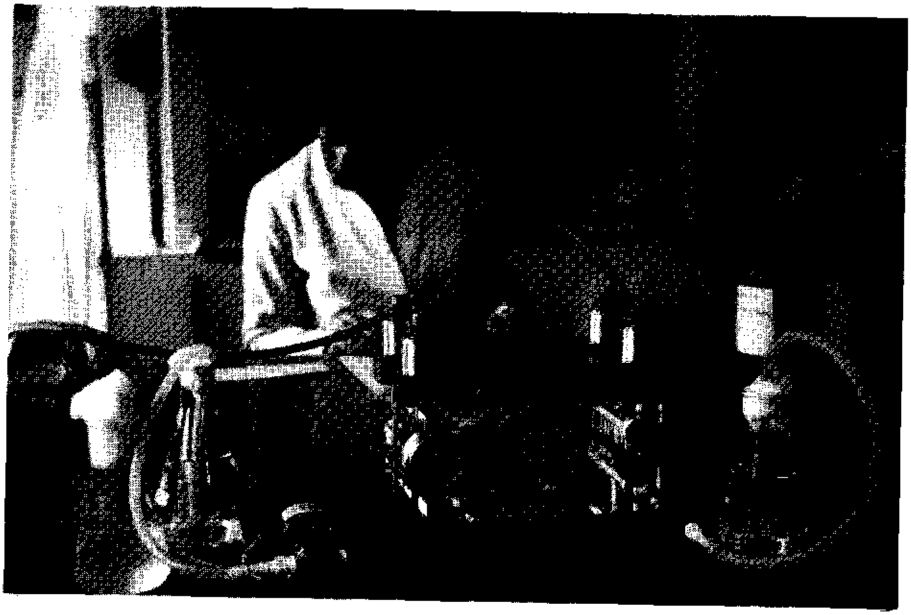

Fig. 3. Molecular filtration of a microbial culture by a Millipore Pellicon recirculating cassette system; Henriëtte Put at the bench.

Glossary and abbreviations

- ABA: abscisic acid,
- abscisic acid: a phytohormone, which acts as growth inhibitor, as an inhibitor of germination, and as an accelerator of leaf abscission.
- abiotic: non-living; of non-biological origin.
- adaptation: changes(s) in an organism, or population of organisms, by means of which the organism(s) become more suited to prevailing environmental conditions.
- adhesion: in nature, micro-organisms often bind specifically or non-specifically to a substratum or other cells, adhesion can be mediated by specialized microbial components or structures.
- aerob: an organism which has the ability to grow in the presence of oxygen.
- aerobic: refers to an environment in which oxygen is present at a partial pressure similar to that in air; or, having the characteristics of aerobe(s).
- aerobiosis: the state or conditions in which oxygen is present; or, life in the presence of air.
- anaerobe: an organism which has the ability to grow in the absence of oxygen; in nature, normally grow - or can only grow - in anaerobic habitats.
- antibiotic: anyu microbial compound which, in low concentrations (µg/ml) can inhibit or kill (susceptible) micro-organisms; or currently, refers to natural, semi-synthetic and wholly synthetic anti-microbial compounds which are effective at low concentrations.
- aseptic technique; precautionary measures taken to prevent the contamination of cultures or plants by extraneous micro-organisms.
- auxins: any of a class of phytohormones which promote stem elongation and play important roles in many other plant processes.
- bacterium (bacteria); prokaryotic micro-organisms: a heterogeneous group of usually single celled organisms; most have a characteristic type of cell wall; free living, occurring in almost every conceivable environment - sometimes under extreme physical conditions.
- bactericidal: bacteriocidal; able to <u>kill</u> at least some types of bacteria.
- bacteristaic: bacteriostatic; able to <u>inhibit</u> the growth and reproduction of at least some types of bacteria.
- biofilm: a film of micro-organisms, usually embedded in extracellular polymer, which adheres to surfaces submerged in, or subjected to aquatic environments; biofilms can frequently cause fouling.

Glossary and abbreviations

- Brownian movement: random movements made by small (ca. 1 μm) particles, or organisms freely suspended in a fluid medium.
- callose: a linear (1→3)-β-D-glucan which occurs associated with sieve plates and sieve tubes in higher plants. Callose may be deposited in plant cell walls in response to wounding and invasion by pathogens.
- capsule: (bacterial) a layer of material external to but contiguous with the cell wall; includes any polysaccharide and/or protein surface layer: macrocapsules; microcapsules; slime layers. The capsular composition varies with species, growth condition etc.

  (mycological) structures analogous to bacterial capsules occur in many fungi as gel-like or mucilaginous layers surrounding hypae or yeast cells, may diffuse into the surrounding medium.
- cavitation: water columns breaking in xylem vessels; a structural breakdown or disruption of water columnm continuity causing a continued decline in xylem vessel conductance (Tyree & Dixon, 1984; Dixon & Peterson, 1989).
- CBS; Centraal Bureau voor Schimmelcultures, Oosterstraat 1, Baarn, The Netherlands. Yeast Division CBS, Julianalaan 67A, Delft, The Netherlands.
- chemotaxis: a taxis in which the stimulus is a cocnentration gradient or a particular chemical (chemo-effector); cells make a net movement towards higher concentrations of some types of chemo-effector (chemo-attrractant) which may or may not be a nutrient, and away from others (chemorepellent)!
- chlorine: (as antimicorbial agent): chlorine is an effective micro-bicidal agent used e.g. for the disinfection of water supplies. It is a strong oxidizing agent, it can react directly with certain groups in cells, chlorine refers to Cl2, hypochlorous acid and other active chlorine compounds.
- chloramines: exert antimicrobial activity by decomposing slowly to release chlorine; their activity is much less rapid than that of hypochlorites; organic chloramines are less toxic and less irritating to biological tissues (human, animal, plants).
- counting methods: (a) the total number of living and dead cells in a given volume or area of a sample is termed the 'total cell count' which usually refers to single-celled organisms, bacteria, spores or yeasts. A total cell count may be determined by microscopy or other instruments (coulter counter, turbidimeter); specific types can be counted by immunofluorescence microscopy; liquid samples containing small numbers can be membrane-filtered and the stained cells counted in situ; (b) viable cell count

refers to the number of living cells per volume or area in a given sample. They can obviously include only those organisms which are detectable by the particular method used. A viable cell count may be determined by: direct examination in a counting chamber, using a vital stain; statistical by a multiple tube method; or by colony counts expressed as CFU, colony forming units, developed on a solid medium on incubation; each colony developing from a single viable cell. The selectivity of the medium and the conditions of incubation may significantly affect the number of viable cells which give rise to colonies. Some types of cells tend to form clumps or to grow in chains or filaments or in microcolonies; in these cases CFU can be any entity capable of giving rise to a single colony.

- cultivar: (cv.) a commercial or cultivated 'variety' of a given species of plant or fungus.
- culture: (1) a liquid or solid medium on or within has grown a population of particular type(s) of micro-organisms as the result of prior inoculation and incubation of that medium; (2) the process of preparing a culture; (3) to encourage growth of particular micro-organisms under controlled conditions.
- cytokinins: phytohormones which stimulate metabolism and cell division; they are 6-N-substituted adenines which are synthesized mainly at the root apex and translocated via the xylem. Certain micro-organisms may produce similar cytokinins.

  Da: dalton ; Atomic Mass Unit.
- disinfectant: any chemical agent used for disinfection; disinfectants which are non-injurious to human, animal of plant tisseus may also be used as antiseptics or preservatives.
- disinfection: the destruction, inactivation or removal of those micro-organisms likely to cause infection or other undesirable effects of the tissue or product to be disinfected.
- DP; degree of polymerizaton.
- electron microscopy: microscopy in which an electron beam interacts with a specimen and subsequently contributes, directly or indirectly to the formation of an image of the specimen. (EM); (SEM); (CryoSEM).
- SEM: scanning electron microscopy: electrons pass through the specimen, and the energies of the emergent electrons are measured in an analyser and used to construct an image of the specimen.
- CryoSEM: the specimen is cooled to very low temperatures without prior

dehydration, such that the water in the specimen forms a 'glass;like' solid without undergoing crystallization, and can be used to construct an image.

- elicitors: molecules capable of inducing phytoalexin accumulation in plants. Biotic elicitors may be derived from or secreted by the plant-invading micro-organisms. Abiotic elicitors are of non-biological origin. Both types of elicitors may act indirectly by releasing phytoalexins or other endogenous elicitors from plants e.g. some plant cell wall polysaccharides (Halverson & Stacey, 1986).
- embolism: air bubbles in xylem vessels. Numerous mechanisms have been proposed for how water stress causes embolism: e.g. (1) xylem pressure becoming increasingly negative; (2) mechanical shock; (3) air coming out of vessel water solutions; (4) the air-seeding hypothesis (Zimmermann, 1983, Sperry & Tyree, 1988).
- endophytic bacteria: those bacteria inhabiting the leaf intercellular spaces or substomatal cavities. They are usually pathogenic (Henis & Bashan, 1986). Extensive exchanges between epiphytic and endophytic populations, especially through stomata, may occur, dependent on the microbiological conditions inside and outside the leaf.
- epiphytic bacteria: those bacteria that can be removed from above-ground plant parts by washing, they may be residents and casually occurring bacteria. They can be either pathogenic or saprophytic (Henis & Bashan, 1986).
- EPS; extracellular polysaccharide.
- ethylene: a phytohormone which, in higher plants, can induce e.g. germination, flowering, senescence. Ethyelene is also produced by plant tissues in response to stress, damage, wounding, infection by pathogens, or to plant tissue hypersensitivity.
- filament: an elongated bacterial or fungal cell, one in which the length exceeds the width by ca. 10 times or more; a sheathed or unsheathed chain of microbial cells. Filamentous fungi.
- fimbriae: (fimbrium) thin, proteinaceous, chromosome- or plasmid-encoded filaments which extend from the cell surface of some types of microbial cell, occurring in large numbers, they are typically able to facilitate cell-cell or cell-substratum adhesion (functionally distinct from pili and flagella).
- flagellum: a thread-like appendage responsible for motility in the majority of motile bacteria.

- fungus (fungi): unicellular, multicellular or coenocytic, heterotrophic, eukaryotic micro-organisms which do not contain chlorophyll and which characteristically form a rigid cell wall containing chitin and/or cellulose. Fungi are widespread in nature.
- germicide: any (bio)chemical agent inhibiting microbial multiplication or inactivating microbial vaibility; any disinfectant, antiseptic, sterilant, biocide.
- gibberellins; a class of phytohormones which are synthezised e.g. in the leaves of plants and which regulates stem elongation seed germination etc.
- hybridization: (in plants) the formation of a hybrid organism by a cross between genetically dissimilar organisms.
- hydroponic cultures: plant cultures which are grown in nutrient solutions as an alternative for soil grown cultures (De Stichter, 1986).
- hypha: (hyphae) in many mycelial fungi and in some bacteria: a branched or unbranched filament, many of which together constitute the vegetative form of the organism and (in some species) form the sterile portion of a fruiting body: a mass of vegetative hyphae is referred to as mycelium.
- IAA: indole 3-acetic acid a photohormone. See: auxins.
- incubation: the maintenance of inoculated media or other types of material at a particulate ambient temperature for a period of time to provide conditions suitable for growth. Specialized incubation permits control of humidity, light, radiation etc.
- infection: the initial entry of a (pathogenic) micro-organism into a host; or a culture which is not handled aspectically.
- isolation: any procedure in which a given species of organism, present in a particular sample or environment, is obtained in pure culture.
- macroscopic: visible to the unaided eye.
- medium: (microbiol.) any liquid or solid preparation made specially for the growth, storage or transport of micro-organisms.
  selective medium: a medium which allows or encourages the growth of some type(s) of organisms in preference to others.
- microbicidal: able to kill at least some types of micro-organisms.
- microbistatic: able to inhibit the growth and reproduction of at least some type(s) of micro-organisms.
- microflora: (1) in a microbiological context: the total of micro-organisms normally associated with a given environment or location; (2) in a broader biological context: the microscopic plants and 'plant-like' organisms

(bacteria, fungi, algae etc.) normally present in given environment or location.

- Mol.wt: Molecular weight.
- motility (microbiol.) locomotion, i.e., an active process in which a cell or organism moves from place to place by expending energy to exert force(s) directly against the surrounding medium or contiguous substratum. It enables an organism to respond to certain environmental stimuli (see taxis).
- mould: an imprecise term for any fungus which forms a visible layer of mycelium and/or spores on the surface of a product, which does not form macroscopic fruiting bodies.
- mucoid: viscous, slimy, mucus-like. The term is often applied to the colonies of certain capsulated bacteria (micro-organisms).
- mucopeptide: any peptide containing mucopolysaccharide (peptidoglycan).
- mucopolysaccharide: (glycosaminoglycan) a polysaccharide which contains a high proportion of amino sugars.
- mucoprotein: a protein covalently linked to amino sugars.
- mycelium: a group or mass of discrete hyphae: the form of the vegetative thallus in many types of fungi and in certain bacteria.
- necrosis: localized death and degeneration of tissues in a living organism - due to an infection or injury (plant leave necrosis).
- ornamental value: a visual parameter of cut flower bud development; expressed in %, compared with the 'normal' bud development of the cut flower when handled aseptically and placed in sterile tap water (or saccharose 1% w/v).
- parasite; parasitism: symbiosis, in which an organism (the parasite) benefits at the expense of the other (the host), the parasite obtains its nutrients from the host, and the association is detrimental to the host (commensalism). Plantparasite: the plant as 'host'.
- pascal (Pa): the SI unit of pressure; it is equal to 1 newton/m^2.
  1 mmHg = 1 torr = 133.3 Pa = 0.133 kPa (1 Bar = 1 atm = 101.325 kPa).
- pathogen: any micro-organism which, by direct interaction with another organism, causes disease in that organism (phytopatogenic, plantpathogenic).
- pectic enzymes: (pectinases): enzymes which degrade pectins, pectinases are produced by a wide variety of micro-organisms.
- pectins: the major component of the pectic polysaccharides, occurring in

plant cell walls, and intercellular regions of plants.

- pectinolytic (pectolytic): able to degrade pectins.
- pellicle: a continuous or fragmentary film, or a mat of organisms, formed at the surface of a liquid culture by certain bacteria or fungi.
- phytoalexin: small lipolytic molecules that have antibiotic activities and are formed as a respons to microbial (elicitor) attack of plants (Albersheim & Valent, 1978; Albersheim & Darvill, 1985; Cervone *et al.*, 1989).
- phyotalexin 'de novo': slaat op aanmaak.
- pili (pilus): filamentous or elongated proteineaceous structures which extend from the cell surface in bacteria (See: fimbriae).
- pit connection: a structure located between two adjacent cells (xylem cell walls), connected by a continuous cytoplasmic (pit)membrane.
- plasmodesma (plasmodesmata): a fine channel in a plant cell wall (or a fungal septum), through which cytoplasma can pass.
- prokaryote: synonymous with bacteria; micro-organisms in which the chromosomes are not separated from the cytoplasm by a membrane.
  eukaryote: micro-organisms (e.g. fungi, algae) in which the chromosomes are separated from the cytoplams by a membrane.
- pulsing: a procedure or pretreatment of cut flowers, directly after harvest, to load flower tissues with certain chemicals (Halevy *et al.*, 1978).
- phytohormones: planthormones; endogenous compounds synthesized at one site and transported to another site of the plant where they exert a physiological effect.
- oligosaccharins: naturally occurring plant cell wall carbohydrates exhibiting biological regulatory functions (Halverson & Stacey, 1986).
- race: physiological race (mycology); a non-specific designation which may refer e.g. to a strain or a variety.
- safety cabinet: sterile cabinet; (laminar flow) cabinet within which microbiological work is carried out so as to prevent contamination (air, personnel etc.).
- secundary metabolism: metabolism which is not essential for, and plays no part in, growth; it commonly occurs maximally under conditions of restricted growth or absence of growth.
- senescence: an irreversible process of gradual degeneration and disintergration of plant tissue, eventually leading to the death of the organism (Bruinsma, 1983).

Glossary and abbreviations

- signal exchange: in plant-microbe interaction; involves the recognition and exchange of specific molecules by the host or microbe or both that elicit (or trigger) physiological, or biochemical responses that affect the development of the plant-microbe interaction (Halverson & Stacey, 1986).
- signal molecules: may be DNA, RNA, protein, lipid or polysaccharides. signals that initiate the process and, signals that maintain and control the interactions (Halverson & Stacey, 1986).
- silver: antimicrobial agent; a heavy metal which in elemental or compound form, is typically microbistatic in low concentrations; the functional antimicrobial moiety is the silver ion, which also may be microbicidal, depending on the Ag^{+} concentration used.
- smoot-rough variations: (S--R) in many types of bacteria; a change in cell-surface composition which occurs spontaneously during in vitro or in vivo growth. S - smooth, glossy colonies; may be capsulated containing lipopolysaccharides; R - rough, dull colonies; may be non-capsulated, missing lipopolysaccharides in the cell wall.
- species: microbiology; one of the smaller taxonomic groupings which display a high degree of mutual similarity populations which are capable of genetic interchange and/or interbreeding, and which are evolving together in a common pattern.
- spore: a differentiated form of a micro-organism which may be: specialized for dissemination; or produced in response to environmental conditions; or produced during an asexual or sexual reproductive process.
- sporicide: any chemical agent which inactivates microbial spores.
- staining: in microbiology: any process which imparts colour, opacity, or electron density, contrast, or confers the ability to fluoresce to parts of a specimen - prior to examination by microscope or electron microscope.
- sterile: refers to the condition of an object, or an environment, which is free of all living cells, all viable spores and all viruses (sterilant, sterilizer, sterilization).
- strain: an organism, or population of organisms, regarded as being genetically similar or identical.
- symbiosis: any stable condition in which two different organisms (symbionts) live in close physical association; or form a close association to their mutual benefit (mutualism).
- taxonomy: the science of biological classification, i.e. grouping of organisms according to their similarities (systematics) used as a key for

the identification of organisms, permits naming (nomenclature).

- taxis: chemotaxis: a locomotive response to an external stimulus (air, light, chemical), exhibiting by certain micro-organisms in which direction of locomotion is related to the direction or oriention of the stimulus (kinesis, phototaxis, tropism, Carlile, 1980).
- toxic: poisonous, harmful (toxin).
- toxigenic: (toxinogenic): refers to an organism which can produce one or more toxins.
- toxin: (microbiological): any microbial product or component which, when present at low concentrations in cells or (plant)tissues of a higher (multicellular) organism, can cause injury by interferring with the structure or functional integrity of those cells or tissues.
- *tropism:* *a response to a directional stimulus exhibiting* by bending or growth of an organism (or part of it; stem or bud of a flower) in an orientation dictated by that of the stimulus (chemotropism; geotropism; phototropism, theotropism, etc.).
- transport systems: systems which permit the uptake or efflux of substances (nutrients, ions) across membranes, which are otherwise impermeable to those substances.
- tween: any of a range of non-ionic surfactants which are polyoxyalkylene derivatives of fatty acid esters of sorbitan (Tween 20; 40; 60; 80).
- tylose: (plant pathology); a balloon-like outgrowth from xylem parenchym cell which expands into, and blocks, the lumen of a xylem vessel or a tracheid; occurs as a respond to wounding or infection (wilt syndrom due to Fusarium, Gilman & Steponkus, 1972).
- vascular wilt: (plant pathology); any of various plant diseases in which infection of the vascular system by a fungal or bacterial pathogen results in wilting (flaccidity) of the plant. Pathogenic factors involved are: gummy polysaccharides; tylose, toxins, vessel blockage by the pathogen itself.
- vase life: the potential longevity (days) of the cut flower at the final consumer's home (Halevy & Mayak, 1979).
- vase life end-point: the first sign of wilting, or the total death of the cut flower (Halevy & Kofranek, 1977).
- water activity: (a_w); the fraction of 'free' or 'available' water in a given substrate.
- water conductivity: in cut flowers; the reciprocal value of the resistance

to water flow through xylem vessels of flowers, expressed as ml water per 30 min per transverse-sectional area of 5 cm vessel length (Gilman & Steponkus, 1972; Chin & Sacalis, 1977; Put & Van der Meijden, 1988).

- water relations: in cut flowers; the balance between the capacity of the flower for: water uptake, water transport, and transpiration (Halevy & Mayak, 1981; Van Meeteren, 1980).
- yeasts: a non-taxonomical category of fungi defined in terms of morphological and physiological criteria. The typical yeast is an unicellular saprotroph which can metabolize carbohydrates and in which asexual reproduction occurs by budding, or sexual by ascospore formation.

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C U R R I C U L U M   V I T A E

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