CHAPTER SEVEN

I. ISOLATION AND IDENTIFICATION OF *PSEUDOMONAS* spp. FROM *ROSA HYBRIDA* 'SONIA' STEMS AND VASE WATER. II. CAVITATION IN *ACACIA AMOENA* STEMS KEPT IN TWO CONCENTRATIONS OF *PSEUDOMONAS FLUORESCENS*

7.1 INTRODUCTION

Many researchers have examined the effects of bacterial numbers and species on vase life and water uptake (Larsen and Scholes 1955; Dansereau and Vines 1975; Accati *et al.* 1980; Marousky 1980; Put and Conway 1986; van Doorn, Buis and de Witte 1986; van Doorn, de Witte and Waltmann 1986; Zagory and Reid 1986a, b; de Witte and van Doorn 1988; Put and Clerkx 1988; Put and van der Meyden 1988; Clerkx *et al.* 1989; Put and Jansen 1989; Put and Rombouts 1989; van Doorn *et al.* 1989; Put 1990; van Doorn and Perik 1990; van Doorn, de Witte and Perik 1990; de Witte and van Doorn 1991; van Doorn and de Witte 1991; van Doorn, Zagory, de Witte and Harkema 1991; van Doorn, Zagory and Reid 1991; Jones and Hill 1993; van Doorn and de Witte 1994). However, the effect on cavitation of bacteria added to the vase water has not been investigated previously. It is hypothesised that high numbers of bacteria added to the vase water will accelerate the rate of cavitation. W. van Doorn (pers. comm. 1991) believed that the 24 h time lag before the rate of cavitation rose in distilled water (Fig. 4.2) was related to a gradual increase in bacterial numbers. Put and Clerkx (1988) found that water uptake was significantly lower during the first 24 h if microbial suspensions were added to the vase water. However, the initial 24 h of vase life was thought to be too short a time for mass colonisation of infiltrated bacterial cells to explain the extent of decreased hydraulic conduction (Put and van der Meyden 1988).

Pseudomonas spp. have been mentioned previously (section 4.4) as the bacteria most commonly isolated from vase water. The predominance of *Pseudomonas* spp. in vase water is likely to be because most of the genus are "nutritionally versatile" (Stanier *et al.* 1966). They utilise over 100 different carbon sources for growth (Buchanan and Gibbons 1974), and multiply rapidly in nutrient-poor vase water (Put 1990). Several studies have been made of the effects of different *Pseudomonas* spp. on vase life, e.g *Pseudomonas aeruginosa* (de Witte and van Doorn 1991); *Pseudomonas putida* (Put and Clerkx 1988; Put and van der Meyden 1988; Put and Jansen 1989); *Pseudomonas fluorescens* (Put and Jansen 1989) and pectic enzymes from *Ps. fluorescens* (Put and Rombouts 1989). Put and Jansen's (1989) study also examined the effect

of other bacterial genera and species, but overall, *Ps. fluorescens* was the bacterium which most frequently decreased water uptake and hence affected flower development and vase life. Bacterial motility by means of flagella is considered a factor aiding infiltration into a plant's vascular system (Put and Clerkx 1988). It is thought that virtually all phytopathogens have flagella and are motile (Billing 1982). *Ps. fluorescens* is motile and has polar multitrichous flagella (Buchanan and Gibbons 1974).

Ps. fluorescens is considered to be an opportunistic plant pathogen because it is not specialised and therefore only infects weakened or damaged plant tissue (Klement *et al.* 1990). Several strains of *Ps. fluorescens* cause soft rot diseases because they possess pectolytic enzymes which degrade pectins in the plant cell wall. Relatively few bacteria are known to synthesise pectate lyases, although *Pseudomonas*, commonly found in vase water (Ford *et al.* 1961; Taplin and Mertz 1973; de Witte and van Doorn 1988; Put 1990; van Doorn, de Stigter, de Witte and Boekestein 1991), is one of the genera which does (Nasuno and Starr 1966). Pectolytic, fluorescent pseudomonads, including *Ps. fluorescens*, require wounding to cause soft rots (Brocklehurst and Lund 1981)—cut flowers are thus vulnerable to attack. Burdett (1970) hypothesised that vascular blockage in cut roses was caused by the secretion of pectolytic enzymes by micro-organisms, which converted cell wall materials into vessel-plugging substances. Furthermore, histochemical studies of senescent roses (Parups and Molnar 1972) revealed that pectinaceous deposits were the major components of xylem blockage. Such deposits were thought to come from the breakdown of secondary wall material (Rasmussen and Carpenter 1974). Put and Rombouts (1989) found that buffered pectic enzymes of *Ps. fluorescens* impaired the water relations of cut roses, and their SEM observations revealed enzymatic degradation of cell walls.

The aims of the experiments described in this chapter were to:

- isolate *Pseudomonas* spp., specifically *Pseudomonas fluorescens*, from vase water and/or stems of flowering *Rosa hybrida* 'Sonia';

- identify the bacteria isolated to the taxonomic level of species;

- determine whether high concentrations (10^6 and 10^8 cfu mL^{-1}) of *Ps. fluorescens* in the vase water would expedite cavitation production in cut stems of *Acacia amoena*; and

- ascertain whether *Ps. fluorescens* produces pectolytic enzymes.

7.2 MATERIALS AND METHODS

7.2.1 Plant material

Plant material used in this chapter ca ne from the sources listed below.

Purchased plants

Cut flowering stems of *Rosa hybrid a* 'Sonia' were ordered from a local florist (Armidale Florist & Nursery, 157 The Mall, Armid: le, N.S.W.). The nearest commercial supplier of 'Sonia' roses was in Queensland (Falcon Court Roses, Goondiwindi, 28°, 33' S; 150°, 18' E), 431 km away. The freshest roses available were already 2 d old when they arrived in Armidale. They are only harvested on a Saturday and delivered to Armidale on a Monday. It was not possible to obtain fresher roses in Armidale. Th e postharvest history of the roses from the time of harvest to use in the laboratory is given below.

Roses were harvested at flowering stages 1 to 2 (Berkholst 1980 = Stages 1 to 2 of Ho and Nichols 1977; see diagrams of flowering stages, section 7.2.2) at about 6.00 a.m. on a Saturday. They were then placed in Chrysal RVB™ (a conditioner) for 12 h at 0 to 4°C. The stems were then transferred to Chrysal Universal™ food (a preservative) and kept in that solution at 2 to 4°C until delivered. The refrigerated truck arrived in Armidale at 8.30 a.m. Monday morning, at which time the stems were transferred to a clean bucket of tap water at the florist's. The stems remained in this water until 9.30 a.m.; they were then kept in air for approximately 10 min during transportation to UNE. At the laboratory, the stems were placed into distilled water, and the basal 10 cm was removed under distilled water, leaving a stem length of 30 cm. Compound leaves were removed until the upper most three compound leaves remained (Put 1990).

Field collections

Stems of *Acacia amoena* were collected as required from the Botany Department garden, UNE, Armidale.

7.2.2 Vase life evaluation of *Rosa hybrida* 'Sonia'

Rose vase life was monitored daily according to the stages of rose floral development described by Berkholst (1980). [The concept of standardised floral development was also recommended by Reid and Kofranek (1980) for o her cut flowers in order to enable inter-laboratory vase life comparisons to be made.] Berkholst's (1980) eight phases of rosebud development were described in Dutch, therefore a translated version is shown below to facilitate understanding.

Stage 1: Too raw;
Stage 2: Summer phase;
Stage 3: Winter phase;
Stage 4: 1 to 4 petals turned down;
Stage 5: 5 to 8 petals turned down;
Stage 6: Half opened - ≥ 9 petals turned down;
Stage 7: Peak of flowering - all petals opened except one or two in the centre;
Stage 8: Totally open flower.

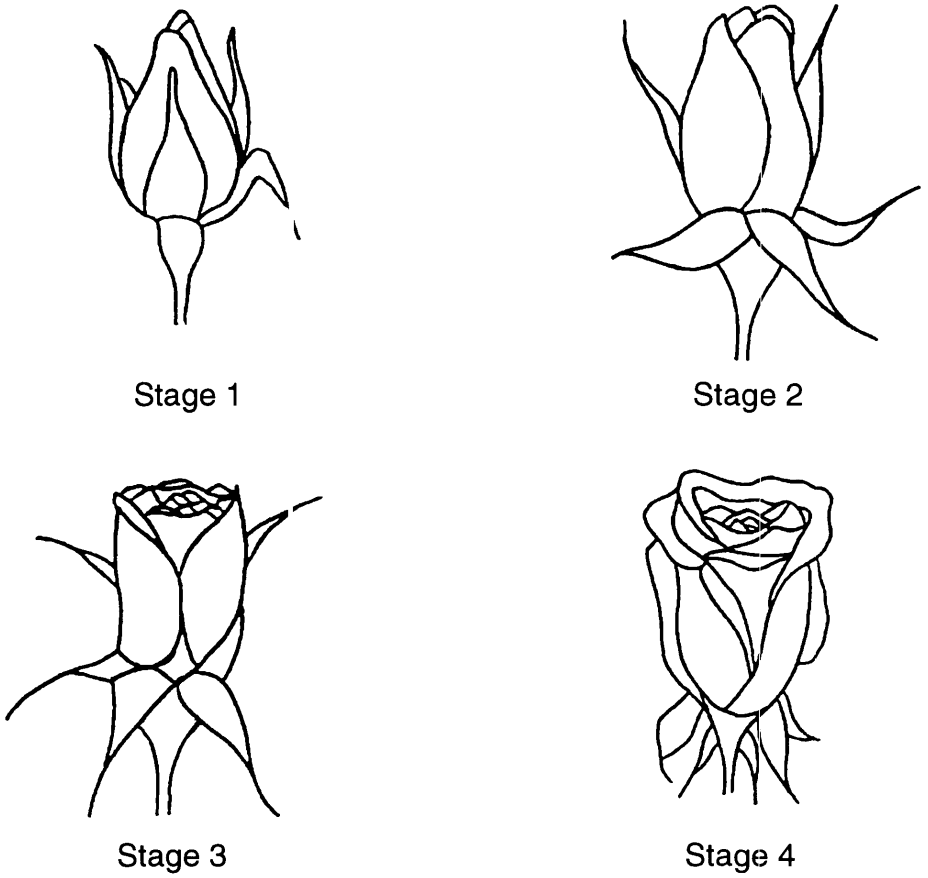

Fig. 7.1. The first four phases of rosebud development, according to Berkholst (1980). (Diagrams adapted from Berkholst 1980.)

The end of ornamental vase life, or onset of visible senescence, was deemed to be reached when any of the following occurred: the flowers did not develop further, petals or leaves wilted, or bent neck occurred (van Doorn, de Witte and Waltmann 1986).

Vase life parameters were measured as described in section 2.13.

7.2.3 Preparation of agar media and dilution series

Agar media and serial dilutions were prepared as described previously (section 6.2.4).

7.2.4 Isolation and identification of *Pseudomonas* spp. from cut *Rosa hybrida* 'Sonia' stems and vase water

Initially, it was hoped to import the bacterium *Pseudomonas fluorescens* Strain 52 from the Netherlands. Put and Jansen (1989) isolated that micro-organism from the vase water and stems of *Rosa*, and determined its effect on water uptake and vase life. Dr. Put was asked (H. Put, pers. comm. 1992) to supply the bacterium, but she thought that some of the Strain's properties might change or be lost during the long flight to Australia. *Ps. fluorescens* Strain 52 has no acronym, therefore it has not been deposited in any official culture collection throughout the world (L. Sly, pers. comm. 1992), so it would not have been possible to obtain the same strain in Australia[1]. Therefore, after several discussions with bacterial specialists in Australia, the author decided to isolate the micro-organism, as *Ps. fluorescens* is ubiquitous and so should be easy to isolate from vase water and cut stems (A.C. Hayward, pers. comm. 1992).

The method of isolation followed Put (1990). *R. hybrida* 'Sonia' stems were used and prepared as described above (section 7.2.1). The stems (10 stems) were placed into separate measuring cylinders (100 mL) containing sterile tap water (100 mL)[2]. The top of the cylinders was sealed with Parafilm to guard against evaporation and atmospheric sources of contamination.

Vase water

Put (1990) found that *Pseudomonas* spp. were the dominant microflora in vase water during the first 3 to 6 d of vase life, so 1 mL samples of vase water were taken from each vase on days 3 and 6 of vase life. The samples were either pour or streak plated onto King's B medium, which is specific for the demonstration of fluorescin production by fluorescent pseudomonads, such as *Ps. fluorescens* (King *et al.* 1954). [Some other fluorescent pseudomonads, such as *Ps. aeruginosa*, which is also found in water, produce the pigment pyocyanin, which is best observed on King's A medium (King *et al.* 1954).] Incubation was at 30°C for 48 h. Fluorescent (yellow-green) colonies were observed under shortwave (366 nm) ultraviolet radiation. Pure cultures were obtained by streaking single fluorescing colonies from the original plates onto solidified King's B medium. After 48 h incubation at 30°C, the procedure was repeated several times to ensure culture purity. The cultures were then ready for first and second stage bacterial tests (Cowan 1974). Tests were carried out on cultures less than 24 h old, in

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[1] It was possible to obtain a typed culture of *Ps. fluorescens*, but there was a risk that the substitute bacterium may have been isolated from other plant material or soil, and thus may have had very different properties.

[2] It should be noted that in Put's experiments (Put 1990), the initial length of harvested stems was approximately 65 cm, however, the longest stems of harvested 'Sonia' roses available in Armidale were 40 cm. This shorter stem length may have had a bearing on the number of micro-organisms on the rose stems, as Put (1990) found that *Rosa* stems (harvested about 1.5 m from the soil) had far fewer micro-organisms than *Gerbera* or *Chrysanthemum* stems (harvested < 10 cm from the soil). She attributed this to the harvested rose stems' greater distance from the soil, although the hairy epidermis of *Gerbera* and the numerous small leaves on *Chrysanthemum* stems, could have also facilitated microbial adherence (Put 1990).

order to avoid inaccuracies in Gram reactions and morphologies (Cowan 1974; Harrigan and McCance 1976). Gram stains, catalase and oxidase tests were performed on all isolates and all test results were the same: gram negative rods, which were both catalase and oxidase positive. Because of the time involved in performing microbial tests on all samples, and the likelihood that many cultures were similar, two representative samples of vase water isolates were chosen (one from day 3 and one from day 6) for comprehensive biochemical testing. The microbial tests performed, together with the results obtained, are shown in Appendix G.

Stem segments

Put (1990) isolated *Ps. fluorescens* from stems as well as vase water of *R. hybrida* 'Sonia' during vase life. The method of rose stem isolation in this thesis followed Put's (1990) method. Thus, stem segments 4 cm long were cut aseptically from the basal end of five rose stems after 3 d of vase life. The segments were transferred into sterile McCartney bottles containing sterilised tap water (20 mL) and Tween 80 (0.05% v/v) (Difco Laboratories, Michigan U.S.A.). The bottles were shaken for 30 min, then 1 mL of each suspension was pour plated onto King's B medium (King *et al.* 1954). The cultures were incubated, examined, subcultured and tested as for the vase water samples above. The procedure was repeated with stem segments cut from the remaining five rose stems after 6 d of vase life. The only difference to the day 3 method was that 1 mL suspensions were streaked onto solidified King's B medium, rather than pour plated. Gram stains, catalase and oxidase tests were again the same for all isolates: gram negative rods, which were catalase and oxidase positive. Therefore, only two representative samples of stem segment isolates were chosen (one from day 3 and one from day 6) for comprehensive biochemical testing. The results of these biochemical tests are detailed in Appendix G.

Final identification of the four isolates was obtained by gas chromatographic fatty acid analysis (Microbial Identification System HP5898A, Hewlett-Packard Company, Pennsylvania) at the Biological and Chemical Research Institute, Rydalmere, Sydney, New South Wales. The isolates were incubated overnight at 30°C on Trypticase Soy Agar (TSA) before being sent to the BCRI for final identification. TSA is an all-purpose agar medium used for the culture of fastidious organisms, and also to monitor the sterility of the microbial workplace (O'Leary 1989). The fatty acid analysis results are set out in Appendix H.

7.2.5 Infiltration of *Ps. fluorescens* (10^6 cfu mL^{-1}) into cut *Acacia amoena* stems

The following procedure was used to determine the amount of *Ps. fluorescens* required to give a concentration of 10^6 cfu mL^{-1}. One tube of the original frozen suspension was removed from a -70°C freezer and unthawed. This tube was divided into 15 tubes (more than enough for all

replicates) by adding three loopfuls of the original suspension to each tube containing 10 mL of sterile Trypticase Soy Broth (TSB) The bacteria were grown for 48 h at 30°C. The original suspension was discarded. Fourteen of the new tubes were refrozen, and one tube was used to do a calibration curve of plate count -v- absorbance (at λ 500 nm). Uninoculated TSB was used as a 'blank' to zero the spectrophotometer (Model 340, Sequoia-Turner Corporation, California). A dilution series was made using TSB, and absorbances were read for those dilutions. A similar dilution series was made with sterile distilled water, from which pour plates were made with Plate Count Agar. These plates were incubated for 48 h at 30°C, then counted to determine the number of bacteria (cfu mL^{-1}) in the original inoculum. A calibration curve was then made of absorbance -v- serial dilutions.

To obtain a concentration of 10^6 cfu mL^{-1}, a dilution series was made, the absorbance was checked, and the required amount of suspension was added to sterile distilled water (200 mL).

Each of the 14 bacterial replicates was removed from the -70°C freezer as required. They were then regrown in TSB for 48 h at 30°C to ensure a standard viability, recovery and growth curve before dilutions were made.

Stems of *A. amoena* to be infiltrated with *Ps. fluorescens* were prepared and brought to full turgor as usual (sections 4.2.2 and 2.2). However, these stems were surface sterilised with ethanol (95% v/v) and rinsed in sterile distilled water before being placed in the bacterial solution. Cavitation events were monitored as described previously (section 4.2.3). The number of bacteria in the solution was determined on days 1, 3 and 5 (section 4.2.8).

7.2.6 Infiltration of *Ps. fluorescens* (10^8 cfu mL^{-1}) into cut *A. amoena* stems

Bacteria were prepared as described above (section 7.2.5). The bacterial solution in the vase was then covered with Parafilm and left for 3 d, 30 cm away from a 100 W incandescent bulb. This procedure had been found to increase the bacterial concentration to a stable 10^8 cfu mL^{-1} (section 7.3.3). Stems were then prepared and placed in the bacterial solution for cavitation monitoring as described above (section 7.2.5).

7.2.7 Pectolytic activity of *Ps. fluorescens*

The presence of pectolytic activity in *Ps. fluorescens* was determined after 5 d using the method of Sands *et al.* (1972). However, it was not necessary to add antibiotics to the medium as a pure culture was being tested. Five single colonies were transferred equidistantly onto the solidified agar medium. The agar plates were then incubated at 30°C for 48 h before being

gently flooded with hexadecyltrimethylammonium bromide aqueous solution (1% w/v), a polysaccharide precipitant (Jayasankar and Graham 1970). The presence of a clear zone around the bacterial colonies within 15 min after flooding is indicative of pectate lyase production (Sands *et al.* 1972).

7.3 RESULTS

7.3.1 Water uptake and vase life of *R. hybrida* 'Sonia' stems kept in sterile tap water

The mean vase life of cut *R. hybrida* 'Sonia' flowers kept in sterile tap water was 2.8 d (Table 7.1). The mean stage of bud development on day 0 of the vase life experiment (which was actually 2 d after harvest) was stage 3 (Table 7.1; see also diagrams, section 7.2.2). By day 3, bud development had reached stage 5.1. Mean water uptake decreased from 18.2 mL d^{-1} on day 0 to 1, to 11 mL on day 1 to 2, and by day 2 to 3, it was 6.5 mL d^{-1}.

Table 7.1

Vase life, water uptake and bud development of cut flowering stems of *R. hybrida* 'Sonia'

Vase life (d) ± SE	Water uptake (mL d^{-1}) ± SE			Stage of bud development ± SE			
	Time (d)			Time (d)			
	0-1	1-2	2-3	0	1	2	3
2.8±0.13	18.2±1.47	11±1.16	6.5±0.93	3±0.30	4.4±0.27	4.6±0.22	5.1±0.41

Data are the means of 10 replicates ± standard error.

7.3.2 Isolation and identification of *Pseudomonas* spp. from cut *Rosa hybrida* 'Sonia' stems and vase water

Pseudomonas spp. were isolated from *R. hybrida* 'Sonia' stems and vase water (Appendices G and H). The results of first- and second-stage bacterial identification tests (detailed in Appendix G) indicated that the bacterium isolated from the day 3 stem culture was either *Ps. fluorescens* or *Pseudomonas putida*. The bacterium isolated from day 3 vase water was also either *Ps. fluorescens* or *Ps. putida*. The bacterium isolated from the day 6 stem culture was identified as *Ps. putida*; and the bacterium isolated from day 6 vase water was either *Ps. fluorescens* or *Ps. putida*.

Two of the bacterial isolates were identified with certainty by fatty acid analysis. The bacterium isolated from day 6 stem culture was identified as *Ps. putida* biovar A, and the bacterium isolated from day 6 vase culture was identified as *Pseudomonas chlororaphis* [formerly *Ps. fluorescens*

biovar D prior to the 8th edition of Bergey's Manual (Buchanan and Gibbons 1974)]. The stem and vase isolates at day 3 were unable to be identified with certainty, but were either biotypes of *Ps. fluorescens* or *Ps. putida*, or *Pseudomonas marginalis*. (See Appendix H for more detail of the results, as well as an explanation of the identification method.)

Because the original aim had been to isolate *Ps. fluorescens*, it was decided to use the day 6 vase isolate, *Ps. chlororaphis* (= *Ps. fluorescens* biovar D, hereafter known as *Ps. fluorescens*) in the following cavitation experiments. This bacterium had the best match with library descriptions of the four isolates identified by fatty acid analysis (Appendix H).

7.3.3 Cavitation of *A. amoena* stems kept in sterile distilled water containing *Ps. fluorescens* (10^6 cfu mL^{-1})

Stems kept in *Ps. fluorescens* (initial inoculum level 10^6 cfu mL^{-1}) had a very low rate of cavitation until 96 h, after which time AAE production rose sharply (Fig. 7.2). Stems did not begin to dry out (phyllode venation prominent; phyllode colour light green) until after 96 h. The average RWC of stems at the end of the experiment was 44%, which was in the next significantly highest grouping after citric acid (Table 7.2). Table 7.2 shows a means separation of the RWC results from all cavitation experiments (some were previously shown in Table 4.1).

Bacterial numbers during the *Ps. fluorescens* cavitation experiment (10^6 cfu mL^{-1}) increased to 10^7 cfu mL^{-1} after 1 d, but stabilised at 10^8 cfu mL^{-1} from 3 to 5 d (data not shown). Bacterial suspensions of *Ps. fluorescens* under the same experimental conditions, but without an *A. amoena* stem, maintained the initial 10^6 cfu mL^{-1} concentration during day 1, but from 3 to 5 d reached and stabilised at 10^8 cfu mL^{-1} (data not shown).

Table 7.2

Relative water content (RWC) of *A. amoena* stems at the end of all cavitation experiments

Treatment	RWC (%) after 120 h
Citric acid (10 mol m^{-3}) (Fig. 4.4)	71.4±4.7[a]
Ps. fluorescens (10^6 cfu mL^{-1}) (Fig. 7.2)	44.3±3.2[b]
Chlorine (DICA 50 mg L^{-1}) (Fig. 4.6)	34.6±4.5[bc]
Degassed distilled water (Fig. 5.4)	27.7±3.9[bc]
Ps. fluorescens (10^8 cfu mL^{-1}) (Fig. 7.2)	25.6±4.1[c]
Distilled water (Fig. 4.2)	19.4±2.3[c]

Means followed by different letters are significantly different ($P < 0.05$).
Standard error is shown as ± value after mean.

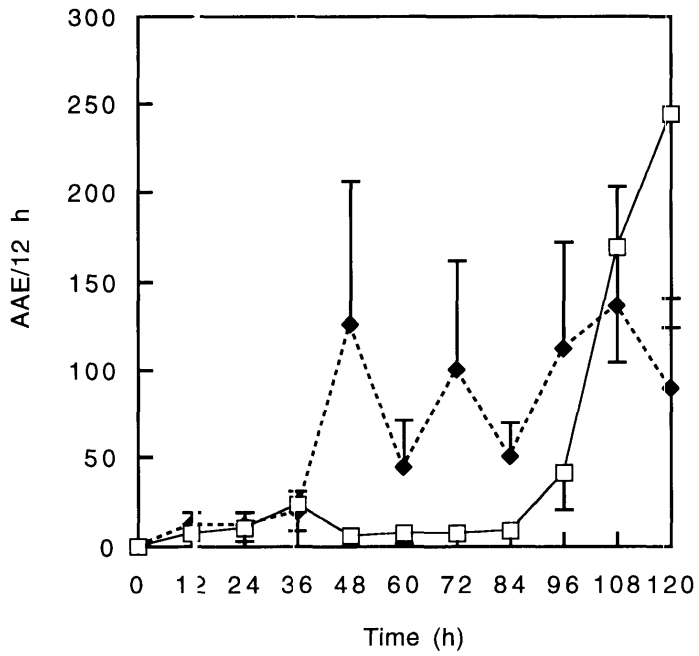

Fig. 7.2. The rate of AAE production while *A. amoena* stems were kept in sterile distilled water containing *Pseudomonas fluorescens* at either 10^6 cfu mL^{-1} (□) or 10^8 cfu mL^{-1} (◆). Each data point is the mean of 10 replicates ± standard error. (Where no error bar appears, the SE was smaller than the size of the symbol.) (See Fig. 4.2 for AAE production in distilled water.)

7.3.4 Cavitation of *A. amoena* stems kept in sterile distilled water containing *Ps. fluorescens* (10^8 cfu mL^{-1})

Stems in a 10^8 cfu mL^{-1} concentration of *Ps. fluorescens* exhibited a 'multi-peak' cavitation profile (Fig. 7.2). The highest peak (at 108 h) was lower than the peak for the 10^6 concentration (at 120 h; Fig. 7.2), but overall more AAE were produced (698 cf. 520) in the 10^8 concentration. The RWC of stems at the end of the cavitation experiments was significantly less in the higher bacterial concentration than in the lower concentration (Table 7.2). Stems began to dry out (phyllode venation prominent; phyllode colour light green) after 72 h in the 10^8 concentration.

The number of bacteria was monitored during the *Ps. fluorescens* cavitation experiments and was found to remain stable at 10^8 cfu mL^{-1} throughout the experimental period (data not shown).

7.3.5 Pectolytic activity of *Ps. fluorescens*

No pectolytic activity was observed in *Ps. fluorescens* on the medium of Sands *et al.* (1972), as no clear zones were evident around the bacterial colonies (data not shown).

7.4 DISCUSSION

The vase life of *R. hybrida* 'Sonia' in sterile tap water was only 2.8 d (Table 7.1), which is much shorter than the 7 to 8 d usually found by Dutch researchers (Put and van der Meyden 1988; Put

and Jansen 1989). However, 2 d elapsed between harvesting and the commencement of the vase life experiment, whereas in Dutch studies the experiments are begun within 4 h of harvest (van Doorn and Perik 1990; Put *et al.* 1992). Although the harvested flowers were kept in Chrysal™ solutions during the 2 d prior to the experiment, perhaps different harvesting practices and conditions occurred which may have favoured short vase life. The amount of water uptake during the first day of vase life (18 mL; Table 7.1) was comparable with that (16 mL) noted by Put and Clerkx (1988), but subsequent water uptake was much lower. In the present experiments, uptake on the second and third days of vase life was 11 and 6.5 mL respectively, whereas in the Dutch experiments it increased to 18 and 20 mL (Put and Clerkx 1988). Put and Clerkx (1988) found that water uptake continued to increase until day 4 of vase life (22 mL). [It was not possible to compare water uptake on a per gram of fresh weight basis because such data were not mentioned by Put and Clerkx (1988).]

The stages of bud development were comparable to those observed by Put *et al.* (1992). Flowers were harvested at stages 1 to 2 (Berkholst 1980) in both experiments, and by day 2 of vase life had reached stage 4 to 5 (Table 7.1). Put *et al.* (1992) also noted that stage 5 was reached after 2 d of vase life. Overall, it was considered that the *Rosa* vase life experiment was comparable enough to Put's experiments to achieve its aim, i.e. of isolating *Pseudomonas* spp. from the vase water and stems of *R. hybrida* 'Sonia'.

The identity of *Pseudomonas* spp. isolated from vase water and stems of *R. hybrida* 'Sonia' was confirmed by both traditional biochemical identification tests (Appendix G) and fatty acid analysis (Appendix H). The biochemical identification tests were unable to separate conclusively *Ps. fluorescens* and *Ps. putida*, except for the day 6 stem culture, which was identified as *Ps. putida* (Appendix G). Identification using fatty acid analysis revealed that the day 6 stem isolate was *Ps. putida* biotype A and the day 6 vase isolate was *Ps. chlororaphis* (= *Ps. fluorescens* biovar D) (Appendix H). Overall, there was good agreement between the two methods of bacterial identification. The traditional biochemical methods were very time-consuming to perform, but they added certainty to the fatty acid analysis results. The advantage of fatty acid bacterial identification is that it is very rapid but the limitation is that the results are dependant upon the organisms stored in its library. Therefore, ideally (time and resources permitting) one should compare the results of the two identification methods, as was performed here.

Because of the single, "excellent" match of the day 6 vase isolate to *Ps. chlororaphis*/*Ps. fluorescens* (Appendix H), that organism was employed in the subsequent cavitation experiments. The cavitation results using a bacterial concentration of 10^6 cfu mL^{-1} were surprising. It was expected that such a high number of bacteria would cause premature xylem blockage and result in an earlier cavitation peak than in distilled water (Fig. 4.2). However, the cavitation rate did not rise appreciably until after 96 h (Fig. 7.2). The high RWC (44%) at the

end of the experiment (Table 7.2) supported this cavitation result and revealed that a high concentration of *Ps. fluorescens* (10^6 cfu mL^{-1}) was the most beneficial treatment for increasing longevity after citric acid. Other researchers have made similar findings. A mixed bacterial population of 10^7 cfu mL^{-1} had no effect on carnation vase life, despite the number of bacteria in basal stem segments being significantly greater in that treatment than in germicidal treatments (van Doorn, Zagory, de Witte and Harkema 1991). In the present experiments, vase solutions containing high bacterial numbers (i.e. > 10^6 cfu mL^{-1}) had a lower dissolved oxygen concentration (data not shown) than distilled water. However, the results of Chapter 5 indicated that oxygen concentration in the vase solution was not strongly correlated with increased longevity (sections 5.3.2 and 5.4). Furthermore, stems in a 10^8 cfu mL^{-1} bacterial concentration exhibited more water stress (increased AAE production and lower RWC) than those in a 10^6 cfu mL^{-1} concentration, even though dissolved oxygen concentration was lower (data not shown).

When *A. amoena* stems were placed in a more concentrated solution of *Ps. fluorescens* (10^8 cfu mL^{-1} cf. 10^6 cfu mL^{-1}), the cavitation rate was much higher from 48 h onwards (Fig. 7.2). The RWC was significantly less in the higher bacterial concentration than in all treatments except distilled water (Table 7.2). The RWC data indicate that a 10^8 concentration of *Ps. fluorescens* was more beneficial to *A. amoena* water relations than distilled water. However, it appears that there is a bacterial concentration above which water relations are impaired, but it would seem that it is the initial bacterial concentration which is important. For example, in the 10^6 concentration experiment, the bacterial level rose to 10^8 after 3 d, but the stems underwent less water stress (i.e. low cavitation rate and high RWC) than when placed immediately into a 10^8 concentration. Put and Jansen (1989) also noted that the higher the initial number of bacteria added to the vase water, the lower the water uptake and the shorter the vase life. The infiltration of *Ps. putida* into the xylem of cut roses increased with higher numbers of bacterial cells in the vase solution, although the number of infiltrated bacteria was much lower than in the vase water (Put and Clerkx 1988).

Van Doorn, Buis and de Witte (1986) noted that water uptake and transpiration were only affected when mixed bacterial levels in the vase water were higher than 10^7 cfu mL^{-1}. Pure cultures of several *Pseudomonas* spp. (*Ps. fluorescens* was not tested) were also found to inhibit water uptake at 10^7 cfu mL^{-1}, but not at 10^5 cfu mL^{-1} (de Witte and van Doorn 1988). It has been observed that different bacterial genera, species and strains added to vase water exerted similar effects on water uptake and vase life (de Witte and van Doorn 1988; Put and Jansen 1989).

The cavitation and bacterial concentration experiments (Fig. 7.2) indicate that a gradual increase in bacterial numbers in uninoculated distilled water (e.g. Fig. 4.2; Table 4.2) is unlikely to cause the delay in the AAE peak. If the AAE peak was the result of a gradual increase in bacterial

numbers, the peak in Fig. 7.2 would have occurred earlier than the 48 h observed in distilled water (Fig. 4.2). Accordingly, the cause of the increase in water stress that leads to the cavitation peak is unknown. Gumnosis has been proposed as a cause of vascular plugging (Parups and Molnar 1972; Put and van der Meyden 1988). A study of the deposition of pectins and callose ('gums') over time is examined in Chapter 9.

Although *Pseudomonas* is one of the few bacterial genera known to synthesise pectate lyases (see section 7.1), Smith (1958) noted that the chief characteristic of pectic enzyme production in *Pseudomonas* was its variability. Some strains of *Ps. fluorescens* have been found to produce pectic enzymes (Fuchs 1965; Huetner and McIntyre 1969; Sands *et al.* 1972). However, the strain of *Ps. fluorescens* isolated from the vase water in the present experiments did not exhibit pectolytic activity on the medium of Sands *et al.* (1972).

Zucker *et al.* (1972) tested for production of extracellular pectate lyase in 33 species of *Erwinia* and *Pseudomonas* and assigned both *Ps. chlororaphis* (CSIRO strain JH-1) and *Ps. fluorescens* Biotype D (UCBB strain 31) to the group which produced only a trace or no activity. The micro-organism used in the present study was identified by fatty acid analysis as *Ps. chlororaphis*, which was formerly known as *Ps. fluorescens* biovar D until the 8th (Buchanan and Gibbons 1974) and present, i.e. 9th (Krieg 1984) editions of Bergey's Manual of Systematic Bacteriology recognised the biovars D and E of Stanier *et al.* (1966) as distinct. The biovars were therefore separated and returned to their original names of *Ps. chlororaphis* and *Ps. aureofaciens* respectively. It should be noted that Bergey's Manual (9th edition, Krieg 1984) lists the infrasubspecific rank of biotype as a synonym for the preferred name of biovar, and so the 'Biotype D' of Zucker *et al.* (1972) is the same as biovar D, and is thus *Ps. chlororaphis*. Whilst the strains of biovar/biotype D are probably different, the results of both Zucker *et al.* (1972) and the present experiment are the same, i.e. no, or only a trace of, pectate lyase activity. De Witte and van Doorn (1988) tested nine strains (six species) of *Pseudomonas* isolated from rose vase water and found that none of them degraded pectin, although *Ps. fluorescens* was not one of the species tested. Thus, if bacteria are responsible for xylem blockage, it is unlikely to be through a bacterially-induced degradation of pectins in the cell wall (see Chapter 9), a conclusion also reached by de Witte and van Doorn (1988).

The following chapter examines whether micro-organisms are visible under SEM in the basal segments of cut *Rosa* and *Acacia* stems during vase life.