

The Presence and Survival of Soft Rot (*Erwinia*) in Flower Bulb Production Systems

J. van Doorn, P.J.M. Vreeburg, P.J. van Leeuwen and R.H.L. Dees
Applied Plant Research
PO Box 85
2160 AB
The Netherlands

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Abstract

Soft rot is causing increasing damage in the flower bulb industry. Bulbous ornamentals such as *Hyacinthus*, *Dahlia*, *Iris*, *Muscari*, *Freesia* and *Zantedeschia* can be infected. Soft rot in flower bulbs is mainly caused by *Dickeya* spp. (*Dickeya* spp.) and *Erwinia carotovora* subsp. *carotovora* (*Pectobacterium carotovorum* spp. *carotovorum*). To identify and detect these soft rot bacterial species in several bulbous ornamentals, standard PCR methods were used.

During the last four years, research was directed to optimize cultural practices in especially *Hyacinthus* to avoid or minimize bacterial soft rot. To evaluate the incidence and infection route of *Erwinia* spp. in the production chain of hyacinth bulbs, field plots were infected by planting diseased hyacinth, iris and *Zantedeschia* bulbs. Subsequently, crop rotation with different flower bulb species was applied on these field plots during two growing seasons. Harvested bulbs were analysed during the handling and storage of the bulbs for *Erwinia* spp. by assessing symptoms as well as by using bacterial culturing- and PCR techniques. The survival of *Erwinia* in water and on materials, used in flower bulb handling, was studied. Results concerning the optimization of bulb handling during the production chain to avoid or to minimize the incidence of bulb soft rot are also presented.

INTRODUCTION

During the last decade problems with soft rot in flower bulbs have increased. Economic losses in this sector may amount up to 8 million Euros yearly; in crops such as seed potatoes the losses due to *Erwinia* are at least twice as much.

Previously *Erwinia carotovora* subsp. *carotovora* (Ecc) was a minor problem and infected especially hyacinth crops after wet field conditions, harvesting during wet field conditions and also after the slow drying of bulbs; Ecc was assumed to be only an opportunistic pathogen (Van Doorn et al., 1993).

Recently it became clear that *Erwinia chrysanthemi* was also present and showed a more aggressive behavior, resulting in increasing problems especially after harvest and during the handling of bulbs. Typical symptoms in the field, although less frequently encountered, are wilting and rotting (Fig. 1a); during handling of the bulbs after harvest the symptoms are softening and rotting of the bulb; often extracellular polysaccharides are oozing out of the bulbs ("resin", Fig. 1b).

Although other *Erwinia* species such as *E. carotovora* subsp. *atroseptica* (Eca), *Erwinia rhapontici* (hyacinth necrosis) and *Erwinia herbicola* (*Pantoeae agglomerans*) have been found occasionally, the main problems are caused by *Erwinia chrysanthemi* and Ecc. Recently, *Erwinia chrysanthemi* has been renamed into *Dickeya* spp. (*D. spp.*; Samson et al., 2005), whereas *Erwinia carotovora* subsp. *carotovora* was renamed *Pectobacterium carotovora* subsp. *carotovorum* (Pcc, Gardan et al., 2003). *E. carotovora* subsp. *atroseptica* has been renamed to *Pectobacterium atrosepticum* (Pa) (Gardan et al., 2003).

In this paper the new names will be used and the term *Erwinia* used to refer to both species.

There are many questions concerning soft rot in flower bulbs. The increase in

incidence of *Erwinia* during the last decade may correlate with changes in the production chain, but may also be connected to the occurrence or introduction of new isolates of *Dickeya* spp. Furthermore, it remains unclear at what stage in the production of flower bulbs *Erwinia* enters the plant. The sudden incidence and devastating symptoms caused by *Dickeya* spp., especially during storage of the bulbs also needs much attention in fundamental research programs.

One of the questions asked frequently by the hyacinth bulb growers is, where *Dickeya* spp. can survive in the environment, and in particular can it survive on equipment used in the production and handling of flower bulbs? Also the incidence of *Dickeya* spp. in the production chain is also of special interest to the flower bulb sector.

In this paper experiments are described in which many samples from different flower bulb species with soft rot symptoms were analysed for *Erwinia*. The survival of *Erwinia* species on materials used in the handling of flower bulbs, and the survival of *Erwinia* in different types of water used “on farm” have also been investigated. In addition the influence of *Erwinia*-contaminated bulbs on other bulbous ornamentals during crop rotation was studied. Finally, data concerning the percentage of soft rot in relation to the stage in the production chain of handling contaminated and *Erwinia*-free hyacinth lots are presented, together with suggestions how to minimize the occurrence of soft rot in flower bulbs.

MATERIALS AND METHODS

Bacterial Isolates and Identification Techniques

Isolates were obtained from bulbous ornamentals by diluting samples from suspected bulb or leaf material using plating techniques (Table 1) and characterized by plating on pectin media (Perombelon and Burnett, 1991). PCR for identification of *Erwinia* was performed using primers ADE1 and ADE2 specific for *Dickeya* spp. *pel* genes (Nassar et al., 1996) or Y1 and Y2 for the *P. carotovora*-group (Darasse et al., 1994). For PCR, DNA was isolated from single colonies using a PureGen Core kit A (Qiagen GmbH, Hilden, Germany). For PCR amplification of DNA, the PCR Master Mix from Promega (Madison, USA) was used. Essentially, 10-50 ng of isolated DNA was added, together with 40 pmol of each of the primers. In addition the 16S ribosomal DNA was isolated using *Erwinia*-specific primers (Toth et al., 1999), the DNA sequence was analyzed by BaseClear (Leiden, the Netherlands) and compared with the data in GenBank (Benson et al., 2008) for identification of the isolate.

Erwinia strains, used for survival studies were spontaneous antibiotic-resistant mutants (van der Wolf et al., 2009) isolated from Pa PRI1887, (Streptomycin-resistant), *Dickeya* spp. PRI 1991 (Nalidixine-resistant) and Pcc PRI1990, (Streptomycin resistant). To assess the rate of survival on materials, antibiotic concentrations of 0.1 mg per 10 ml agar (Nutrient agar, Oxoid, Hampshire, UK) were added.

Survival of *Erwinia*

For survival studies, the spontaneous antibiotic-resistant bacterial isolates of *D.* spp., Pa and Pcc were used.

Test materials (PVC, rubber, plywood, stainless steel and concrete) were cut into pieces of 12.5×12.5 cm and surface-sterilized by incubation with 70% alcohol and rinsed with sterile tap water. The test squares were divided into 56 smaller squares, on which 100 µl bacterial suspensions of approximately 10⁸ ml were applied. The test squares were incubated in a humid atmosphere (45, 60 or 95% RH) at 23°C, an average temperature during on farm handling and sorting of flower bulbs where contact surfaces are mostly PVC and rubber. During storage materials as concrete (floors of barns), wood (pellets) or stainless steel (machines parts) are present and might be soiled with *Erwinia*. In some experiments, hyacinth resin (a secondary sugar metabolite produced by *Dickeya* spp. in infected hyacinth bulbs especially during storage, (Fig. 1b) was collected in an Eppendorf tube, disinfected by heating to 100°C for 20 min and then added to the bacterial

suspension in PBS buffer in a ratio of 1:1. Analysis of surviving bacteria was performed by taking samples after 1, 3, 6, 24, 30, 48 and 120 hours or even longer. The test squares were processed by swabbing the test materials with a cotton swab and incubating this in 300 µl of PBS in an Eppendorf tube. Dilutions of this sample were tested by culturing on nutrient agar with the appropriate antibiotic for 2 days at 27°C after which the bacterial (*Erwinia*) colonies were counted. The identity of the bacterial colonies was confirmed by PCR as described above.

The survival of the three different *Erwinia* species was assessed by adding 10⁹ bacterial cells of the same strains described above to 100 ml tap water, basin water (rain water, collected on farms) and ditch water at temperatures of 4, 12 and 24°C (control= PBS at the same temperatures). The survival rate was estimated by plating dilutions of the samples on nutrient agar with the appropriate concentration of antibiotics; the bacterial counts were estimated in triplicate. To assess whether cells were viable but not cultivable, 10 ml of the water samples were centrifuged (3 min., 2500 ×g), the pellet resuspended in 100 µl PBS and prepared according the manufacturer's prescription (LIVE/DEAD[®] BacLight[™], INVitrogen, Breda, the Netherlands).

Crop Rotation Studies in *Erwinia*-Infected Plots

To assess whether infected soil could cross-infect planted bulbous crops, an area of 300 m² of alluvial sandy soil, (representative of the soil type used in cultivation of bulbous crops along the dunes at the coastal area of the North Sea) was planted with *D.* spp. infected hyacinth and iris, and with Pcc-infected *Zantedeschia*. Plots of 1×1 m² (in three replicates) were then planted with *Iris* ('Blue Magic'), *Muscari armeniacum*, hyacinth 'Carnegie', *Dahlia* 'Sandra' and *Zantedeschia* 'Treasure'; the following year the crops were rotated. The infection rate was estimated visually after harvest in summer (hyacinth, *Muscari*, *Iris*) and in winter (*Zantedeschia*, *Dahlia*); control samples with symptoms were tested by PCR for the presence of *Dickeya* spp. and for Pcc as described previously.

Five different planting conditions were used: no infected plants, infected hyacinth, infected hyacinth with compost (GFP), infected iris and infected *Zantedeschia*, respectively. In the second year (planting season) an identical infection experiment was performed; the same crops were planted but in a different rotation scheme. In the first year the planting scheme was *Zantedeschia*, *Dahlia*, *Muscari*, hyacinth and iris; in the second year it was rotated to hyacinth, *Muscari*, iris, *Zantedeschia* and *Dahlia*.

The Production Chain of Hyacinth

After harvest, two individual bulb farms in the Netherlands were visited and hyacinth samples taken. The bulb samples were taken from a planting stock and from the sale stock of the same cultivar ('Delfts Blue' and 'Carnegy', respectively). The hyacinth bulbs were sampled before harvesting, after harvest during drying and cleaning, at grading, and at the moment of delivery or after storage and heat treatment, respectively. The infection rate was estimated visually for rotting spots, soft bulbs or oozing of secondary metabolites; PCR was used as previously described to confirm for the presence of Pcc or *Dickeya* spp. if soft rot symptoms were observed.

RESULTS AND DISCUSSION

Characterization of Bacterial Isolates from Infected Flower Bulbs

The bacterial isolates were obtained from 18 different bulbous ornamentals which were collected during the years 2004 to 2007. Typical symptoms in the field, displayed by infected hyacinth bulbs (Fig. 1a) are wilting and sometimes decline and collapsing of the plants. In storage hyacinth bulbs infected with *Erwinia* generate resin that oozes from the bulb (Fig. 1b).

Most of the isolates were obtained from hyacinth (Table 1) and PCR confirmed these isolates to be mostly *Dickeya* spp. In approximately 70 % of the samples *Erwinia*

was identified; in about 30% no reaction was found in PCR with the primers, which amplify *Dickeya* spp. or the *Pectobacterium* group. Identification of *Erwinia*'s was done using PCR conditions as described above. It is therefore likely that there are other bacteria which can induce soft rot in bulbous ornamentals and it is also possible that *Erwinia* is present as a part of mixed infections with other bacterial species.

In *Zantedeschia*, only Pcc was found in the tuber. This is in agreement with other findings (Snijder et al., 2002). However, in plants with rot symptoms in the leaves and stems, no *Erwinia* strains could be isolated. The agent responsible for rot in these tissues seems to belong to the species *Pseudomonas*. Although *Erwinia* species appear to be the major cause of bacterial rot in flower bulbs other bacterial species such as pseudomonads have the potential to induce rot. This is consistent with studies of other crops suffering from bacterial rot, for instance chicory, *Pseudomonas marginalis*, *P. viridiflava* and other pseudomonads (Schober 1998) can infect these crops. No *Pectobacterium atrosepticum* was found. Therefore in the future primers will be developed to test samples not only for *Dickeya* spp. and Pcc, but also for pseudomonads. Fungi as *Fusarium* can also give rise to rotting, due to its capability to produce pectinolytic enzymes, although the symptoms (dry rot) which it produces can be differentiated in most cases from bacterial (soft) rot.

In 2007, two samples of infected daffodil ('Tête à Tête') were received showing soft rot symptoms and analyzed by PCR. *Dickeya* spp. was found; the 16S DNA sequence analysis and alignment in GenBank showed that this isolate belonged to *D. dadantii*. To our knowledge, there exist no earlier reports of daffodil, infected by *Dickeya* spp. It remains to be established whether or not this is an isolated incident or the prelude to more new host plants, sensitive to *Dickeya* spp. among bulbous ornamentals.

Survival of *Dickeya* spp., Pa and Pcc on Farm Materials and in Water

The materials tested for the survival of *Erwinia* are found for instance on sorting machines, containers, floors and storage shelves for flower bulbs on farms. The relative humidity influences the rate of survival of *Erwinia*. This has been shown for a *Dickeya* strain, Pa and Pcc on PVC (polymerized vinyl chloride) and concrete. Pa (*Pectobacterium atrosepticum*) is not found in flower bulbs but was also tested since it is a pathogen, found in potato and a closely related *Erwinia* species (Perombelon and Kelman, 1980). On concrete, only at a RH of 95% showed surviving bacteria; the survival rate on PVC increased from 6 hours (RH of 45%) to about 48 hours at a RH of 95% (Fig. 2a,b). This was also tested for Pcc and Pa on concrete and PVC (Fig. 2c-f). Stainless steel, plywood, rubber and concrete were tested for the survival of three *Erwinia* strains at 25°C and a relative humidity of 60% (Fig. 3).

To assess whether *Erwinia* could survive better in the presence of secondary metabolites, bacterial samples were mixed with hyacinth resin and applied on plywood, concrete (not shown) and PVC (Fig. 3).

On PVC, in resin the bacteria managed to survive for a prolonged period of time; only *Dickeya* spp. died after approximately 35-50 days (Fig. 3b); after 100 days bacterial cells of Pa and Pcc could still be cultured on nutrient agar (Fig. 3b). Without resin, *Dickeya* spp. died within 2 day (Fig. 3a). On stainless steel, only Pa lived up to 85 days whereas the other *Erwinia* strains died off after 7-13 days (Fig. 3c). The survival rates on rubber, concrete and wood showed a corresponding picture; on average *Dickeya* spp. died after about 6 days, Pcc after 8-13 days whereas Pa survived until 27 days (Fig. 3d,e,f). In comparison, the bacteria survived much shorter periods when applied in PBS only (Fig. 2a-f); Pcc did not survive long on any material and died within 24 hours; *Dickeya* spp. survived about 24 hours on stainless steel whereas Pa could survive 48 hours on concrete and stainless steel (results not shown). At an RH of 95% *Dickeya* spp. survived about 24 hours on concrete; Pcc and Pa survived for 48 hours. On PVC, at 95% RH the survival rated up to 70 hours (Fig. 2b,d,f).

These results showed that Pa is the most resilient, and that the RH influences the rate of survival. The resin is also important. Without resin, the bacteria survived only for a few hours at the most. These polysaccharides are likely to have an important role

because their presence influences the environment immediately surrounding the bacteria, providing a humid layer which acts as a biofilm. The mucoid layer might protect the bacteria and help to conserve the cells until better (more humid) conditions prevail for the bacteria to start growing again. Little is known if desiccation brings the bacteria into a special survival state.

These results made it clear that hygiene measures, to be taken in the production chain, are very important to prevent contamination of healthy bulbs with *Erwinia*'s during the handling on farm.

The *Pectobacterium* group is dependent of humid conditions to colonize and survive in plants. As water is used in different moments in the production chain of hyacinth, it is important to assess if, and for how long these plant pathogens can survive in water.

Three types of water were used. In tap water the *Erwinia* strains did not survive long; *Dickeya* spp. died within a few hours whereas Eca and Pcc survived for 24 hours (Fig. 4a). In basin water, the *Erwinia* species survived longer (Fig. 4b) whereas in sterilized ditch water (Fig. 4c) the *Erwinia* lived longest. The temperature had a slight effect, but the concentration of minerals and buffering substances seem to be crucial in the PBS buffer.

To assess whether bacteria were present in a viable but not cultivable state, samples were concentrated by centrifugation and stained with a dye that will fluoresce green if the cells are viable and red if the cells are dead. No green cells were found. However, when the sites of the flasks with basin water or ditch water were swabbed, *Erwinia* cells were found which could be cultured on nutrient agar or CVP (results not shown). These findings indicated that although the bacteria were not present in the supernatant, it appeared that *Erwinia* still exists in a biofilm. This may have consequences for bulb growers who obtain water for rinsing flower bulbs from basin water, or water their crops with ditch water.

The most important question for the flower bulb industry is how to prevent infection of the plants. The main routes of infection can be infected materials, the soil and water.

In comparison, *Dickeya* spp. survived in sandy soil (without plant material) approximately for 23 days, whereas Eca and Pcc survived up to 84 days (Van der Wolf et al., 2009).

In tap water, *Erwinia* appeared to be short-living, probably due to osmotic shock as shown by their prolonged survival (at least of Pcc and Pa) in PBS buffer.

Transmission of *Erwinia* by Crop Rotation of Flower Bulb Species

In Table 2 the results of the percentages of diseased flower bulbs are shown. Especially in *Muscari* in 2005 a high percentage of *Dickeya* spp. was found. However, compared with the control plots without any diseased plant material there was no significant increase in the rotated crops in respect to the percentage of *Erwinia* infection. Clearly the estimated percentage was already present in the planted crops, and not caused by any soil-transmitted infection. This is in agreement with the study of the survival of *Erwinia* in the soil; *Erwinia* cannot survive for a prolonged time (f.i. one year) in the soil, at least not without plant material. The question remains when and where *Erwinia* enters the production chain.

Influence of the Production Chain on the Infection Rate of *Dickeya* spp. in Hyacinths

Two hyacinth lots (planting stock and sale stock) appeared to be free of *Erwinia* as assessed in samples, taken before harvesting and showed no infection during the production chain (Fig. 5). Two other hyacinth bulb lots however were infected; handling during the production chain (cleaning, sorting, storage and packaging) resulted in an increase of soft rot symptoms. Thus, the (latent) presence of *Erwinia* is crucial for the development of soft rot symptoms later in the production chain, probably dependent of the rate of (miss-)handling for example damaging by falling, humid conditions and other

factors.

Recommendations to curb soft rot problems in the production chain especially for hyacinth are hygiene precautions and avoidance of humid or wet conditions during storage or cleaning of flower bulbs.

Future research will be directed in the identity of the isolated *Dickeya* strains to species level. In infected daffodil bulbs, *D. dadantii* has been found. It is not yet clear whether certain *Dickeya* strains are host specific infecting specific crops such as *Dahlia* or *Freesia*. In respect to this, the flower bulb industry is in need of routine diagnostic tests to assess whether plant lots or propagation bulbs are free of *Erwinia*. This would help to start with *Erwinia*-free planting stock which will reduce the incidence of *Dickeya* and *Pectobacterium* during the production chain.

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Tables

Table 1. Isolates from soft rot symptoms of bulbous ornamentals as obtained during 2004-2007.

Sample	Amount of isolates	<i>Dickeya</i> spp.	<i>Pectobacterium car.</i> subsp. <i>car.</i>	Other organisms
Hyacinth	134	61	33	<i>Pseudomonas</i> , <i>Fusarium</i>
<i>Muscari</i>	9	5		
<i>Zantedeschia</i>	7	2		
<i>Iris</i>	11	4	2	
<i>Allium</i>	5		1	<i>Pseudomonas?</i>
<i>Dahlia</i>	3	3		
<i>Freesia</i>	3	1		<i>Fusarium</i>
Daffodil	3	1		<i>Fusarium</i>
<i>Crocus</i>	1			Not determinated
<i>Brodea</i>	1	1		
<i>Amaryllis</i>	3	2		
<i>Ornithogalum</i>	2		2	
<i>Colocasia</i>	1		1	
<i>Triteleia</i>	1	1		
<i>Gladiolus</i>	1			Not determinated
<i>Oxalis</i>	1			Not determinated
Anemone	3	2		

Table 2. Percentage of plants with soft rot symptoms by soil, infected in different ways with *Dickeya* spp.

Infection	Hyacinth		<i>Muscari</i>		<i>Iris</i>		<i>Dahlia</i>		<i>Zantedeschia</i>	
	2005	2008	2005	2008	2005	2008	2005	2008	2005	2008
none	0.7	17.9	44.4	0	0.6	5	0	0	13.4	16.7
Hyacinth	2	31.7	50.0	0	1.0	4.3	0	1.1	23.9	2.9
Hyacinth + compost	0.3	11.9	43.2	1.3	1.2	3.9	0	0	15.9	0.0
<i>Iris</i>	0	25.0	52.0	0	1.3	3.9	0	3.3	29.4	11.1
<i>Zantedeschia</i>	0	4.4	46.4	0	0.8	6.1	0	0	21.7	13.3

Figures



Fig. 1a. Wilting and rotting of *Hyacinthus*, caused by *Dickeya* spp.



Fig. 1b. Production of extracellular polysaccharides (“raisin”) in hyacinth bulbs, inoculated with *Dickeya*.

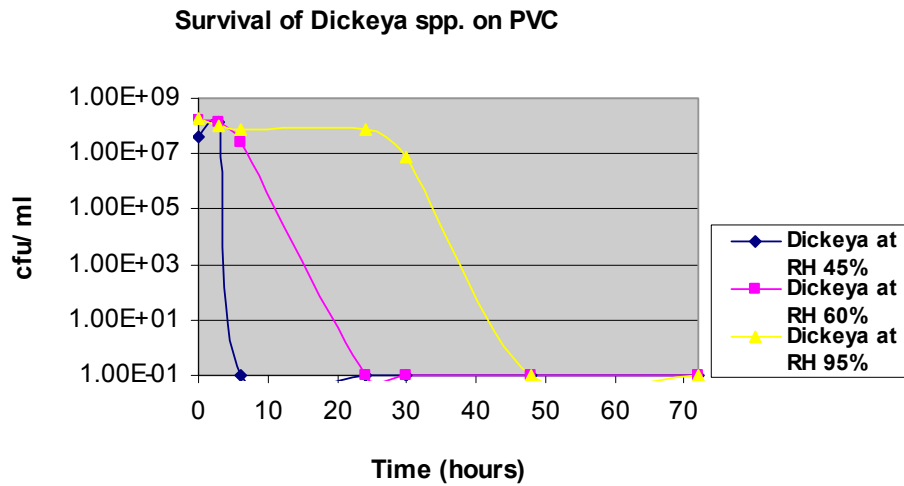


Fig. 2a. Survival of *Dickeya* spp. on PVC in time in relation to different RH.

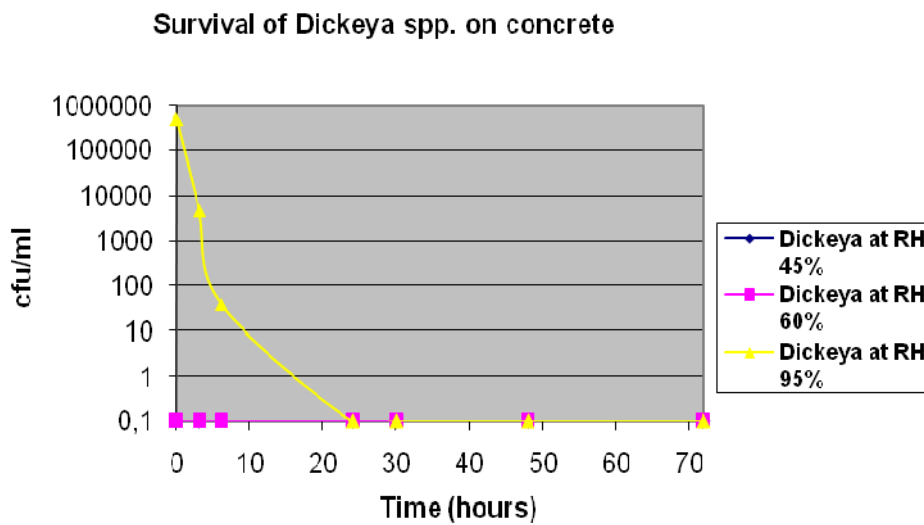


Fig. 2b. Survival of *Dickeya* spp. on concrete in time in relation to the relative humidity (RH)

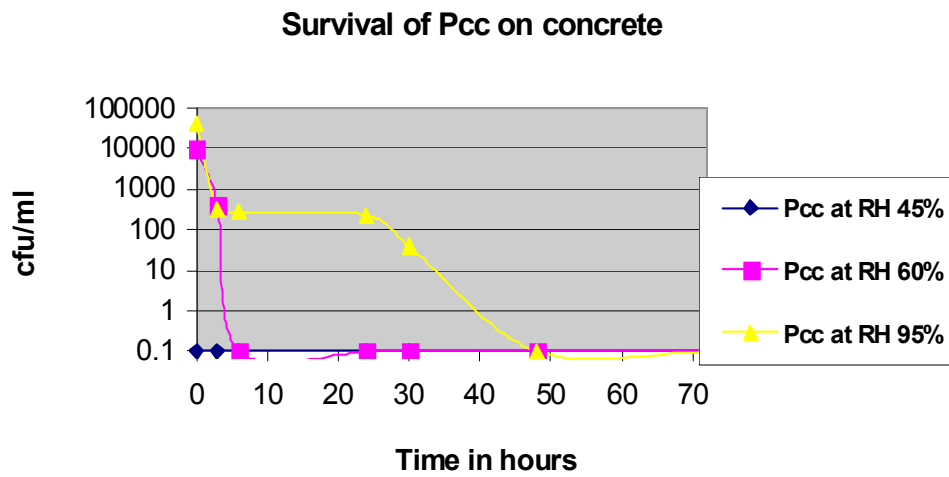


Fig. 2c. Survival of Pcc on concrete in time in relation to different RH.

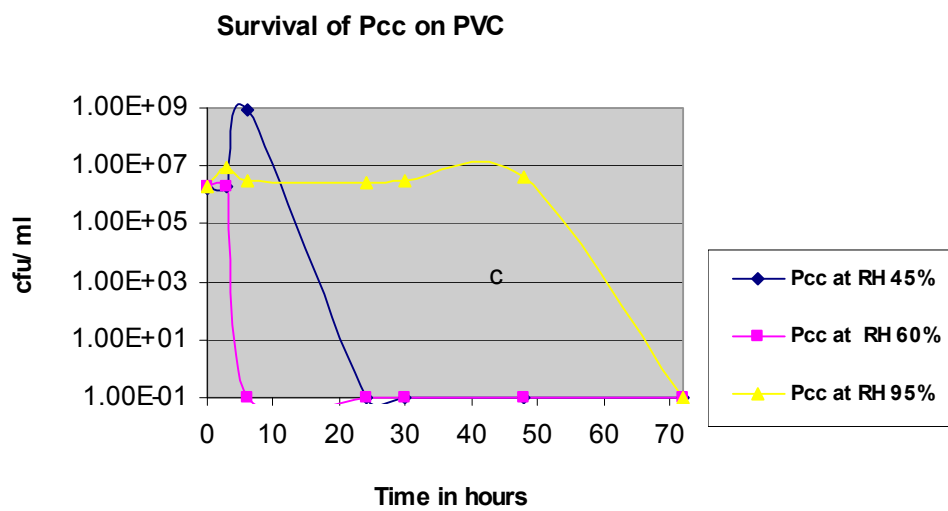


Fig. 2d. Survival of Pcc on PVC in time in relation to different RH.

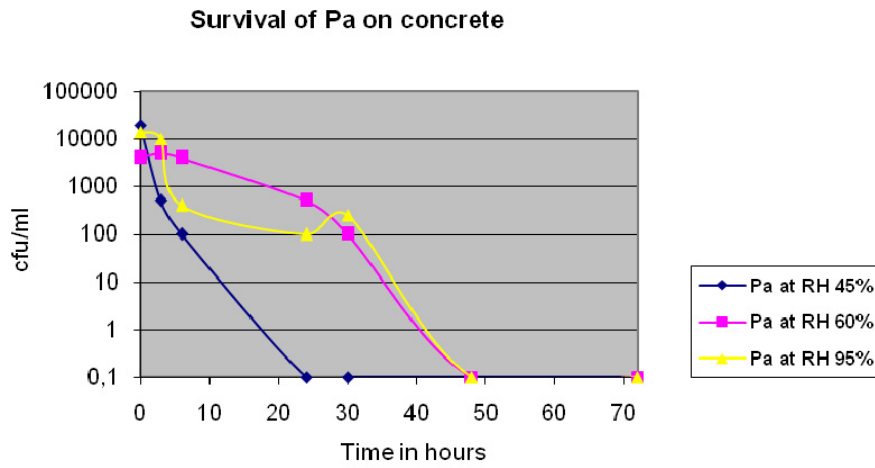


Fig. 2e. Survival of Pa on concrete in time in relation to different RH.

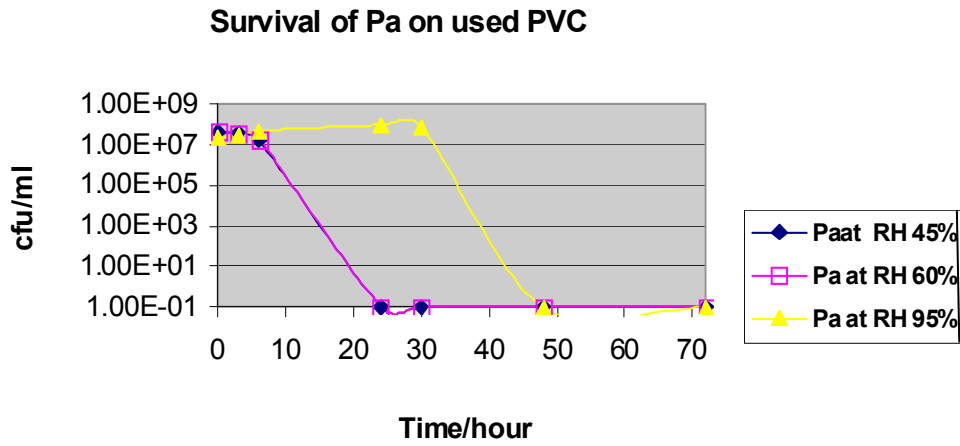


Fig. 2f. Survival of Pa on PVC in time in relation to different RH.

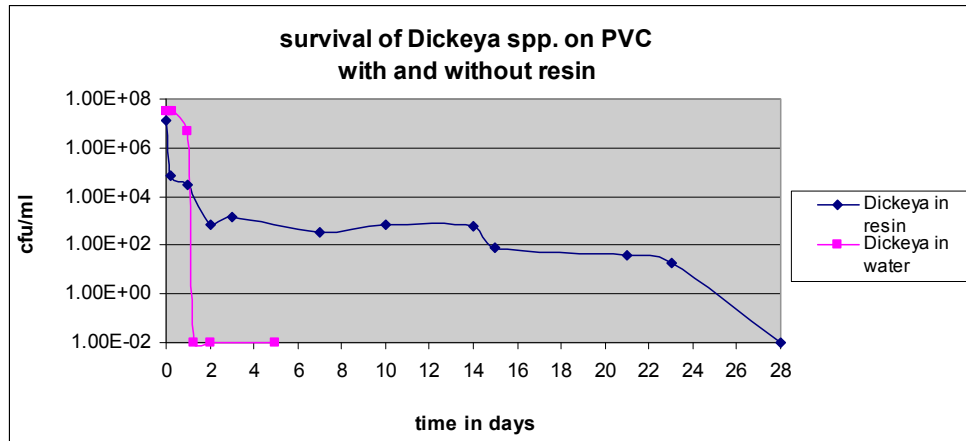


Fig. 3a. Survival of *Dickeya* spp. with and without resin on PVC.

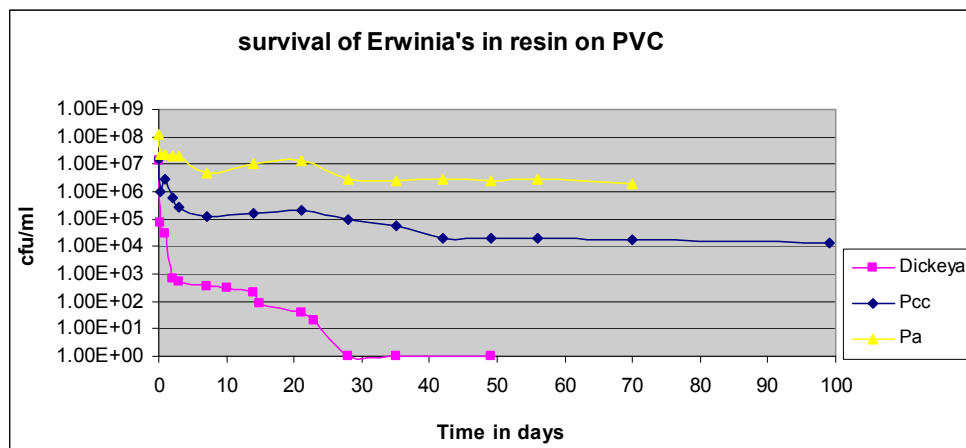


Fig. 3b. Survival of *Pcc*, *Dickeya* spp. and *Eca* in resin on PVC.

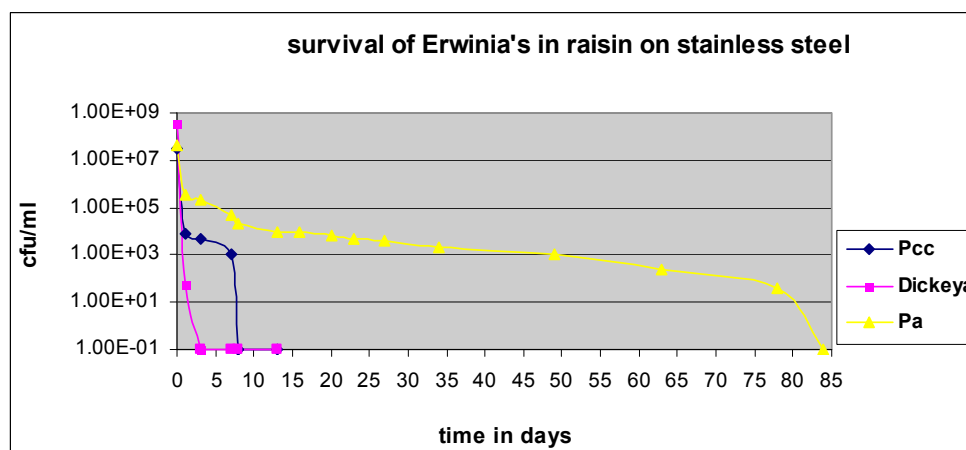


Fig. 3c. Survival of *Pcc*, *Dickeya* spp. and *Eca* in resin on stainless steel.

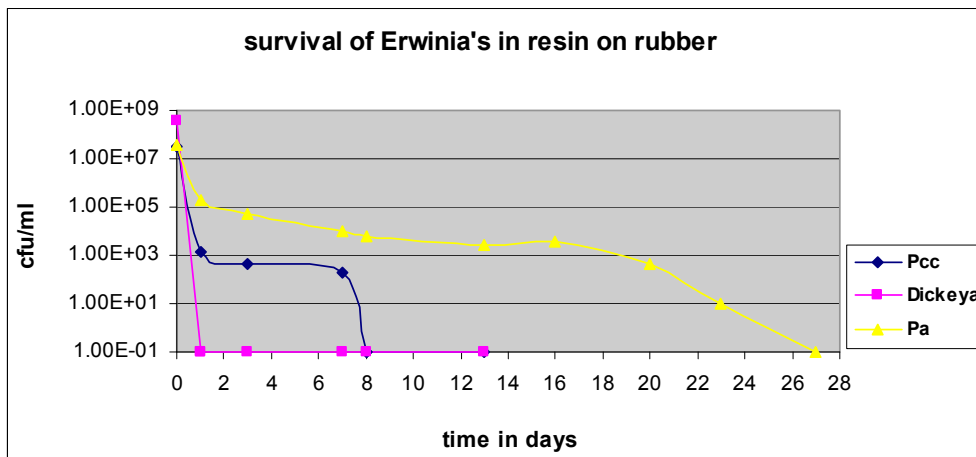


Fig. 3d. Survival of Pcc, *Dickeya* spp. and Eca in resin on rubber.

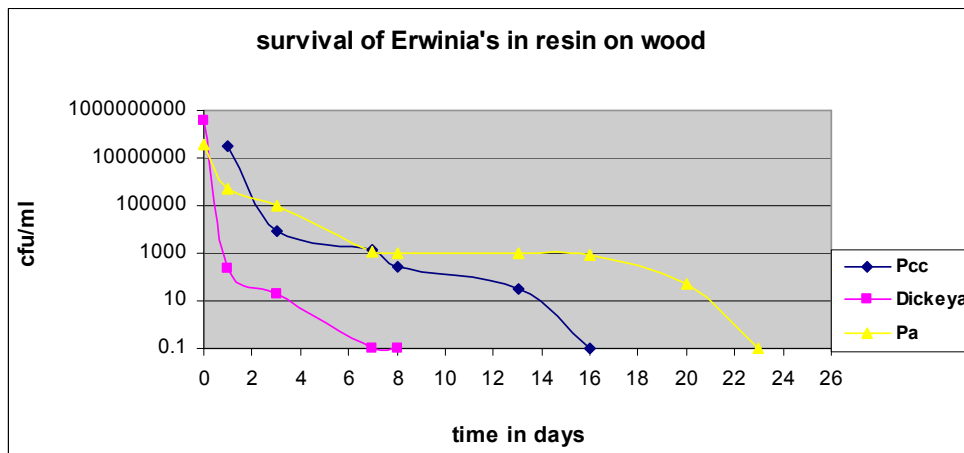


Fig. 3e. Survival of Pcc, *Dickeya* spp. and Eca in resin on wood.

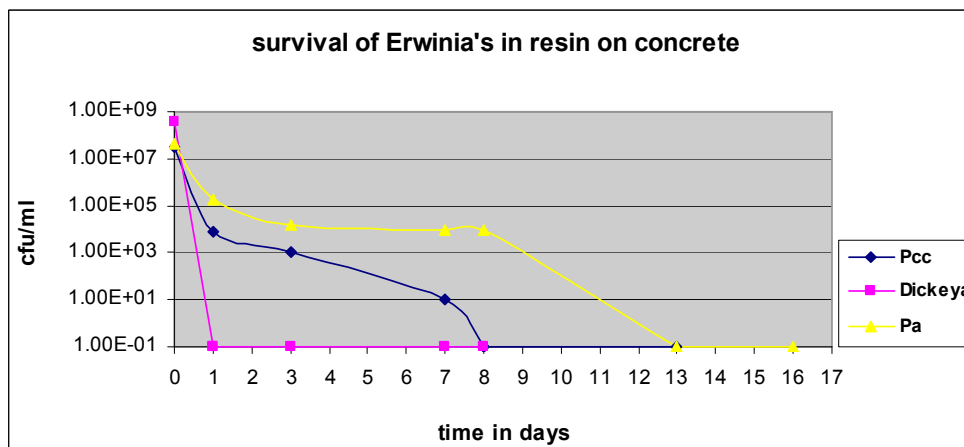


Fig. 3f. Survival of Pcc, *Dickeya* spp. and Eca in resin on concrete.

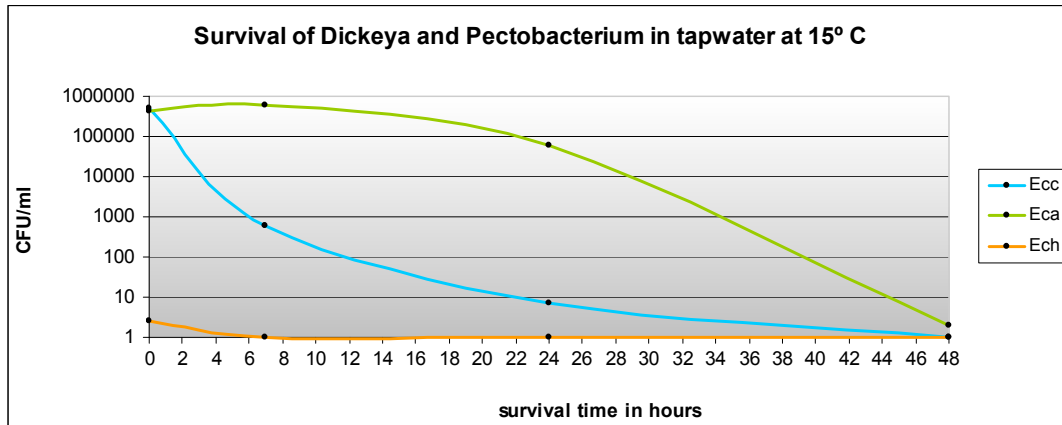


Fig. 4a. Survival of *Erwinia* in tapwater.

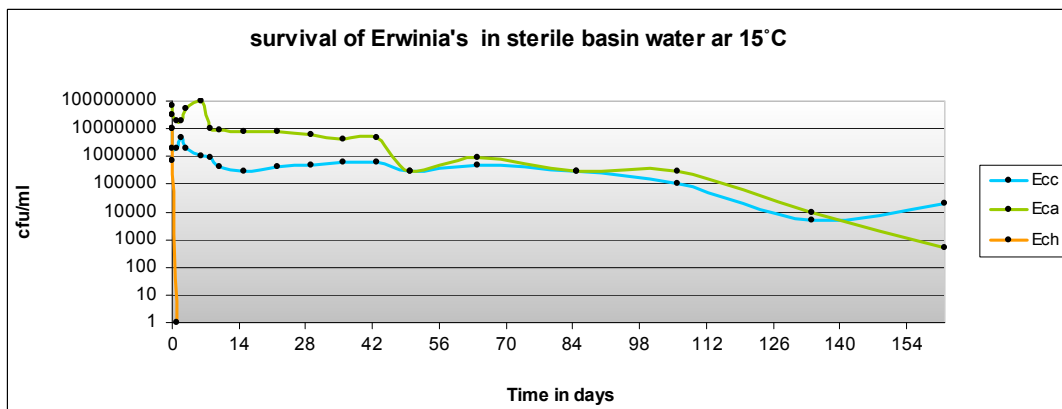


Fig. 4b. Survival of *Erwinia* in sterile basin water.

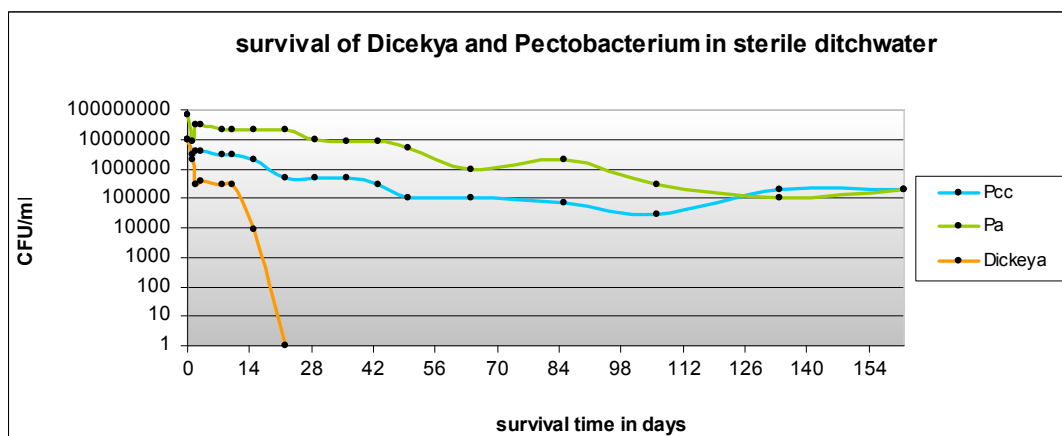


Fig. 4c. Survival of *Erwinia* in sterile ditch water.

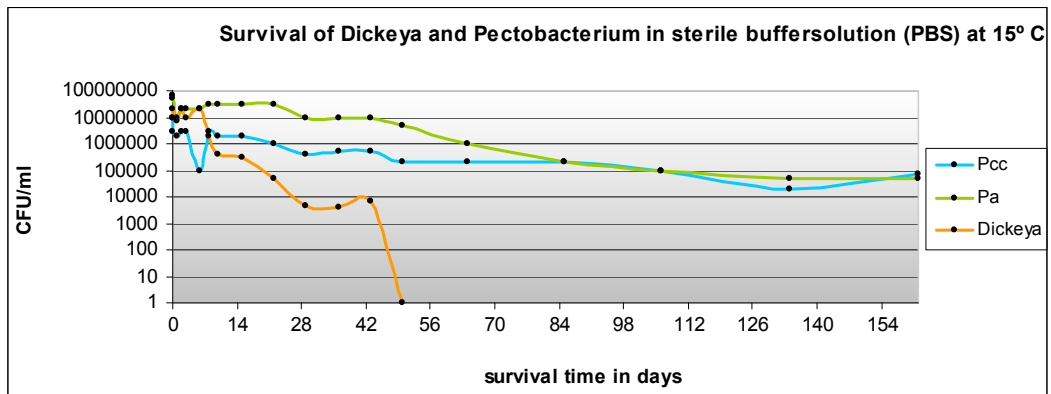


Fig. 4d. Survival of *Erwinia* in PBS at 15°C.

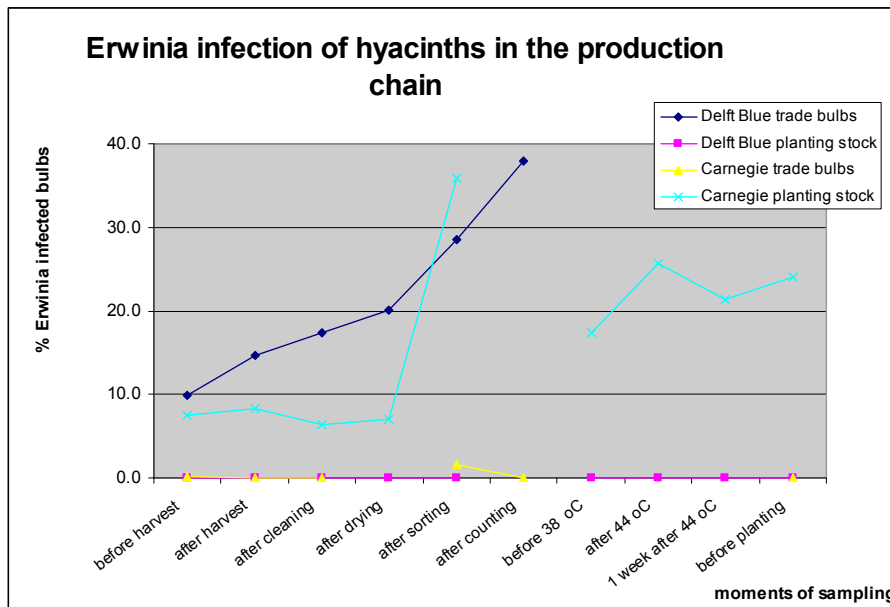


Fig. 5. *Erwinia* infection during the production chain of hyacinths.

